Cysteine String Protein Monitors Late Steps in Cystic Fibrosis Transmembrane Conductance Regulator Biogenesis⁺

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We examined the role of the cysteine string protein (Csp) in cystic fibrosis transmembrane conductance regulator (CFTR) biogenesis in relation to another J-domain protein, Hdj-2, a recognized CFTR co-chaperone. Increased expression of Csp produced a dose-dependent reduction in mature (band C) CFTR and an increase in immature (band B) CFTR. Exogenous expression of Hdj-2 also increased CFTR band B, but unlike Csp, Hdj-2 increased band C as well. The Csp-induced block of CFTR maturation required Hsp70, because a J-domain mutant (H43Q) that interferes with the ability of Csp to stimulate Hsp70 ATPase activity relieved the Csp-induced block of CFTR maturation. Nevertheless, Csp H43Q still increased immature CFTR. Csp-induced band B CFTR was found adjacent to the nucleus, co-localizing with calnexin, and it remained detergent-soluble. These data indicate that Csp did not block CFTR maturation by promoting the aggregation or degradation of immature CFTR. Csp knockdown by RNA interference of CFTR, therefore targeting the protein for proteasome-mediated degradation if folding does not succeed (15–17). The co-translational association with other CFTR domains, particularly the R-domain (12). In contrast, prolonged or excessive interactions with Hsp70 and Hdj-2, represent the ubiquitin ligase, CHIP, which poly-ubiquitinates Hsp70-ladened...
rnts (18). Ostensibly, this finding was compatible with the known action of Csp, identified originally as a synaptic vesicle-associated protein that plays an essential role in neurosecretory vesicle insertion into the plasma membrane (19). Subsequent studies of Csp demonstrated that it is required for other regulated exocytic processes, including insulin secretion (20). Nevertheless, although Csp localized partially to the apical membrane domain in polarized CFTR expressing epithelia, as expected for its participation in regulated exocytosis, its co-localization with calnexin also suggested the potential for Csp to function in the endoplasmic reticulum (ER). Further studies showed that the Csp-induced inhibition of CFTR currents was explained by a primary effect on the production of mature CFTR protein (18), implying that Csp may also function at early steps in the protein secretory pathway.

In this study, we examined the physiological role of Csp in CFTR protein processing by experimentally increasing or decreasing its expression level. The effects of varying Csp expression on CFTR biogenesis were compared with those of Hdj-2, an acknowledged CFTR co-chaperone (12). Our results are consistent with the concept that these J-domain proteins play different roles in the processing of CFTR; Hdj-2 facilitates CFTR biogenesis, whereas Csp regulates the export of CFTR to post-ER compartments.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Wild type or mutant Csp constructs were tagged with the Myc epitope as described (20). Anti-CFTR monoclonal antibodies (M3A7) were kindly provided by Dr. John Riordan (Mayo Clinic, Scottsdale, AZ) or purchased from Chemicon (MAB3480). Anti-Csp was also from Chemicon. Anti-CFTR polyclonal antibodies (R3195 and MD1314) were generated and affinity-purified, as described previously (21), and kindly provided by Dr. Christopher Marino, University of Tennessee, Memphis. The monoclonal antibody to human c-Myc developed by Dr. Michael Bishop was obtained from the Developmental Studies Hybridoma Bank, under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa. Monoclonal anti-Hsp70 and Hsp90 antibodies were purchased from Sigma. Anti-tubulin antibody was a gift from Dr. Linton Traub (University of Tennessee, Memphis). Rabbit anti-green fluorescent protein (GFP) was purchased from Abcam (Ab6556). Mouse anti-Hsp70 (SPA-810) was obtained from Stressgen. Goat antibody to Grp78 was purchased from Santa Cruz Biotechnology (SC-1050). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase was from U. S. Biochemical Corp. Mouse anti-calnexin was purchased from Affinity Bioreagents (MA3-027).

Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Sigma. Double-stranded RNAs (dsRNA) to interfere with the expression of endogenous mammalian Csp (CCUCG-GAUGCAUAUAAAGA) or Xenopus Csp (GAUACGCCGCAA-GAAC) mRNAs were designed by Oligogene software and obtained from Dharmacon Research. Lipofectamine was obtained from Invitrogen. RedVue Pre-mix L-355 was obtained from Amersham Biosciences. Protein A/G-agarose beads and Taq polymerase were purchased from Invitrogen; glutathione-Sepharose® 4B was purchased from Amersham Biosciences. DNA restriction endonucleases were from New England Biolabs. DNA purification kits were obtained from Qiagen. Reagents for analytical gel electrophoresis were from Bio-Rad. Pre-stained protein standard used for immunoblotting was purchased from Invitrogen. BCA protein assay, Supersignal West Dura chemiluminescent substrate, and Restore stripping reagent were from Pierce. Other reagent grade chemicals were purchased from Sigma.

**Cell Culture and Transient Transfection**—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) and supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 mM HEPES, and antibiotics (Invitrogen). For transient transfection of cDNAs encoding Myc-tagged Csp and CFTR, 10^6 cells per well in 6-well plates were plated and transfected using Lipofectamine 2000 the following day according to the manufacturer’s instructions and the methods described by Zhang et al. (18). Calu-3 cells were cultured as described (18, 22).

**Immunoblots and Pulse-Chase Assays**— Transfected cells were lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA supplemented with complete protease inhibitor mixture (Roche Applied Science) by rocking for 30 min at 4 °C. To determine the distribution of CFTR between soluble and insoluble fractions, we used the following protocol. The Triton X-100-insoluble fraction was separated by centrifugation at 16,000 × g for 15 min at 4 °C. The soluble fraction was decanted, and the insoluble pellets were washed twice with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and re-solubilized in SDS-PAGE loading buffer by sonication.

Crude membranes and membrane extracts from cell lines were prepared as described (18). Briefly, 50 μg of membrane extract per lane was separated on 12% SDS-polyacrylamide minigels and transferred to polyvinylidene difluoride membranes. Blots were probed with the indicated antibodies and diluted as follows: anti-Csp, 1:2000; anti-Hsp70, 1:3000; anti-Hsp90, 1:2000; and anti-CFTR, 1:200. Immunoblots were scanned and quantitated using ImageJ, a public domain Java image-processing program (downloaded from the National Institutes of Health, Bethesda). Statistics and t test calculations were done with Excel 2000 (Microsoft, Redmond, WA). Metabolic labeling and immunoprecipitation (IP) for detection of protein binding to CFTR were performed using methods described previously (9, 18).

**Immunofluorescence**—Cells were replated on 12-mm poly-l-lysine-coated coverslips (BD Biosciences) in 24-well plates 1 day after transfection and then fixed and permeabilized on day 2. Antibody incubations were done in phosphate-buffered saline containing 2% BSA, and all washes were done with phosphate-buffered saline containing 0.5% BSA. Cy3- and Cy5-labeled secondary antibodies were from Jackson ImmunoResearch (West Grove, PA), and nuclei were stained with the DNA dye Hoechst 33342 (bisbenzamide) purchased from Sigma. Images were obtained with an Olympus IX80 100× confocal microscope (see also Refs. 22 and 23).

**In Vitro Binding Assays and Co-immunoprecipitation**—In vitro binding assays were performed as described by Sun et al. (24). Briefly, 10 μg of GST or GST-R domain fusion protein (GST-CFTR-R) were incubated with 20 μL of pre-equilibrated glutathione-Sepharose 4B beads in 200 μL of DIGNAM-D buffer containing 0.1% BSA at 4 °C for 1 h (18, 25). In vitro competitive binding assays were performed as described (24) with modifications. Glutathione beads were incubated with 35S-labeled Csp or Hdj-2 at the indicated concentrations. After five washes with binding buffer, samples were resuspended in SDS sample buffer, and the proteins were resolved by SDS-PAGE; the signals were visualized by autoradiography.

Co-IPs were performed using either 3 μL of anti-Csp or anti-CFTR (M3A7) antibody as described (18, 22). As a control for efficiency in the Csp/CFTR IPs, supernatant fractions obtained after IP of myc-Csp were incubated overnight with 3 μL of anti-CFTR (M3A7) antibody in the presence of protein G-agarose at 4 °C. The next day, samples were washed four times with DIGNAM-D buffer and separated by 5% SDS-PAGE. Blots were probed for CFTR (re-IP) using polyclonal anti-CFTR (R3195; 1:2000).

**RNA Interference**—Six μg of dsRNA was transfected using 20 μL of Lipofectamine 2000 into 50% confluent 3T3 cells stably expressing CFTR (kindly provided by Dr. Michael Welsh, University of Iowa), which had been seeded onto 35-mm dishes 1 day earlier. Two and 4 days
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later, the transfections were repeated with the same procedure. Three days thereafter, cell lysates or membrane extracts were prepared and analyzed for CFTR, chaperone protein, and control protein expression, as described previously (18). Western blots were probed simultaneously with anti-Csp and anti-Hsp70 antibodies or with anti-CFTR and anti-Hsp90 antibodies. The blots were stripped and re-probed for tubulin as control.

**SPQ Assays of Regulated Anion Transport**—Experimental procedures for monitoring forskolin-stimulated halide transport across the plasma membranes of HEK293 cells using SPQ fluorescence were performed as described (26) with modifications. HEK293 cells were seeded onto coverslips pre-coated with poly-L-lysine and cultured for 24–36 h. When cells reached ~60% confluence, 1.5 μg of cDNA encoding human CFTR and/or wild type or mutant Csp was co-transfected using Lipofectamine, as described (18). After 1 day, the cells were loaded with SPQ using hypotonic loading conditions as follows. Cells were incubated at room temperature for 5 min in a 1:1 mixture of CI- buffer (126 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 1.0 mM MgCl2, 20 mM HEPES, 0.1% bovine serum albumin, 0.1% d-glucose, pH 7.2) and distilled H2O, containing 5 mM SPQ. After a 30-min recovery period in culture medium, the coverslips were then mounted in a chamber heated to 37 °C on the stage of a fluorescence microscope. The chamber was supplied continually with warmed buffer solution, and individual cells (regions of interest) were selected for study. Fluorescence intensity was collected as the perfused solutions were changed from sodium iodide to sodium nitrate to dequench SPQ fluorescence and expressed relative to intensity at time 0. Further fluorescence dequenching elicited by forskolin (10 μM) plus IBMX (100 μM) was indicative of cAMP-stimulated iodide efflux from preloaded cells and provides an assay for CFTR channel activity. Re-addition of iodide buffer then quenches SPQ fluorescence to baseline levels. Data were collected from at least 20 cells per coverslip, with images acquired at 0.2 Hz. The blank field obtained by closing the camera shutter was used as background and subtracted prior to image acquisition.

**Oocyte Expression**—Oocyte preparation and cRNA injections were carried out essentially as described by Takahashi et al. (27). A total of 1 ng of cRNA encoding CFTR with or without 100 ng of dsRNA targeting endogenous Xenopus Csp were injected, and expression was allowed to proceed for 4–5 days before current recordings. All data are provided as the mean ± S.E.

**Hsp70 ATPase Assays**—The ability of WT or H43Q Csp to stimulate Hsp70 ATPase activity was determined as described previously (28, 29) using the yeast homologue, Ssa1p. Briefly, Ssa1p-ATP complexes were formed as described (30) by incubating Ssa1p (25 μg) with 100 μCi of [α-32P]ATP (PerkinElmer Life Sciences) and 25 μM ATP in complex buffer (25 mM HEPES-KOH, pH 7.5) into a refolding buffer (50 mM KCl, 2 mM MgCl2, 20 mM HEPES, pH 7.2) to a final concentration of 2 μM. Protein aggregation was measured by light scattering as a function of time at a wavelength of 320 nm at 30 °C in a 14DS UV-visible IR spectrophotometer (Aviv Associates, Inc.) with and without Csp or Csp H43Q. The results were plotted as relative aggregation normalized to values at 10 min for NB1D alone (13).

**RESULTS**

Csp Block of CFTR Maturation Requires Hsp70—The influence of Csp expression on steady-state levels of CFTR was determined by cotransfection of HEK293 cells with plasmids encoding both proteins (Fig. 1A). As observed previously (18), overexpression of Csp reduced the expression of mature, glycosylated (band C) CFTR and increased the expression of nascent, core glycosylated (band B) CFTR. This finding suggests that excess Csp interferes with the ability of CFTR to progress to Golgi-localized glycosylation sites. In our prior studies (18), a block of CFTR maturation was observed with the co-expression of either Csp isoform, Csp1 or Csp2. The latter variant represents a C-terminal truncation of 31 amino acids from Csp1. It is expressed at lower levels than Csp1 (32, 33) and is functionally indistinguishable from it (34), as shown in our prior work (18). Therefore, we elected to focus the present studies on the predominantly expressed form, Csp1. We first examined several

**FIGURE 1. Effect of Csp and Csp mutants on steady-state CFTR levels.** Wild type and mutant Csp1 (H43Q, E93V, or H43Q/E93V double mutant [DM]; see lane designations) were transiently co-transfected with CFTR into HEK293 cells (cDNA ratio 1:1). A, membrane extracts were resolved using 15% SDS-PAGE and blotted with polyclonal anti-CFTR (R3195, 1:2000). B, Csp was immunoprecipitated with anti-Myc monoclonal antibody (9E10, 1:2000), and immunoblot (IB) was performed as in A. C, supernatant from Myc IP was re-immunoprecipitated by using anti-CFTR (R3195, 1:2000) and blotted for CFTR as in A. The results are representative of at least three experiments. C and B in right margin indicate bands C and B.
Csp mutations shown previously to interfere with regulated exocytic events (20).

As illustrated in Fig. 1A, the Csp HPD mutant, H43Q, restored the ability of CFTR to progress to Golgi glycosylation sites, as evidenced by the reappearance of CFTR band C. Mutation of the conserved HPD motif within the J-domain interferes with the ability of Csp to stimulate Hsp70 ATPase activity (26) (see also Fig. 1A), and in general, mutations in the conserved Hsp70-interacting HPD motif impair the ability of J-domain proteins to serve as co-chaperones (35). Despite the recovery of CFTR maturation, immature CFTR continued to accumulate during the expression of Csp H43Q.

A so-called linker region lies between the N-terminal J-domain and the cysteine-rich membrane-anchoring region of Csp (36). Previous studies have implicated a functional role for the phylogenetically conserved glutamate within the linker region at position 93 in regulated exocytosis (20). Nevertheless, the results obtained from expressing Csp E93V with CFTR did not differ from those of WT Csp (Fig. 1A). Consistent with this, the Csp double mutant, H43Q/E93V, yielded results qualitatively similar to those observed with Csp H43Q. Thus, although the linker region is implicated functionally in exocytosis, we did not detect a significant role for this residue in CFTR biogenesis, either in band B production or in its maturation to band C.

Immunoprecipitation assays performed in Calu-3 airway cells were consistent with a physical interaction between Csp and CFTR in vivo (18). As shown in Fig. 1B, we examined these interactions in HEK cells co-expressing CFTR and Myc-tagged Csps. After Csp IP with anti-Myc, the pellets were blotted for CFTR to detect binding. Then the supernatants from the Csp IP were re-immunoprecipitated with CFTR antibodies and blotted for CFTR to judge the extent of CFTR depletion resulting from removal of Csp complexes and to verify the forms of CFTR present during the Csp IP.

The absence of CFTR in the 1st lane of Fig. 1B confirms the specificity of the CFTR co-immunoprecipitation with Myc-tagged Csp, as shown in the other lanes. As suggested by our earlier studies of airway cells endogenously expressing Csp, it is apparent that WT and mutant Csps interact with both immature and mature CFTR, but with one exception. The Csp H43Q/E93V double mutant did not interact significantly with mature CFTR, despite the presence of band C during the Csp IP (Fig. 1C). The absence of a band C interaction suggests that Glu-93 and the linker region may be involved in a selective association of Csp with mature CFTR, perhaps reflecting its known role in exocytosis at the plasma membrane. Thus, a CFTR interaction with the linker region of Csp may be involved in the trafficking and insertion of mature CFTR at epithelial cell apical membranes. A fraction of Csp co-localizes with CFTR at the apical domain of Calu-3 cells (18). Nevertheless, both Csp E93V and the double mutant (H43Q/E93V) interacted strongly with immature CFTR, because band B was virtually removed from the supernatant by the Csp IP (compare Fig. 1, B and C). This finding implies that the Csp-band B interaction reflects a significant role for Csp in CFTR biogenesis in the ER.

Dose-dependent Actions of WT and H43Q Csp on CFTR Biogenesis—

The data from H43Q Csp expression suggested the possibility of two separate actions of Csp in the ER handling of band B, and its subsequent maturation. To examine these effects we performed dose-response relations for WT and H43Q Csp. The data of Fig. 2A illustrate that overexpression of Csp reduced the expression of mature CFTR in a dose-dependent manner, and this was accompanied by the accumulation of core glycosylated (band B) CFTR. These effects were maximal at Csp:CFTR plasmid ratios of 1:4 or higher, and ratios between 1:4 and 1:1 were used in the studies reported here. Quantitation of the blots from all experiments (Fig. 2B) showed reciprocal changes in bands B and C, and thus a marked decrease in the C:B ratio, as the dose of Csp was increased; this persisted at least 3 days after transfection (data not shown).

To better evaluate the Csp-induced increase in immature CFTR, similar dose-response experiments were performed using Csp H43Q. As shown in Fig. 2C, H43Q Csp did not markedly alter the steady-state expression of mature CFTR; however, it increased the levels of immature CFTR in a dose-related manner. Indeed, Csp H43Q increased band B, but it failed to block CFTR maturation at doses four times higher than the WT Csp level that produced a maximal block of band C formation. Fig. 2D provides quantitation of bands B and C from all experiments with H43Q Csp. The data show that the actions of Csp on the steady-state levels of mature and immature CFTR can be separated experimentally using this Csp mutant. Thus, the buildup of band B resulting from
overexpression of WT Csp is not solely a consequence of blocking CFTR maturation; rather, it suggests another mechanism of action.

Action of Csp Is Not Associated with the Formation of Insoluble CFTR Aggregates—Wild type CFTR is inefficiently folded in cells exogenously expressing the protein (37, 38). Thus, overexpression of CFTR or treatment of cells with inhibitors of its proteasomal degradation leads to the intracellular accumulation of CFTR in detergent-insoluble aggregates that are protected from degradation (39). To determine whether the increase in immature CFTR observed with Csp co-expression represents an accumulation of insoluble CFTR, cell lysates were separated into detergent-soluble and detergent-insoluble fractions, as described under “Experimental Procedures.” As illustrated in Fig. 3, Csp expression did not increase the detergent-insoluble fraction of CFTR relative to GFP co-expressing controls. In control cells, ~17 ± 7% of total CFTR (bands B plus C) after 1% Triton extraction sedimented in the pellet with a molecular weight corresponding to immature CFTR. With Csp co-expression, only immature CFTR was present, and 7 ± 2% was found in the Triton-insoluble fraction. This finding indicates that the immature CFTR that accumulated during Csp expression remains in a soluble and presumably folded or foldable state.

We determined also whether Csp overexpression was associated with a cellular stress response, which is expected generally to increase chaperone expression and stabilize immature CFTR. As shown in the supplemental data (Fig. 1), we detected no differences in Hsp70 or Grp78 (BiP) levels in HEK cells co-expressing CFTR with Csp or GFP, whereas the expected increases in Hsp70 and Grp78 were observed in response to heat stress or blockade of N-linked glycosylation with tunicamycin. Thus, the Csp-induced failure to mature CFTR and the accumulation of band B during Csp expression were not the result of a cellular stress response.

Csp Expression Inhibits CFTR Function—Prior studies indicated that Csp co-expression decreased CFTR currents in Xenopus oocytes (18). To determine whether Csp affects CFTR channel function in mammalian cells and to examine the influence of the Csp mutants, we measured the CFTR- and cAMP-dependent anion permeability of the plasma membrane in HEK cells using the halide-sensitive indicator, SPQ, under the expression conditions used in the steady-state measurements (Fig. 1). In cells expressing CFTR alone, we noted an upward deflection in the time course of relative fluorescence intensity upon the addition of forskolin plus IBMX (Fig. 4). This response reflects dequenching of SPQ, which is associated with cAMP/protein kinase A-stimulated iodide efflux from preloaded cells (see “Experimental Procedures” for details). The typical response to cAMP agonists observed in control cells was virtually abolished in cells expressing WT Csp or Csp E93V. Fluorescence time courses similar to these were obtained from HEK cells lacking CFTR expression (data not shown), suggesting that the slight fluorescence dequenching observed with bath iodide/nitrate exchange in cells expressing WT Csp is because of a background anion conductance expressed in HEK cells (cf. Ref. 40). A significant CFTR response was absent in cells expressing Csp or E93V Csp (Fig. 4), but the response to cAMP stimulation was recovered in cells expressing Csp H43Q or the double mutant, H43Q/E93V. These findings are consistent with the effects of Csp and Csp mutants on mature CFTR protein expression, indicating that the band C formed in the presence of Csp H43Q reflects the presence of functional, mature CFTR.

Csp Leads to CFTR Accumulation in the ER—The cellular location of CFTR was evaluated by indirect immunofluorescence microscopy in an attempt to identify the compartment where immature CFTR resides in cells overexpressing Csp. Markers of the ER and Golgi were used to establish landmarks in HEK cells; the results from immunostaining of these cells are provided in Fig. 5. A diffuse plasma membrane pattern for CFTR is observed in control cells expressing CFTR plus GFP. A relatively small fraction of CFTR co-localized with calnexin, but most CFTR had progressed beyond this compartment, in accord with the relative amounts of immature and mature CFTR observed at steady state (Figs. 1A and 3). In contrast, co-expression of CFTR with Csp condensed the CFTR signal into large perinuclear accumulations that contained calnexin. This location is consistent with the absence of mature and functional CFTR under these conditions (Figs. 3 and 4). In the Csp co-expressers, CFTR was generally localized to one side of the nucleus, occupying only a fraction of the calnexin-positive compartment. Cells co-expressing CFTR and Csp showed no discernable CFTR co-localization with the Golgi marker, giantin, and no alteration in the morphology of cellular Golgi distribution (data not shown). The ER localization of CFTR in response to Csp co-expression is consistent with the biochemical and functional data shown above.

Csp Knockdown Promotes CFTR Maturation—We next determined the effect of decreasing Csp expression on steady-state CFTR levels. NIH 3T3 cells stably expressing WT CFTR were treated with dsRNA corresponding to mammalian Csp, as described under “Experimental Procedures.” Preliminary pulse-chase experiments performed in HEK cells indicated that Csp may be relatively stable; its levels decreased modestly even after a 16-h chase (data not shown). Accordingly, it was
necessary to perform repeated dsRNA transfections over a 3-day period in order to effect, on average, an ~40% reduction in endogenous Csp expression (see below). Control cells were subjected to the same transfection protocol, except for omission of the dsRNA. Immunoblot data from an experiment showing the largest decrease in endogenous Csp (~60%) are illustrated in Fig. 6, and mean data from multiple plates in three independent experiments are provided in the corresponding panels. On average, a 40% reduction in Csp resulted in a 5-fold increase in mature CFTR levels.

To verify that the broad band produced by Csp knockdown represented the complex glycosylated (mature) form of CFTR, cell lysates from dsRNA-treated cells were incubated with \( \text{N-glycosidase} \). Subsequent immunoblot of this material showed that this band condensed to a more discrete and mobile band that corresponded to the size of non-glycosylated CFTR (~150 kDa; data not shown). As shown in Fig. 6, this level of Csp knockdown did not significantly alter the expression levels of the CFTR chaperones Hsp70 and Hsp90. These results suggest that Csp functions normally to limit CFTR maturation. Together with the overexpression data of Figs. 1 and 2, the findings indicate that the extent of mature CFTR expression varies inversely with the Csp expression level.

Csp Knockdown Enhances Functional CFTR Expression—To evaluate the effect of reduced Csp on CFTR activity, we examined the effect of Csp RNA interference on the activity of CFTR using \( \text{Xenopus} \) oocytes, a common system for functional studies of CFTR. Oocytes were co-injected with a dsRNA corresponding to \( \text{Xenopus} \) Csp together with the cRNA employed to express WT CFTR. Recordings were performed 3–4 days post-injection, and cAMP-stimulated currents obtained from control and dsRNA injected oocytes are illustrated in Fig. 7. On average, the CFTR currents were roughly doubled in oocytes injected with Csp dsRNA. Immunoblots of oocyte extracts obtained from parallel preparations showed a 1.6-fold increase in CFTR expression. These data suggest that the increase in mature CFTR induced by Csp knockdown represents a functional cAMP/protein kinase A-stimulated \( \text{Cl}^- \) channel. Although it may be difficult to compare results from oocytes and mammalian cells quantitatively, the increase in CFTR protein expression with Csp knockdown appears to be greater than the increase in CFTR current. This may reflect a limit imposed by the reduced Csp level on plasma membrane CFTR expression, which would be consistent with the impairment of exocytosis observed when Csp expression is decreased (19, 36).

Hdj-2 Enhances CFTR Biogenesis—Earlier studies suggest that another J-domain containing protein, Hdj-2, cooperates with Hsp70 to facilitate early steps in CFTR biogenesis (12). To compare its effects with those of Csp, we determined the effect of Hdj-2 co-expression on steady-state levels of CFTR, analogous to the Csp studies shown in Fig.
1. The CFTR immunoblots of Fig. 8 indicate that Hdj-2 facilitates overall CFTR biogenesis, increasing the levels of both immature and mature CFTR (band B, 6-fold; band C, 2.1-fold). This effect did not require an intact HPD motif, i.e. Hdj-2 H34Q was as effective as WT Hdj-2 in promoting the production of both immature and mature CFTR. Thus, the effect of Hdj-2 on band B was similar to that of Csp; however, these J-domain containing proteins had opposite effects on the level of mature CFTR.

Hdj-2 and Csp Compete for R-domain Binding—The similar actions of Hdj-2 and Csp on the level of immature CFTR raised the possibility that the interactions of these chaperones with CFTR may be similar. To assess this possibility, we evaluated the association of in vitro translated, metabolically labeled Hdj-2 and Csp with a GST-R domain fusion protein using pull-down assays. The results are illustrated by the autoradiogram shown in Fig. 9. As observed previously for Csp (18), Hdj-2 H34Q was as effective as WT Hdj-2 in promoting the production of both immature and mature CFTR. Thus, the effect of Hdj-2 on band B was similar to that of Csp; however, these J-domain containing proteins had opposite effects on the level of mature CFTR.

FIGURE 6. Knockdown of endogenous Csp enhances mature CFTR expression. dsRNA for Csp was transfected into 3T3 cells stably expressing CFTR. Left panel, cell lysates were analyzed by immunoblotting with anti-CFTR and anti-Hsp90 antibodies together, with anti-Csp and anti-Hsp70 antibodies together. Data from the experiment giving the largest Csp knockdown (~60%) are shown. Right panel, density quantitation obtained from three independent experiments. +/- (100) indicates the change in expression induced by dsRNA treatment relative to control.

FIGURE 7. Csp RNA interference increases CFTR currents. cAMP-stimulated Cl− currents were recorded from oocytes 3 to 4 days after a single injection with 1 ng of CFTR cRNA with or without 100 ng of dsRNA corresponding to Xenopus Csp. CFTR current values were taken following plateau of the response to 10 μM forskolin plus 1 mM IBMX, at a holding potential of −60 mV; *, p < 0.05. Results from two independent experiments, each using at least 10 oocytes, demonstrated that Csp RNA interference induced a 1.6-fold increase in CFTR expression, paralleling the increase in CFTR current.

FIGURE 8. Combined effects of co-expressed Csp and Hdj-2 on steady-state CFTR. HEK cells were co-transfected with CFTR with or without Csp and/or Hdj-2 as indicated. CFTR IB was performed as for Fig. 1, and cell extracts were probed with FLAG antibody as a control for FLAG-Hdj-2 expression. Data are representative of three experiments.

Combined Effects of Hdj-2 and Csp—The similar actions of these chaperones on the production of immature CFTR, and their opposite actions on CFTR maturation, raised the question of which action predominates if they are co-expressed with CFTR. Fig. 10 provides steady-state data from HEK cells transiently transfected with varying amounts of Hdj-2 at constant Csp. The absence of band C under these conditions indicates that the negative effect of Csp on CFTR maturation predominates over the positive influence of Hdj-2 to increase band C production. Nevertheless, it is apparent from these data that the actions of
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Hdj-2 and Csp on the level of immature CFTR were additive, as indicated by densitometry of band B (Fig. 10, numbers in parentheses). Thus, Csp can block CFTR maturation even when the production of immature CFTR is augmented by Hdj-2.

Csp Suppresses NBD1 Aggregation—Previous studies have shown that Hdj-2 increases Hsp70 ATPase activity and functions as a molecular chaperone, suppressing the aggregation of NBD1 folding intermediates (12). To determine whether Csp shares this property, we first determined the ability of Csp to augment the ATPase activity of Ssa1p, the yeast homologue of Hsp70 that is 70% identical to human Hsp70. The yeast chaperone is purified in-house and is of higher purity than commercially available Hsp70; however, its ATPase activity and its stimulation by mammalian J-domain chaperones are similar (41). As shown in Fig. 11A, Ssa1p had low intrinsic ATPase activity, but in combination with Csp, ATP hydrolysis increased 6.8-fold. As anticipated, this stimulatory action of Csp was not shared by the HPD mutant Csp H43Q.

Next, we determined the capacity of Csp to serve directly as a molecular chaperone for CFTR by assaying its ability to suppress NBD1 aggregation, determined as described under “Experimental Procedures.” As illustrated in Fig. 11B, when purified NBD1 was diluted into a folding buffer at a final concentration of 0.5 μM, it underwent time-dependent aggregation. In the absence of added Hsp70 or ATP, Csp suppressed NBD1 aggregation, and this property was shared by Csp H43Q. These findings suggest that Csp acts as a direct chaperone of CFTR folding, independent of Hsp70, and they are consistent with the ability of both Csp and Csp H43Q to augment the production of immature CFTR (Figs. 1 and 2).

DISCUSSION

Our findings implicate Csp in two aspects of CFTR biogenesis, the production of immature CFTR and the subsequent progression of CFTR to Golgi glycosylation sites. Steady-state levels of immature band B CFTR increased, whereas CFTR maturation decreased, with increases in the level of Csp expression. Similarly, decreasing Csp augmented the expression of mature CFTR. These actions of Csp in protein biogenesis represent novel functions for this J-domain containing protein, which was identified initially as an essential factor in calcium-triggered neurotransmitter exocytosis at nerve terminals (19, 42). Csp is anchored on the cytoplasmic surface of synaptic vesicle membranes by multiple palmitoylation within its cysteine-rich region, and although the mechanism is not entirely clear, Csp is thought to cooperate with Hsp70 at this site to chaperone the protein interactions necessary for synaptic vesicle fusion (43, 44). Indeed, Csp is now recognized to facilitate a variety of regulated exocytic events (for review see Ref. 45). In agreement with this view, we showed previously that Csp partially localized with CFTR at the apical domain of airway epithelial cells (18), which likely reflects a role for Csp in the apical membrane insertion of CFTR.

In addition to its documented role in the late protein secretory pathway, Csp localizes with CFTR at the apical domain of airway epithelial cells (18), which likely reflects a role for Csp in the apical membrane insertion of CFTR.

Csp Facilitates the Production of Immature CFTR—Several findings indicate that Csp can function as a CFTR chaperone, similar to Hdj-2. First, WT Csp increased the steady-state level of immature CFTR in a dose-dependent manner. This action of Csp did not correlate with its ability to block the progression of CFTR to the mature form, because the H43Q mutation in the J-domain of Csp fully restored mature CFTR levels without altering the ability of Csp to promote the accumulation of immature CFTR. In these and prior studies (46), Csp H43Q had little or no effect on the ATPase activity of Hsp70 (Fig. 11A). A minor effect on Hsp70 ATPase would not account for the ability of Csp H43Q to increase CFTR band B, because the dose dependence of this effect was similar to that of WT Csp (Fig. 2, B and D).

These steady-state findings are consistent with the results of pulse-chase experiments (18). Normally during CFTR biogenesis, band C formation is detected at 30–60 min of chase, but during co-expression with Csp, no band C CFTR formation was detected up to 4 h. Labeled band B has normally disappeared by this time, but in Csp co-expressing cells, 70–80% of band B persisted up to 4 h. In the present studies, pulse-chase experiments performed with cells co-expressing CFTR and Csp H43Q showed no difference in the kinetics of mature CFTR formation relative to cells expressing CFTR alone; however, immature CFTR was stabilized, as observed with WT Csp expression (data not shown). Thus, the steady-state data (Figs. 1 and 2) reflect the action of Csp in CFTR biogenesis in the ER. The Csp-induced increase in immature CFTR was not associated with a general stress response, and the accumulated band B did not result from the formation of CFTR aggregates that would be refractory to degradation (Fig. 3).

Second, the Csp-induced increase in immature CFTR resembles the effect of Hdj-2, implicated in prior studies as a CFTR co-chaperone (12). Indeed, the co-expression of these J-domain proteins further increased the levels of immature CFTR (Fig. 10). Hdj-2 appears to stabilize NBD1, permitting its assembly with other CFTR domains (12). A CFTR fragment comprising the N terminus through the R-domain associates with about half as much Hdj-2 as one that terminates at the end of NBD1,
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suggesting that the assembly of an NBD1-R domain interface is facilitated by Hdj-2. It is likely that Csp shares this chaperone function, because it competed with Hdj-2 for binding to an isolated R-domain fusion protein (Fig. 9). Prior studies detected Csp binding to the R-domain in vitro, with an apparent dissociation constant in the low micromolar range, consistent with the affinities of other chaperone interactions (18).

Third, Csp reduced the aggregation tendency of NBD1, a property that is also shared with Hdj-2 (12). Consistent with its role as a DnaJ-like protein, we showed that Csp stimulated Hsp70 ATPase activity ~7-fold, and this stimulation was eliminated by mutation of the conserved Hsp70-interacting site (HPD) within the J-domain. This mutation interferes with the ability of Csp to stimulate Hsp70 ATPase, impairing its activity as a co-chaperone (46). Thus, it is of interest that the ability of Csp to increase band B CFTR, as well as the ability of Hdj-2 to augment both bands B and C, is not influenced by mutations that block their functional interactions with Hsp70. Consistent with these findings, DnaJ has been shown to associate with unfolded substrates, preventing their aggregation (47, 48). Other findings indicate that the chaperone and co-chaperone actions of DnaJ can be separated experimentally (49).

In addition to the chaperone action of Csp on NBD1 demonstrated in this study, Csp was shown earlier to reduce the aggregation of denatured firefly luciferase in the absence of Hsp70 (46), consistent with its direct effect on NBD1 aggregation (Fig. 11).

The finding that the HPD mutants of Hdj-2 and Csp are as effective in CFTR biogenesis as the parent proteins suggests that their inherent chaperone functions are sufficient for this purpose, and their ability to augment the ATPase cycle of Hsp70 is not necessary to support their roles in CFTR biogenesis. Consistent with this concept, Meacham et al. (12) found that Hdj-2 produced a dose-dependent suppression of NBD1 aggregation in the absence of Hsp70 and ATP. Csp shares this property (Fig. 11B). Thus, although both proteins have the ability to bind and activate Hsp70, the synthesis and folding of CFTR likely occur at rates that do not require the accelerated Hsp70 association-dissociation cycle provided by their co-chaperone functions.

Csp Regulates CFTR Exit from the ER—In addition to its action as a CFTR chaperone, our data indicate a second role for Csp in regulating the exit of CFTR from the ER. First, elevated Csp interfered with the steady-state expression of mature CFTR (Figs. 1 and 2), and pulse-chase experiments have shown that band C CFTR failed to appear when Csp was co-expressed (18). The effect of Csp on band C level was paralleled by a loss of cAMP-stimulated anion efflux (Fig. 4). Because Csp augmented the steady-state levels of immature CFTR and stabilized immature CFTR in pulse-chase studies (18), the Csp-induced inhibition of CFTR maturation is not attributable to an increase in the degradation of nascent CFTR.

Second, treatment of CFTR-expressing cells with Csp dsRNA, which reduced Csp expression ~40%, increased the steady-state level of mature CFTR ~5-fold. Similarly, the injection of dsRNA into Xenopus oocytes elicited an increase in CFTR expression and a 2-fold increase in cAMP-stimulated CFTR currents. These findings, together with the results from Csp overexpression, indicate that this J-domain containing protein normally has an inhibitory effect on the amount of CFTR that is permitted to exit from the ER. This action of Csp represents a qualitative divergence from the chaperone function of Hdj-2, which augmented the steady-state levels of mature CFTR (Fig. 8) and increased CFTR currents in Xenopus oocytes (data not shown). In addition, the negative influence of Csp on CFTR maturation was dominant over the positive action of Hdj-2 when the two proteins were co-expressed. The ability of Csp to interfere with CFTR progression requires its interaction with Hsp70, and perhaps stimulation of its ATPase activity, because the H43Q mutant abolished this action of Csp and rescued CFTR maturation.

Third, when co-expressed with Csp, CFTR accumulated in a perinuclear compartment where it co-localized with calnexin. Consistent with the biochemical and functional data, no CFTR was detected at the plasma membrane of cells co-expressing CFTR and Csp. Although the precise mechanism remains unclear, the H43Q mutant of Csp has also been shown to interfere with vesicle fusion in neurosecretory and other cell types where Csp has been implicated in regulated exocytosis. Therefore, Csp is thought to employ Hsp70 to facilitate interactions among trafficking regulatory proteins, and it may enhance their sensitivity to Ca^{2+} as a mediator of this process. In these systems, Csp overexpression blocks exocytosis (50–52). An analogous block of CFTR trafficking may account for the effect of increased Csp on CFTR maturation; however, reducing Csp augmented CFTR maturation, whereas studies of neurosecretory processes indicate that Csp knockdown has the opposite effect, impairing exocytosis (53).

These opposing actions of reduced Csp suggest that it plays different roles in exocytosis at the plasma membrane and in the early steps of CFTR biogenesis. The results obtained from expression of the Csp E93V mutant support this idea; Csp E93V inhibited insulin exocytosis (20), whereas its effect on CFTR expression did not differ from that of WT Csp (Fig. 3). This suggests that the E93V mutation may selectively affect distal trafficking events. Although the precise mechanisms remain to be defined, it is likely that the Csp-Hsp70 chaperone complex facilitates different protein interactions at the ER than those with which it interacts at the plasma membrane.

The action of Csp on CFTR maturation and the physical interaction detected between Csp and CFTR suggest that an association between these proteins may govern the transfer of newly synthesized CFTR to post-ER compartments. In general, this step is mediated by the formation of carrier vesicles that bud from the ER (54), a process that is regulated by the coat protein type II (COPII) complex (for review see Ref. 55). In addition to the biosynthetic cargo, membrane targeting and fusion components (i.e., Rab and Rab effector proteins, including soluble NSF attachment protein receptors) are also recruited to this site, and they ensure fidelity in the targeting of budded membranes to the appropriate acceptor destination.

It is now clear that coat proteins assist in sorting and selecting cargo (e.g., CFTR) for export (56–58). Whether Csp plays a role in this process is unknown; however, Csp has been implicated in GDP dissociation inhibitor-mediated Rab protein recycling at the synapse (59). Csp was found to be a component (together with Hsc70 and Hsp90) of a Rab3a regulatory complex that facilitates GDP dissociation inhibitor-mediated extraction of Rab3 from pre-synaptic acceptor membranes following vesicle fusion and returns it to synaptic donor vesicles for additional rounds of neurotransmitter release. Thus, it is possible that a similar specialized membrane-associated recycling chaperone system operates during bud formation and vesicle trafficking from the ER in cooperation with Rab1 (60, 61). If this is true, then the association of Csp with CFTR may provide a cargo-associated regulator of Rab1 recruitment that controls CFTR exit from the ER. Moreover, in its role as a CFTR chaperone, this arrangement may allow Csp to monitor the conformational properties of CFTR prior to exit to the native, folded protein.

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