Cysteine String Protein Interacts with and Modulates the Maturation of the Cystic Fibrosis Transmembrane Conductance Regulator*

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-regulated chloride channel whose phosphorylation regulates both channel gating and its trafficking at the plasma membrane. Cysteine string proteins (Csp) are J-domain-containing, membrane-associated proteins that have been functionally implicated in regulated exocytosis. Therefore, we evaluated the possibility that Csp is involved in regulated CFTR trafficking. We found Csp expressed in mammalian epithelial cell lines, several of which express CFTR. In Calu-3 airway cells, immunofluorescence colocalized Csp with calnexin in the endoplasmic reticulum and with CFTR at the apical membrane domain. CFTR co-precipitated with Csp from Calu-3 cell lysates. Csp associated with both core-glycosylated immature and fully glycosylated mature CFTRs (bands B and C); however, in relation to the endogenous levels of the B and C bands expressed in Calu-3 cells, the Csp interaction with band B predominated. In vitro protein binding assays detected physical interactions of both mammalian Csp isoforms with the CFTR R-domain and the N terminus, having submicromolar affinities. In Xenopus oocytes expressing CFTR, Csp overexpression decreased the chloride current and membrane capacitance increases evoked by cAMP stimulation and decreased the levels of CFTR protein detected by immunoblot. In mammalian cells, the steady-state expression of CFTR band C was eliminated, and pulse-chase studies showed that Csp coexpression blocked the conversion of immature to mature CFTR and stabilized band B. These results demonstrate a primary role for Csp in CFTR protein maturation. The physical interaction of this Hsc70-binding protein with immature CFTR, its localization in the endoplasmic reticulum, and the decrease in production of mature CFTR observed during Csp overexpression reflect a role for Csp in CFTR biogenesis. The documented role of Csp in regulated exocytosis, its interaction with mature CFTR, and its coexpression with CFTR at the apical membrane domain of epithelial cells may reflect also a role for Csp in regulated CFTR trafficking at the plasma membrane.

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§ The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; Cm, capacitance current; Csp, cysteine string protein; ER, endoplasmic reticulum; GST, glutathione S-transferase; GST-C, GST-CFTR C terminus; GST-N, GST-CFTR N terminus; GST-R, GST-CFTR R-domain; Hek, human embryonic kidney; Hsc, heat shock cognate; Ic, cAMP-induced membrane current; NBD, nucleotide binding domain; R-domain, regulatory domain; SNARE, soluble NSF attachment protein receptor; VSV-G, vesicular stomatitis virus glycoprotein; CHIP, C terminus of Hsc70-interacting protein.

The primary step in CFTR activation involves cAMP/protein kinase A-mediated phosphorylation of its central regulatory (R) domain, which not only permits the channel to gate, but also alters pathways for CFTR trafficking at the plasma membrane (7). In recent years, protein interactions at several CFTR domains have been implicated in channel function and trafficking. The N-terminal cytosolic tail of CFTR is a site of syntaxin 1A binding (8), and syntaxin 1A overexpression is thought to inhibit CFTR currents by reducing channel gating (9) and by interfering with regulated CFTR trafficking to the plasma membrane (10). A motif promoting CFTR endocytosis has been identified at the C terminus, and this region interacts physically with the plasma membrane endocytic adapter (AP-2) complex (11, 12). The C terminus of CFTR corresponds to a PDZ (PSD-95/Disc-large/ZO-1) binding motif, which interacts with ezrin radixin moesin-binding phosphoprotein 50 or E3KARP to bring ezrin into proximity with CFTR (13, 14). Ezrin can anchor protein kinase A at a position physiologically appropriate for CFTR phosphorylation (15, 16), and ezrin interactions with...
the cytoskeleton permit retention of CFTR at the apical membrane as a mechanism to establish its polarity in epithelial cells (17). Here, we report the interaction of CFTR with another multidomain protein, the cysteine string protein (Csp).

The Csp were first discovered in Drosophila and Torpedo (18, 19), and their expression has now been documented in a variety of tissues from several mammalian species (20, 21). Csp null mutants in Drosophila produce a temperature-sensitive block of synaptic transmission followed by paralysis and premature death. These findings demonstrate a crucial role for Csp in neurosecretion (22). In neurons, Csp is associated predominately with synaptic vesicles (19), and in secretory cells it is found in large dense core or secretory granules (23, 24). Studies of membrane dye labeling (25) and neurotransmitter release (26, 27) indicated that Csp knockout exhibit defective neurotransmitter exocytosis; presynaptic endocytosis and vesicle recycling appear to be intact. Studies in neuroendocrine (28) and endocrine cells (24) have confirmed that altered Csp expression elicits an impaired secretory phenotype.

Csp contain an N-terminal “J-domain,” a central cysteine-rich “string” region, and a phylogenetically more variable C-terminal domain. The J-domains of Csp are structurally conserved among different species, and this region provides a fingerprint of Csp as a member of the DnaJ/Hsp40 (heat shock protein) chaperone family. As observed for other chaperone proteins (29), the HPD motif of the Csp J-domain mediates its binding to and activation of the Hsc70 chaperone ATPases, which influence protein conformation/binding. Structural predictions indicate that the C terminus of Csp1 may form a coiled-coil region, and this structure may be important in view of the emerging evidence that Csp can interact with proteins involved in exocytosis, such as vesicle-associated membrane protein and syntaxin 1A and 4 (21, 30, 31). Thus, prior results suggest that Csp could link functions of the SNARE complex with those of Hsc70. The concept that assembly of the core fusion complex requires chaperone activity has been raised previously (21); however, the explicit roles of Csp and Hsc70 in the assembly or disassembly of SNARE components are not yet known. The C terminus of Csp contains the only primary structural difference that exists between the mammalian isoforms: full-length Csp1, and its C-terminal truncated variant, Csp2 (32, 33).

Csp expression in epithelial cells has not been characterized previously. We found that various mammalian epithelial cell lines express two Csp isoforms and that the expression of Csp1 predominated over that of Csp2. In Calu-3 airway epithelial cells, Csp partially colocalized with CFTR in the ER and apical plasma membrane domains. Csp coprecipitated with CFTR from Calu-3 cell lysates and bound selectively to the N terminus and R-domain of CFTR with high affinity. Coexpression of Csp isoforms with CFTR inhibited the functional responses associated with its cAMP stimulation, and exogenous Csp expression decreased the production of mature CFTR. These findings indicate that Csp is a CFTR-binding partner that can influence the biogenesis and trafficking of CFTR.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Protein A/G-agarose beads and Tq polymerase were purchased from Invitrogen. Glutathione-Sepharose 4B was purchased from Amersham Biosciences. DNA restriction endonucleases were purchased from Invitrogen. Glutathione-Sepharose 4B was purchased from Amersham Biosciences. DNA purification kits were obtained from Qiagen. Csp1 and Csp2 cDNAs were subcloned into pcDNA3 with a N-terminal Myc tag (34). Mouse hippocampus neuronal cell (SY5S) lysate, employed as a Csp protein positive control, was kindly provided by Dr. Chaohua Yan (Children’s Hospital, Pittsburgh). Protein kinase A and other reagent grade chemicals were purchased from Sigma.

**Antibodies**—Anti-Csp serum was raised in rabbits against recombinant bovine Csp1 (32). Monoclonal anti-GST and anti-Golgi 58K antibodies were purchased from Sigma. Anti-CFTR polyclonal antibodies (R3195) were generated and affinity purified as described (35). Anti-CFTR (mAb3484 and mAb3482) and anti-Csp monoclonal antibodies were obtained from Chemicon. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Sigma. monoclonal and polyclonal antibodies were obtained from Affinity Bioreagents.

**Protein Expressions**—Mouse hippocampus neuronal cell (SY5S) and mouse Csp1 (Csp1b) and Csp2 (Csp2b) cDNAs were subcloned into pcDNA3 with a N-terminal Myc tag (34). Mouse hippocampus neuronal cell (SY5S) were from New England BioLabs. DNA restriction endonucleases were purchased from Invitrogen. Mouse hippocampus neuronal cell (SY5S) were from New England BioLabs. DNA restriction endonucleases were purchased from Invitrogen. Mouse hippocampus neuronal cell (SY5S) were from New England BioLabs. DNA restriction endonucleases were purchased from Invitrogen. Mouse hippocampus neuronal cell (SY5S) were from New England BioLabs. DNA restriction endonucleases were purchased from Invitrogen. Mouse hippocampus neuronal cell (SY5S) were from New England BioLabs. DNA restriction endonucleases were purchased from Invitrogen. Mouse hippocampus neuronal cell (SY5S) were from New England BioLabs. DNA restriction endonucleases were purchased from Invitrogen. 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After 24 h, the cells were rinsed with phosphate-buffered saline and either prepared for pulse-chase assays or lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) or Nonidet P-40 lysis buffer (0.09% Nonidet 40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 10 mM NaMoO4). Samples were incubated for 2 h in the appropriate lysis buffer and centrifuged at 16,000 × g for 30 min at 4 °C. Cell extracts were utilized for immunoblot analysis (see figure legends).

**Pulse-Chase Assays**—Metabolic labeling and immunoprecipitation of CFTR were performed using methods described previously (39, 40), with modifications. Transfected HEK293 cells were starved in methionine- and cysteine-free Dulbecco’s modified Eagle’s medium for 30 min and then metabolically labeled with Redivue Pro-mix L-35S (140 μCi/ml; Amersham Biosciences) for 30 min at 37 °C. Cells were washed twice with phosphate-buffered saline and lysed immediately or incubated in complete Dulbecco’s modified Eagle’s medium for the indicated chase periods. Cell lysates were precleared with protein A-agarose, and immunoprecipitation was performed as described (40) using M3A7 anti-CFTR, kindly supplied by Dr. John R. Riordan. Immunoprecipitates were analyzed using 7% SDS-PAGE and autoradiography.

Electrophysiology—CFTR, Csp1, and Csp2 cRNAs were synthesized using the mMessage mMachine kit (Ambion) from linearized plasmids. The quality of synthesized cRNA was determined by spectrophotometry and agarose gel electrophoresis. Oocyte preparation and cRNA injection were carried out as described by Takahashi et al. (41). Expression proceeded for 2–6 days before current and capacitance recordings, which were performed as described (10, 41). All data are provided as the mean ± S.E.

**RESULTS**

**Csp Is Expressed in Epithelial Cells**—Immunoblotting was performed to determine whether Csp is expressed in several epithelial cell lines. Crude membrane extracts from the mammalian cells HEK293, Madin-Darby canine kidney (type 2), CFPAC-1, Calu-3, and T84, and from the *Xenopus* A6 cell line were resolved on 12% SDS-PAGE and probed with an anti-Csp antiserum that does not distinguish between Csp isoforms (32). As a positive control, membranes from the *SY5Y* human neuronal (hippocampal) cell line were applied to the gel. As shown in Fig. 1A, all of the mammalian epithelial cells examined expressed two Csp isoforms. The upper bands in Fig. 1A are appropriate in size for Csp1 (33–35 kDa), whereas the lower bands in mammalian cells correspond to the size expected for Csp2 (26–28 kDa); this isoform was uniformly less abundant. In contrast, A6 cells expressed only one Csp isoform. It is more closely related to Csp1 than Csp2 (33), although its molecular size is slightly smaller than the Csp1 in mammalian cells. The Csp antiserum also recognized in vitro synthesized Csp1 and Csp2 (data not shown).

To confirm the expression of Csp isoforms in these cells, reverse transcription-PCR was employed, and the results are shown in Fig. 1B. The sizes of the PCR products were as expected for the cDNAs encoding Csp1 and Csp2. These products are not derived from contaminating DNA because the primers employed span Csp exons. Csp2 protein is truncated relative to Csp1 because of the insertion of a stop codon-containing RNA splice fragment (32); this accounts for the smaller size of the Csp2 protein but its larger cDNA and PCR product (Fig. 1B). Although the PCR results are not quantitative, the Csp amplicon ratios from different cell lines generally agreed with the pattern of isoform expression observed by immunoblot. As with the protein analysis, Csp isoforms were expressed at lower levels in T84 cells. Only one isoform of Csp was detected in A6 cells, in accord with the protein data (Fig. 1A) and information in the data base. Sequencing of these PCR products confirmed the expression of Csp1 and 2 in Calu-3 cells, and of the single isoform (33) reported in *Xenopus* A6 epithelia (data not shown).

**Csp Are Partially Colocalized with CFTR at the ER and Apical Membranes**—In non-epithelial cells, Csp are membrane-associated and are localized predominantly on large dense core vesicles or synaptic-like microvesicles (22–24, 42, 43). A small fraction of Csp is located on the plasma membranes of neurons, neuroendocrine or endocrine cells. Large dense core vesicles are present in epithelial cells having defined secretory products (e.g. pancreatic acinar cells), but Csp distribution in epithelial cells has not been defined previously.

To examine the subcellular distribution of Csp in airway epithelial cells, immunofluorescent staining was performed in polarized Calu-3 cells and visualized using confocal microscopy. Sections taken at various cell depths (Fig. 2, A–D) showed a predominantly punctate and vesicular distribution pattern for Csp (green fluorescence). Csp and CFTR (red fluorescence) colocalized in the most apical sections, as indicated by the yellow staining pattern of Fig. 2A. As seen in the xz scan (Fig. 2E), a fraction of Csp colocalized with CFTR at the apical region of some cells, but the apex of other cells showed little apparent colocalization. Although Csp colocalized with CFTR in apical membrane compartments, significant fractions of both proteins showed no apparent overlap by immunofluorescence in their steady-state cellular distributions (Fig. 2). This is partly because of the presence of Csp in what appear to be large secretory granules that lack CFTR (44). These granules are likely to contain the secretory products characteristic of airway submucosal gland serous cells, which Calu-3 cells express (e.g. mucins, mucosal defense substances and protease inhibitors) (45). CFTR was not detected in their membranes (Fig. 2), in contrast to the localization of CFTR found in the mucin granules of canine gallbladder cells (46). This observation suggests that Csp may play a CFTR-independent role in secretory granule exocytosis from Calu-3 cells, and this possibility will require further study.

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**Fig. 1. Csp are expressed in various epithelial cell lines.** Panel A, homogenates (15 μg of protein) from the indicated cell lines were probed with Csp antiserum. Panel B, reverse transcription-PCR was performed as described under “Experimental Procedures.” No cDNA was added in the PCR designated Control.
Fig. 2. Colocalization of Csp with CFTR or with calnexin in Calu-3 cells. Calu-3 cells were labeled with polyclonal Csp antiserum and monoclonal CFTR R-domain or calnexin antibodies. Labeling was detected by Alexa488 (green indicating Csp) or Alexa568 (red indicating CFTR in panels A–E or calnexin in panel F). Serial images in the xy plane were collected every 0.5 μm through the specimen depth; xy images (panels A–D), taken from cell apex to base, are separated by 2.5 μm. Panel E shows the xz scan of the region indicated in panel A. Scale bar, 10 μm. Images were collected by confocal microscopy and analyzed using Metamorph (Universal Imaging) software.

Interaction of CFTR with Csp—To determine whether a physical interaction between Csp and CFTR could be detected in vivo, we performed coimmunoprecipitation experiments (Fig. 3). In these studies, the initial precipitation was performed with either Csp antiserum or with antibodies against the first nucleotide binding domain (NBD1) of CFTR. As shown in Fig. 3A (lane 3), Csp antibody coimmunoprecipitated the mature, glycosylated CFTR (band C, upper) as well as the immature, core-glycosylated CFTR (band B, lower) from Calu-3 cell extracts. As a negative control, no CFTR was precipitated by a nonimmune IgG (lane 2). As a control for the coimmunoprecipitation, we performed direct CFTR immunoblotting on the same Calu-3 membrane preparations (Fig. 3A, lane 1). Both CFTR bands were present, but the ratio of the B and C bands found in the Csp immunoprecipitation differs from that constitutively expressed. Together with the results from the cellular localization experiments, the immunoprecipitation results suggest that in the steady state, Csp interacts with both the immature, core-glycosylated form of CFTR in the ER and with mature, fully glycosylated CFTR at the apical membrane domain. As shown in Fig. 3B, a CFTR NBD1 antibody coprecipitated Csp from Calu-3 membranes (see positive control, Calu-3 cell lysate, lane 1). An N-terminal CFTR antibody did not precipitate significant Csp (data not shown). The lower band denoted Csp in panel B likely represents deacylated Csp, produced by the coimmunoprecipitation conditions, which include overnight incubation with detergent, as noted by others (47). This lower band is not observed under the conditions used for immunoblotting (Fig. 1). As a negative control, Csp did not coprecipitate with a GST antibody (Fig. 3B, lane 2). The absence of Csp coprecipitation by anti-CFTR-N could represent a problem of antibody affinity, or it may reflect impaired antibody access to its epitope at the N terminus of CFTR.

Csp Interacts with the R-domain and N Terminus of CFTR—The results from coimmunoprecipitation experiments indicate that Csp and CFTR interact in vivo. To evaluate CFTR domain interactions with Csp, we performed pull-down experiments in which GST or GST-CFTR fusion proteins were bound to gluta-
thione-Sepharose beads and incubated with Calu-3 or CFTR-expressing HEK293 membrane extracts. Protein complexes were washed, resolved on SDS-PAGE, and immunoblotting was performed using Csp antibodies. To verify that comparable amounts of GST or GST fusion proteins were applied in these assays, the Western blots were subsequently stained with Coomassie Blue. The similar amounts of fusion proteins present are illustrated in Fig. 4A. As seen in Fig. 4B, both the CFTR R-domain and the N terminus showed significant interactions with Csp. A weak interaction between CFTR-C and Csp was detected here, but this was not observed consistently. No binding was detected between Csp and GST, GST-CFTR-N random, or GST-syntaxin 1A in these pull-down assays. The interaction of Csp with CFTR-N is consistent with the finding from the above coimmunoprecipitation studies and suggests that the interaction with CFTR-N could be masked by Csp binding.

As indicated above, two Csp isoforms have been identified in various mammalian species (21); Csp2 is truncated by 31 amino acids at the C terminus relative to Csp1. The Csp isoform(s) that interact with CFTR or CFTR domains in Calu-3 cells (Figs. 3 and 4) cannot be determined because of the lack of isoform specific Csp antibodies. Csp1 may be the more physiologically significant isoform because of its predominant expression and the presence of only Csp1 in some species (Fig. 1). In further characterizing CFTR-Csp interactions, we evaluated the influence of both Csp1 and Csp2; however, in the discussion following we refer to CFTR interactions with Csp, without attempting to discriminate whether they are mediated by a specific Csp isoform.

**Relative Affinities of Csp for CFTR Domains**—To determine the relative binding properties of Csp1 and 2 for CFTR, we performed binding assays with in vitro synthesized 35S-labeled Csp1 and Csp2. Csp interactions were documented using autoradiography (Fig. 5A) and liquid scintillation counting (Fig. 5B). As indicated by the data from both assays, the interaction of Csp1 with the CFTR R-domain (lane 2) was stronger than that with CFTR-N (lane 4), consistent with the Calu-3 cell pull-down experiments of Fig. 4. In the present experiments, a weak interaction between CFTR-NBD1 and Csp1 (lane 5) was also detected relative to the GST negative control (lane 1). The data for CFTR-C (lane 3) were not different from the GST control. Unlike Csp1, Csp2 showed significant but low level binding to CFTR-NBD1 relative to the GST control. The lower panel provides pooled data from three independent experiments.

To determine the relative binding affinities of Csp with the CFTR R-domain and N terminus, we performed in vitro competitive binding assays as described under “Experimental Procedures.” In these pull-down experiments, 35S-labeled Csp1 or 2 was mixed with various amounts of unlabeled Csp before mixing with immobilized CFTR domains. As shown in Fig. 6A, unlabeled Csp1 competed with labeled Csp1 for binding to CFTR-R, such that 1 μM unlabeled Csp1 eliminated 51% of
The influence of Csp coexpression on the steady-state levels of CFTR expressed in mammalian cells was determined by cotransfecting HEK293 cells with plasmids encoding Csp and CFTR. CFTR levels were determined by immunoblot after 24 h. The Csp cDNAs used for transfection included an N-terminal Myc-tag, consistent with the A6 cell line data shown in Fig. 1A. The exogenous expression of Myc-tagged Csp1 and 2 are shown in lanes 2 and 3 of the upper panel. We do not know whether the endogenous Xenopus Csp, which most closely resembles mammalian Csp1 (42), contributes to differences in the extent of inhibition by these isoforms or whether the different potencies relate to their structural features.

**Csp Inhibits Functional CFTR Responses by Decreasing Steady-state CFTR Expression**—To determine whether the observed inhibition of CFTR current and capacitance changes was the result of a Csp-induced change in CFTR expression levels, we immunoprecipitated CFTR from oocytes expressing CFTR alone or CFTR plus Csp1, Csp2 or the Csp2 double mutant, H43Q/E93V. CFTR was precipitated from oocyte membrane extracts, resolved on SDS-PAGE, and immunoblotted (see “Experimental Procedures”). CFTR currents recorded from these populations of oocytes at the time of harvest are also provided. As seen in Fig. 8 (upper panel), the coexpression of Csp1 or Csp2 produced significant decreases in the level of CFTR expression (to 52 and 36% of the control level by densitometry, lanes 3 and 4, respectively). As a negative control, CFTR was not detected in noninjected oocytes (lane 1). As a positive control, we blotted CFTR expressed transiently in HEK293 cells (lane 6). The apparent molecular mass of CFTR B and C bands expressed in oocytes was similar to those observed in HEK cells (a longer exposure demonstrated migration of HEK cell band B at the same location as observed in oocytes). An increase in CFTR expression was observed with the Csp2 double mutant, but this did not result in currents greater than those of the CFTR control. This degree of CFTR expression increase was not observed in all experiments, and it was not present in mammalian cells (see below). The basis of this stimulatory effect of Csp2 double mutant has not been pursued. As to the double mutant mutation sites, His-43 is a conserved site in the J-domain of Csp which is essential for Hsc70 binding and ATP hydrolysis (48), and Glu-93 is located in the Csp linker region and has been shown previously to abrogate the inhibitory effect of Csp overexpression on insulin release from pancreatic cells (24). These results indicate that the effects of exogenously expressed Csp on CFTR activity are specific for known Csp functional domains, and they suggest that the inhibition of CFTR functional response is due primarily to a Csp-induced reduction in CFTR protein expression.

**Csp Coexpression Inhibits CFTR Biogenesis**—The influence of Csp coexpression in the steady-state levels of CFTR expressed in mammalian cells was determined by cotransfecting HEK293 cells with plasmids encoding Csp and CFTR. CFTR levels were determined by immunoblot after 24 h. The Csp cDNAs used for transfection included an N-terminal Myc-tag, permitting exogenous Csp expression to be detected using an anti-Myc antibody (lower panel, Fig. 9A). As shown in the top panel of Fig. 9A, the coexpression of either Csp1 or Csp2 with CFTR (lanes 3 and 4) eliminated the expression of mature, glycosylated CFTR (band C) and produced an accumulation of immature CFTR (band B). This finding suggests that Csp over-
expression interferes with the processing of CFTR to its mature form and that it stabilizes the immature form of CFTR. The effect of exogenous Csp on CFTR biogenesis is specific because the Csp2 double mutant (H43V/E93Q) eliminated its inhibitory effect on CFTR maturation. In other experiments, a similar result was obtained with the Csp2 single mutant, H43Q, implying that a functional J-domain is critical for the inhibitory effect of Csp overexpression. The enhancement of CFTR protein level by the Csp2 double mutant found in oocytes was not observed in mammalian cells.

Because Csp is an Hsc70-binding protein, we examined the levels of Hsc70 by immunoblot under these conditions. A similar result was obtained with the Csp2 single mutant, H43Q, implying that a functional J-domain is critical for the inhibitory effect of Csp overexpression. The enhancement of CFTR protein level by the Csp2 double mutant found in oocytes was not observed in mammalian cells.

Expression of Csp alone did not affect the levels of Hsc70, as shown in the middle panel of Fig. 9A. The effect of Csp1 or 2 on CFTR expression was not associated with a change in Hsc70 levels when this Hsc70-binding protein was expressed. Thus, the failure of cells to express mature CFTR is not caused by a change in the expression of this important CFTR chaperone. In addition, the specificity of the Csp effect for CFTR biogenesis was determined by coexpressing Csp with the VSV-G. Csp coexpression did not affect the production of the mature VSV-G, in experiments performed in a manner similar to those with CFTR (Fig. 9B). This result suggests that the effect of expressed Csp is specific for maturation of CFTR.

The effect of Csp on the steady-state expression of mature CFTR was supported by the results of pulse-chase assays. As shown in Fig. 10, Csp1 coexpression in HEK293 cells blocked the conversion of immature CFTR to mature CFTR. As in prior studies, ~30% of wild-type CFTR matures to the fully
glycosylated band C protein (lower panels); however, no band C was detected in Csp-coexpressing cells. In addition, the half-life of immature CFTR was prolonged, consistent with the accumulation of band B observed in the steady-state data (Fig. 9A).

Similar effects on the biogenesis of mature CFTR resulted from the coexpression of Csp2 (data not shown). With Csp coexpression, the rise in band B production at chase periods of 30 and 60 min is probably attributed to the lack of cold methionine and cysteine in the chase media; nevertheless, the experiments with CFTR alone were performed identically. The results indicate that Csp interferes with CFTR biogenesis when Csp is overexpressed. The implications of these findings for a physiological role of Csp in the production of CFTR will be discussed below.

**DISCUSSION**

The results of this study show that Csp is expressed in a variety of epithelial cell lines, several of which are models for CFTR-mediated chloride secretion. These cells expressed predominantly Csp1, as observed previously in neurons and secretory cells (21) (e.g., see SY5Y result, Fig. 1). Immunofluorescent labeling showed a membrane-limited distribution of Csp in ER and apical membrane compartments. Significant amounts of CFTR coprecipitated with Csp from Calu-3 cell lysates; in protein binding assays, Csp associated with both the CFTR R-domain and the N terminus. The overexpression of Csp decreased CFTR biogenesis, and it correspondingly reduced the functional response of CFTR to cAMP stimulation. These results indicate that Csp interacts physically and functionally with CFTR in epithelial cells. This is the first report of an action of Csp on protein maturation, as discussed below.

**Role of Csp in Regulated Exocytosis—**Csp is expected to influence CFTR directly by virtue of its localization at the cytosolic surfaces of intracellular membranes that contain CFTR. A defining feature of the Csp family is their central cysteine-rich (string) domain, where palmitoylation at multiple sites

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**Fig. 8.** Csp coexpression reduces oocyte CFTR protein levels. cRNAs encoding CFTR (1 ng) and Csp isoforms (5 ng) were injected into Xenopus oocytes as indicated. Upper panel, results from CFTR immunoprecipitation. Immunoprecipitation was performed with anti-CFTR monoclonal M3A7, and the immunoblot was probed with anti-CFTR polyclonal R3195 antibodies. Lane 1, un.injected control; lane 2, CFTR alone; lane 3, CFTR plus Csp1; lane 4, CFTR plus Csp2; lane 5, CFTR plus Csp2 double mutant; lane 6, HEK293 cells transiently expressing CFTR used as a positive control. Lower panel, cAMP-stimulated chloride currents recorded from the oocyte populations used for the immunoprecipitations. Data are from five pooled oocytes for immunoprecipitation (50 μg of protein/lane) and four or five oocytes for ΔIc/l under each condition. Recordings/immunoprecipitations were performed 4 days after injection.

**Fig. 9.** Effect of Csp on CFTR biogenesis. Panel A, HEK293 cells were transfected with CFTR (lane 2), CFTR plus Myc-Csp1 (lane 3), CFTR plus Myc-Csp2 (lane 4), and CFTR plus Myc-Csp2 double mutant (lane 5). Lane 1, nontransfected control. After 24 h, 50 μg of cell extract was resolved by 5% or 15% mini-SDS-PAGE, and blots were probed with polyclonal anti-CFTR (R3195, 1:2,000), monoclonal anti-Hsc70 (1:2,000), or monoclonal anti-c-Myc (9E10, 1:2,000, Sigma) antibodies. B and C indicate the positions of immature and mature CFTR, respectively. Panel B, HEK293 cells were transfected with VSV-G (lane 1) or VSV-G plus Csp1 (lane 2); blots were probed with anti-VSV-G polyclonal (1:5,000). Experiments were otherwise performed as in panel A.
permits Csp to associate with membrane lipid (49). Immunofluorescence demonstrated that Csp is distributed in membrane-limited compartments, including the apical membrane domain, in Calu-3 cells (Fig. 2). The CspS were discovered in Drosophila, and from their cellular distribution and knockout studies, a role for these proteins in neurotransmitter secretion became apparent (18). Studies in neuroendocrine (28) and endocrine cells (24) have confirmed the role of Csp in regulated exocytosis. Several studies have implicated a regulated exocytic event in the control of plasma membrane CFTR channel density (50). In Xenopus oocytes expressing CFTR, cAMP stimulation enhances the chloride current and elicits increases in membrane capacitance and cell surface CFTR labeling, consistent with cAMP-regulated insertion of CFTR into the plasma membrane (10, 41, 51). In adenovirus-transduced Madin-Darby canine kidney cells, CFTR was distributed in both intracellular and plasma membrane compartments under basal conditions and was recruited to the plasma membrane during cAMP stimulation (52). The partial colocalization of Csp with CFTR at the apical membrane domain of Calu-3 cells (Fig. 2E) and its interaction with mature (band C) CFTR (Fig. 3A) is consistent with the concept that this protein may participate in post-Golgi CFTR trafficking events. It will be interesting to determine whether Csp interacts with CFTR in a regulated trafficking compartment because this location would be consonant with the documented role of Csp in regulated exocytosis. To perform such studies, however, it will be necessary to obviate the influence of Csp on CFTR maturation.

A Role for Csp in CFTR Biogenesis—We found Csp to be localized in airway epithelial cells in a compartment marked by the ER resident protein, calnexin. Coimmunoprecipitation experiments showed that more of the core glycosylated, immature CFTR was associated with Csp than was the mature, fully glycosylated protein, particularly in proportion to the steady-state expression levels of the mature and immature forms of CFTR. In addition, Csp coexpression reduced the production of mature CFTR, and this effect of Csp appears to require a functional J-domain. These results imply that Csp and CFTR interact during CFTR biogenesis in the ER. As a member of the DnaJ family of proteins, Csp could play a role in CFTR processing via its association with Hsc70. The DnaJ proteins can serve as co-chaperones that recruit Hsc70 ATPases to specific substrates, where they activate the ATPase activity of the chaperone to modulate protein conformation (folding) or protein-protein interactions (4, 29). Hsc70 has long been recognized as a CFTR chaperone, which coprecipitates with CFTR in the presence of ATP (39). Hsc70 has been shown to assist in preventing the aggregation of NBD1, the domain in which the common CFTR folding mutant, ΔF508, is located (4, 53, 54). Normally, wild-type CFTR will be released from chaperones as it achieves its native conformation, but Hsc70 is thought to remain attached to misfolded CFTR where it can recruit the ubiquitin ligase, CHIP, to facilitate proteosome-mediated CFTR degradation (40, 55).

Csp is a J-domain protein that interacts with Hsc70 and enhances its ATPase activity (56). The interaction of Csp with Hsc70 and CFTR is likely to affect CFTR processing in one of two ways. First, Csp may be a CFTR co-chaperone that stabilizes intermediate forms of CFTR to promote their folding and maturation. This model suggests that Csp contributes positively to CFTR maturation, similar to the Hsc70 co-chaperone, Hdj-2 (53). Second, Csp interactions with nonfolded CFTR intermediates may contribute to the degradation of nascent CFTR. This model suggests that Csp may contribute to CFTR degradation, similar to the Hsc70-interacting protein, CHIP (see below). Either model could explain how Csp overexpression reduces the steady-state expression levels of mature CFTR in mammalian cells or oocytes and therefore, the cAMP-stimulated CFTR currents (Figs. 8 and 9). In the first model, Csp overexpression causes a prolonged association of Hsc70 with CFTR, which can target CFTR for degradation (55). Hsc70 has been shown to promote the ubiquitination and degradation of several cellular proteins (57). For example, overexpression of Hsc70 leads to ubiquitination and degradation of apolipoprotein B (58). Thus, a prolonged Csp-mediated association of CFTR with Hsc70, because of Csp overexpression, could lead to CFTR degradation, despite the possibility that the physiological role of Csp is to facilitate CFTR biogenesis.

The second model is trivial, in that augmenting the level of a protein involved in CFTR degradation would reduce CFTR expression. Illustrative of this model is the Hsc70-binding protein CHIP, a ubiquitin ligase whose overexpression promotes proteosome-mediated CFTR degradation (40). CHIP appears to recognize slowly folding or misfolded forms of CFTR and promotes their destruction by binding to CFTR-associated Hsc70. CHIP then targets CFTR to ubiquitin-conjugating enzymes. The finding that proteosome inhibitors fail to promote trafficking of class II CFTR mutants suggests that mechanisms exist.

**FIG. 10.** Pulse-chase analysis of CFTR biogenesis in HEK293 cells, performed as described under “Experimental Procedures.” TTF indicates the transient transfection conditions. [35S]Labeled CFTR was immunoprecipitated using 3 μg of monoclonal CFTR antibody M3A7. Samples were resolved on SDS-PAGE, and CFTR was revealed by autoradiography. The intensities of CFTR C and B forms at the indicated times were quantified by densitometry and are expressed as a percentage of CFTR B form at chase time = 0 (taken as 100%).
for irreversible targeting of protein to intracellular degradation processes before their cellular destruction. CHIP appears to be a component of this pathway; in principle, Csp could function in this manner as well.

Several findings favor the co-chaperone model. First, the predominant interaction of Csp with CFTR band B in Calu-3 cells (Fig. 3) suggests a physiological role for Csp in the maturation of CFTR because the majority of the CFTR expressed in these cells is the fully glycosylated mature form. Second, the direct interaction of Csp with CFTR subdomains in the absence of Hsc70 (Figs. 4 and 5) suggests that Csp may localize Hsc70 at specific sites to facilitate CFTR folding. In contrast to CHIP (40), Csp does not require Hsc70 for its interaction with CFTR. Third, Csp overexpression stabilized CFTR band B, which is contrary to the result expected for a protein that promotes degradation. Finally, previous data indicate a positive role for Csp in protein folding processes, also favoring the first model of Csp interaction. As is true of other DnaJ proteins, Csp can bind to unfolded proteins and prevent their aggregation (49). This activity is synergistic with the similar action of Hsc70. To-
