Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 9480–9484, October 1993
Cell Biology

The common variant of cystic fibrosis transmembrane conductance regulator is recognized by hsp70 and degraded in a pre-Golgi nonlysosomal compartment

(recombinant adenoviruses/molecular chaperones/immunoelectron microscopy)

YIPING YANG*†, SONIA JANICH*, JONATHAN A. COHN‡, AND JAMES M. WILSON*†§

*Departments of Internal Medicine and Biological Chemistry, and Howard Hughes Medical Institute, University of Michigan Medical School, Ann Arbor, MI 48109; and ‡Department of Medicine, Duke University, Durham, NC 27710

Communicated by Michael S. Brown, July 9, 1993

ABSTRACT **The most common cause of cystic fibrosis is deletion of Phe-508 (ΔF508) from the cystic fibrosis transmembrane conductance regulator (CFTR). Previous studies have suggested that ΔF508 CFTR is an unstable protein that retains a pattern of glycosylation specific to the endoplasmic reticulum. This report examines the mechanism responsible for the mislocalization of ΔF508 CFTR in a human cystic fibrosis epithelial cell line overexpressing recombinant CFTR by virtue of adenovirus-mediated gene transfer. Immunoelectron microscopy confirmed that wild-type CFTR is delivered to the plasma membrane of these cells and that ΔF508 CFTR is retained in the endoplasmic reticulum. Pulse–chase studies showed that newly synthesized CFTR complexes with the chaperone hsp70. The wild-type protein dissociates from hsp70 before its transport to the Golgi, and the protein is subsequently degraded in lysosomes. By contrast, the complex formed between ΔF508 CFTR and hsp70 is retained in the endoplasmic reticulum and ΔF508 CFTR is rapidly degraded in a pre-Golgi nonlysosomal compartment. Thus, hsp70 discriminates between the normal form of CFTR and the form of the protein that most commonly causes cystic fibrosis (ΔF508). These findings clarify the mechanism by which mutation causing ΔF508 affects the intracellular trafficking of CFTR and suggest another function for hsp70 in ensuring quality control during the biosynthesis of plasma-membrane proteins.**

Cystic fibrosis (CF) is a common lethal inherited disease caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR, refs. 1–3). A variety of studies have indicated that CFTR is localized to the apical surface of epithelial cells (4, 5), where it functions as a cAMP-regulated Cl^- channel (6, 7). It is likely, however, that CFTR is involved in other cellular functions (8, 9). Over 220 disease-causing mutations have been identified in the CF gene (10), the most common of which is the deletion of 3 nt that results in loss of Phe-508 (ΔF508, ref. 3). An important clue to the molecular pathogenesis of CF was provided by Cheng *et al.* (11), who studied the biosynthesis of CFTR in transfected COS cells. They demonstrated that ΔF508 CFTR retains a pattern of glycosylation specific to the endoplasmic reticulum (ER) and proposed that this mutation destabilizes the protein, leading to its retention in the ER and its accelerated degradation. Additional studies indicate that processing of ΔF508 CFTR is sensitive to temperature, further supporting defective protein folding in the pathogenesis of this genotype (12, 13). These studies suggest several essential questions that need to be tested: Is ΔF508 CFTR localized to the ER? Does ΔF508 CFTR exhibit accelerated degradation and does this degradation occur in compartments separate from the site of wild-type (WT) CFTR degradation? What proteins are involved in the folding of CFTR and selective recognition of the ΔF508 mutation?

CF is one of several inherited diseases in which mutant proteins malfunction because they exhibit defective intracellular trafficking. Other diseases sharing this mechanism include the most common familial forms of hypercholesterolemia and emphysema, resulting from mutations of low density lipoprotein receptor and α-1-antitrypsin, respectively (14, 15). In each disease, available evidence indicates that mutant proteins are retained in the ER. Within ER, chaperone proteins normally facilitate folding and assembly of newly synthesized proteins (16, 17). Furthermore, it seems plausible that chaperones may play a role in intracellular surveillance whereby mutated or misfolded proteins are recognized and degraded (18, 19). This report presents evidence implicating the chaperone hsp70 in the folding and intracellular trafficking of the common CFTR variant ΔF508.

MATERIALS AND METHODS

Recombinant Adenoviruses. Ad.CB-CFTR WT and ΔF508 viruses, both of which are based on adenovirus 5 (Ad5), were produced by homologous recombination in 293 cells (20). The vectors used to produce the CFTR viruses called pAd.CB-CFTR WT and ΔF508 contain the following relevant sequences (described in 5′ to 3′ sequence, description of sequences in refs. 2, 21, and 22): 5′ ITR (inverted terminal repeat) of Ad5 spanning 0–1 map units; *Tha* I–*Sna*BI fragment (−521 to −339 nt) of the immediate-early gene of cytomegalovirus; promoter from the chicken β-actin gene spanning *Xho* I at nt −275 to *Mbo* I at nt +1; human CFTR cDNA (WT or ΔF508) containing 60 nt of 5′ untranslated sequence, the entire coding sequence, and 80 nt of 3′ untranslated sequence; simian virus 40 late gene polyadenylylation signal (nt 2533–2729); 9.2–16.1 map units of Ad5; and plasmid sequences. These vectors were linearized and cotransfected into 293 cells with *Cla* I-digested dl7001 viral genome, which is an Ad5/Ad2 recombinant that has been deleted from most E3 sequences spanning 78.4–86 map units (from William Wold, Washington University). Recombinants were isolated, subjected to two rounds of plaque purification, and expanded in 293 cells, as described (20). Virus was purified from 293 cell lysates by two rounds of CsCl density centrifugation and gel filtration through Sephadex G-50 in phosphate-buffered saline (PBS) and stored at 4°C for imme-

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Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; ER, endoplasmic reticulum; ΔF508, deletion of Phe-508; WT, wild type; Ad5, adenovirus 5.

†Current address: Institute for Human Gene Therapy, University of Pennsylvania Medical Center, and The Wistar Institute, Room 204, 36th and Spruce Streets, Philadelphia, PA 19104–4268.
§To whom reprint requests should be addressed.

diate use or, with addition of 10% (vol/vol) glycerol, at −70°C before use. The concentration of virus was determined by measuring absorbance at 260 nm, and the titer of virus was estimated by plaque assay with 293 cells. Presence of replication-competent virus was detected by infecting HeLa cells with a portion of the viral stock and evaluating the cells for cytopathic effects after extended cultivation. None of the stocks of virus used in the experiments contained detectable replication-competent virus. CFPAC cells, passaged 1:5 onto 10-cm^2 plates, were exposed to diluted stocks of Ad.CB-CFTR (WT and ΔF508) with multiplicity of infection (m.o.i.) equal to 500. Two days later, the infected cells were used for immunoelectron microscopy and immunoprecipitation studies.

Immunoelectron Microscopy. Immunoperoxidase staining for EM was done as described with some modifications (23). CFPAC cells were fixed with 2% formaldehyde/10 mM sodium metaperiodate/75 mM lysine/37.5 mM sodium phosphate, pH 5.8, for 1 hr and permeabilized with 0.06% saponin/10% normal goat serum/PBS (GS/PBS) for 30 min. Cells were incubated for 1 hr with polyclonal antibody to CFTR at 5 μg/ml (24) in 2% GS/PBS containing 0.01% saponin, and the primary antibody was detected by indirect immunoperoxidase staining using the Vectastain ABC kit (Vector Laboratories). Cells were postfixed with 1.5% glutaraldehyde/5% sucrose in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min and washed three times with 7.5% sucrose/50 mM Tris, pH 7.4 (buffer A). Peroxidase activity was visualized by incubation in 0.2% diaminobenzidine in buffer A/0.03% H_2O_2 for 5 min, after which they were washed with buffer A and further fixed for 45 min with 1% OsO_4/0.1 M sodium cacodylate buffer, reduced with 1% potassium ferrocyanide. Cells were dehydrated by sequential washings in 70, 95, and 100% ethanol, followed by two washes with 100% propylene oxide. Dehydrated cells were then placed in 50% Epon/propylene oxide for 1 hr and embedded in 100% Epon overnight at 60°C. Thin Epon-embedded sections were cut with an RMC MT-7 microtome and stained with lead citrate. Micrographs were taken on a Phillips CM-10 EM.

Metabolic Labeling and Immunoprecipitation of CFTR. Cells were labeled for 30 min at 37°C with [^{35}S]methionine at 200 μCi/ml (Amersham; 1 Ci = 37 GBq) in methionine-free medium and chased for various periods of time in complete medium. For regular immunoprecipitation, labeled cells were lysed for 20 min at 4°C in RIPA buffer (1% Triton X-100/1% sodium deoxycholate/0.1% SDS/PBS, pH 7.4) with addition of protease inhibitors as described (24); for immunoprecipitation under native conditions, labeled cells were lysed for 15 min at 4°C with 0.1% Triton X-100 containing apyrase at 10 units/ml of PBS, and the lysates were then adjusted to RIPA buffer conditions (25, 26). After clarification at 10,000 × *g* for 10 min at 4°C, lysates were precleared with protein A-agarose

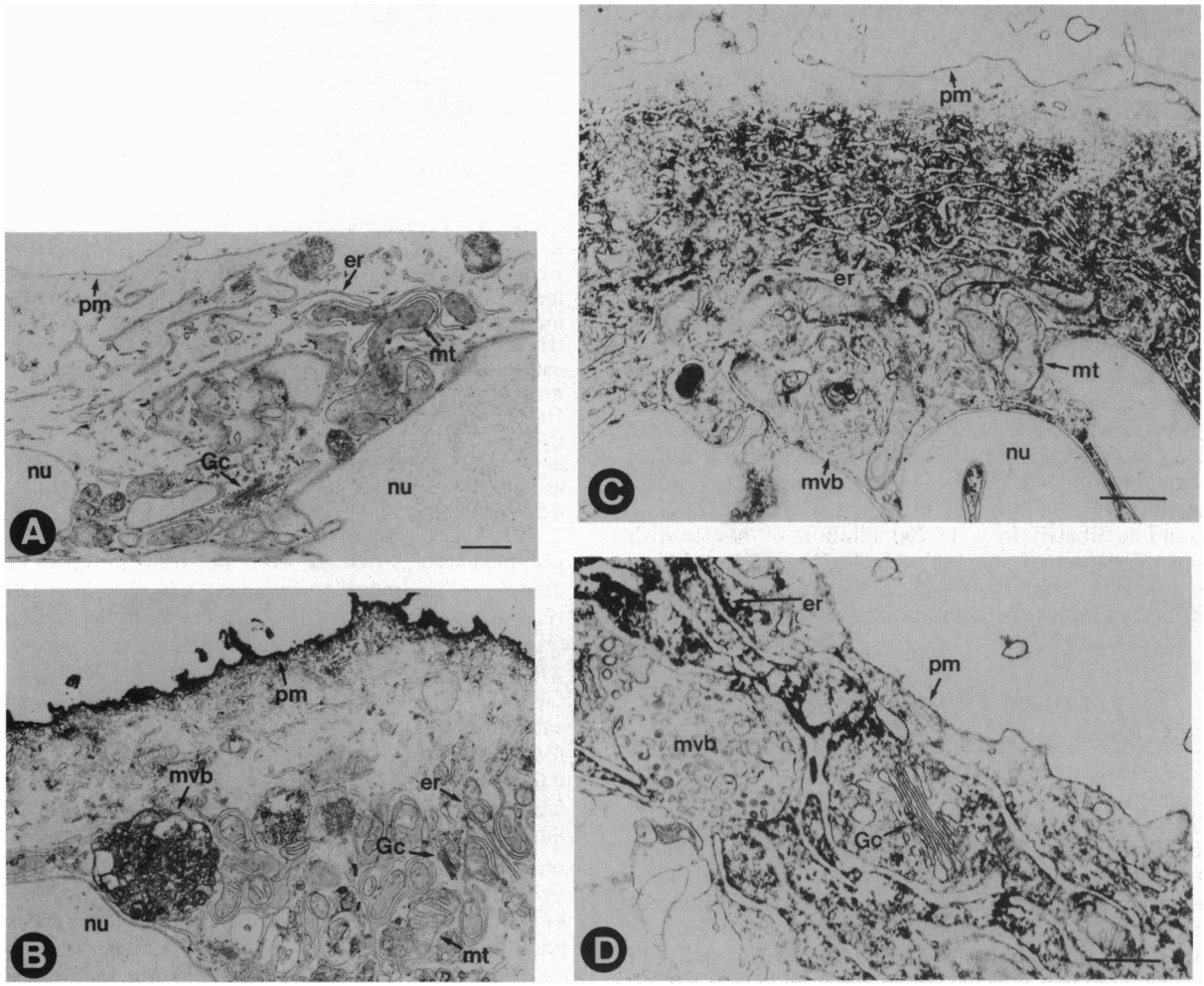

FIG. 1. Ultrastructural localization of WT and ΔF508 CFTR in CFPAC cells. Cells were fixed, permeabilized with saponin, and labeled with CFTR antibody followed by biotinylated goat anti-rabbit IgG and ABC method. After reaction with diaminobenzidine and H_2O_2, the cells were prepared for EM. EM photographs are shown of mock-infected (*A*), Ad.CB-CFTR WT-infected (*B*), and Ad.CB-CFTR ΔF508-infected (*C* and *D*) CFPAC cells. nu, Nucleus; er, ER; mt, mitochondrium; Gc, Golgi complex; mvb, multivesicular body; pm, plasma membrane. [Bars = 1 μm (*A* and *B*); 1 μm (*C*); and 0.5 μm (*D*).]

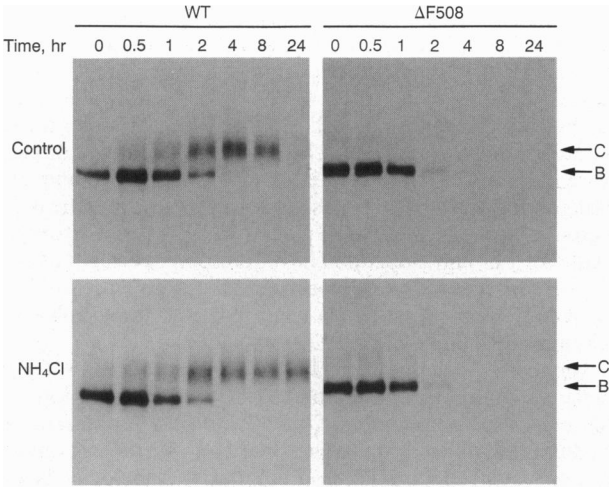

FIG. 2. Fates of CFTR proteins in CFPAC cells: Differential effect of NH_4Cl. CFPAC cells expressing WT and ΔF508 CFTR were treated with (NH_4Cl) or without (Control) NH_4Cl (50 mM) for 1 hr before pulse-labeling with [^{35}S]methionine at 200 μCi/ml. Cells were chased in complete medium for the indicated times (0.5, 1, 2, 4, 8, and 24 hr) at 37°C with or without NH_4Cl. Labeled cells were lysed, and CFTR protein was isolated by immunoprecipitation. Immunoprecipitates were analyzed by 7% SDS/PAGE under reducing conditions. To quantify radioactivity incorporated into protein, band intensities on fluorography were determined by scanning densitometry. The mature form (labeled C) and endoglycosidase H-sensitive form (labeled B) are indicated.

for 30 min at 4°C and incubated with antibody either to CFTR, hsp70 (hsp72/73, an affinity-purified monoclonal antibody from Boehringer Mannheim), or the ER luminal protein BiP (17, 23) and protein A-agarose for 1 hr at 4°C. Immunoprecipitates were washed three times in RIPA buffer to remove nonspecifically adsorbed proteins. Bound antigen was eluted from the beads by incubating in SDS sample buffer for 10 min at 37°C and analyzed by 7% SDS/PAGE.

Immunoprecipitates from unlabeled cell lysates incubated with hsp70 and BiP antibodies were subjected to immunoblot analysis using antibody to CFTR. Briefly, immunoprecipitates were resolved on 7% SDS/PAGE and transferred to a nitrocellulose filter. The filter was quenched with 5% nonfat milk/PBS buffer, pH 7.4/0.1% Triton X-100 (quench buffer) and incubated with antibody to CFTR in quench buffer. After primary-antibody reaction, the filter was rinsed with quench buffer and incubated in a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit IgG (The Jackson Laboratory) for 1 hr. The filter was rinsed as described and exposed to enhanced chemiluminescence detection solution (ECL, Amersham).

RESULTS AND DISCUSSION

The intracellular localization and metabolism of CFTR was studied by using adenovirus-transduced populations of CFPAC cells expressing high levels of WT and ΔF508 CFTR. The CFPAC cell line, which was derived from a pancreatic adenocarcinoma of a CF patient homozygous for ΔF508, expresses little if any endogenous CFTR (27, 28). Recombinant adenoviruses deleted of E1 and E3 sequences were used to efficiently transduce WT and ΔF508 CFTR alleles into the CFPAC cell line. Analysis of the infected cells revealed high levels of recombinant-derived CFTR RNA and protein (data not shown).

The critical first step in delineating the molecular pathogenesis of the ΔF508 genotype was to precisely define the intracellular localization of the mutant protein. Indirect evidence, based on patterns of glycosylation, suggested in

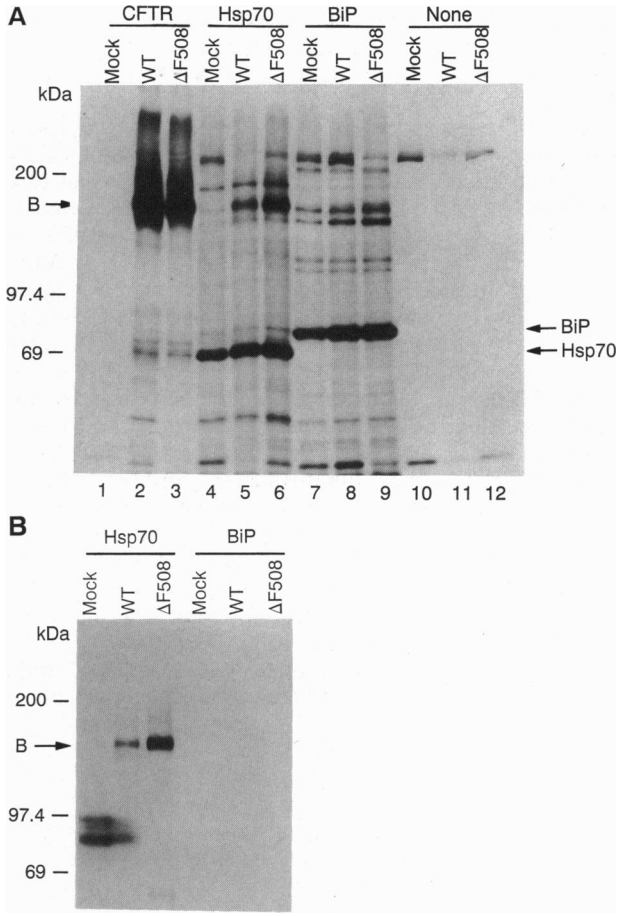

FIG. 3. Interaction of hsp70 with newly synthesized CFTR proteins. (*A*) Mock, WT, and ΔF508 CFTR-infected CFPAC cells were labeled for 30 min with [^{35}S]methionine. Cells were lysed immediately under native conditions, as described, and lysates were immunoprecipitated with antibody either to CFTR, hsp72/73 (Hsp70), BiP, or without antibody (None). The precipitates were analyzed by SDS/PAGE under reducing conditions. Band B and molecular size markers are indicated at left in kDa, and BiP and Hsp70 proteins are indicated at right. (*B*) Mock, WT, and ΔF508 CFTR-infected CFPAC cells were lysed as described above, immunoprecipitated with antibody to either hsp72/73 (Hsp70) or BiP, and analyzed by immunoblot using antibody to CFTR. Molecular size markers and band B are indicated at left.

previous studies that ΔF508 is not transported to the Golgi complex (11). Immunoperoxidase EM was done to localize recombinant-derived CFTR proteins in the adenovirus-transduced CFPAC cells (Fig. 1). Cells were permeabilized with saponin and incubated with the polyclonal antibody to CFTR protein (topologically, its epitope is on the cytosolic side of the membrane structures). No peroxidase staining was found in mock-infected CFPAC cells (Fig. 1*A*) or in WT CFTR-infected cells incubated with an irrelevant antibody or with the CFTR antibody preabsorbed to excess CFTR C-terminal peptide (data not shown). The peroxidase staining was predominantly detected in the plasma membrane and multivesicular bodies in cells expressing WT CFTR (Fig. 1*B*); an occasional cell also demonstrated peroxidase staining in additional structures such as the ER cisternae, nuclear envelope, and Golgi complex (data not shown). No staining of mitochondria was observed. Cells expressing ΔF508 CFTR exhibited a qualitatively different distribution of reaction product. The peroxidase staining was restricted to the ER and nuclear envelope (Fig. 1*C*); no staining of the Golgi complex, plasma membrane, multivesicular bodies, or mito-

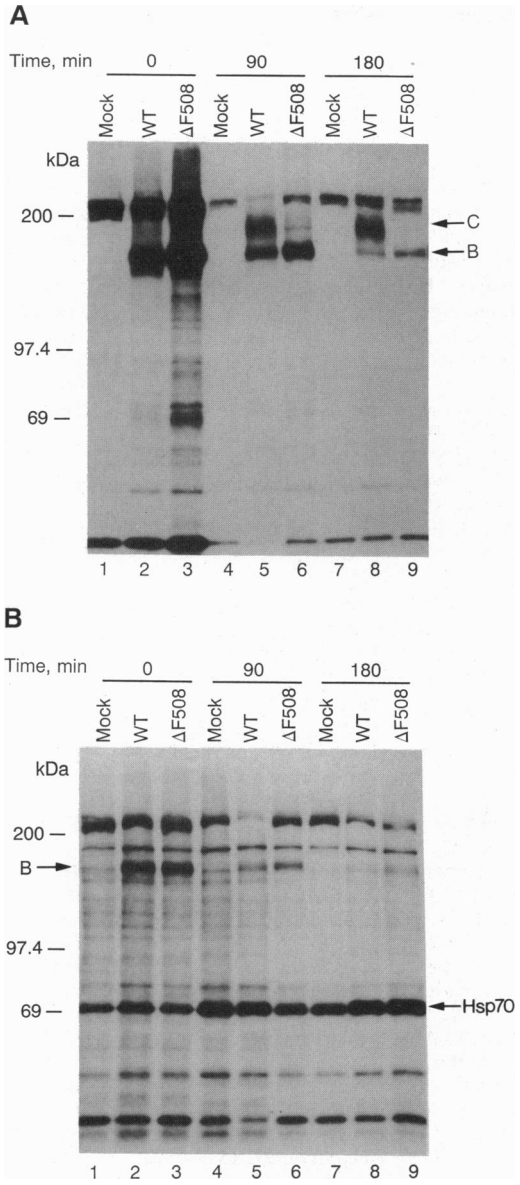

FIG. 4. Prolonged association of ΔF508 CFTR with hsp70. (*A*) Mock, WT, and ΔF508 CFTR-infected CFPAC cells were labeled for 30 min with [^{35}S]methionine and chased for 0, 90, and 180 min. Cell lysates were prepared as described for Fig. 4, immunoprecipitated with anti-CFTR, and analyzed by SDS/PAGE under reducing conditions. Molecular size markers are indicated at left, and bands B and C are indicated at right. (*B*) The same lysates described in *A* were immunoprecipitated with anti-hsp72/73 and analyzed by SDS/PAGE under reducing conditions. Molecular mass markers and band B are indicated at left, and Hsp70 is indicated at right.

chondria was detected (Fig. 1 *C* and *D*). These data confirm that ΔF508 CFTR is retained in ER of CFPAC cells.

Biosynthesis of CFTR protein was studied in cells metabolically labeled with [^{35}S]methionine. After a chase in medium free of radioactivity, cell lysates were prepared, and CFTR was isolated by immunoprecipitation (Fig. 2). Consistent with previous studies, WT CFTR was initially synthesized as an endoglycosidase H-sensitive protein (called band B in ref. 11), which was converted to an apparently larger endoglycosidase H resistant band representing the fully mature form of CFTR (called band C in ref. 11). In contrast, ΔF508 CFTR was found exclusively as a single endoglycosidase H-sensitive form (i.e., band B) that was rapidly degraded without conversion to band C (Fig. 2). To study the mechanisms of CFTR degradation, pulse–chase experiments were done in the presence of the lysosomal inhibitor NH_4Cl, a weak base that raises intravesicular pH and thereby inactivates lysosomal enzymes (Fig. 2, ref. 29). Degradation of WT CFTR was substantially inhibited by NH_4Cl treatment (i.e., differences at the 24-hr time point suggest the half-life was increased by at least 3-fold in the presence of NH_4Cl), whereas degradation of ΔF508 CFTR was unaffected. These results point to lysosomes as the primary site of degradation of WT CFTR and a pre-Golgi nonlysosomal compartment as the site of ΔF508 degradation.

In an attempt to determine the molecular mechanism(s) responsible for the different intracellular processing of normal and variant CFTR, experiments were done to identify chaperone proteins that complex with CFTR. Molecular chaperones are proteins distributed in various compartments of the cell that mediate the proper folding and assembly of newly synthesized proteins (16, 17, 25). The stress 70 protein family of chaperones was studied, including an ER luminal protein called BiP and the cytosolic proteins hsp72/73 (subsequently referred to as hsp70). Adenovirus-transduced CFPAC cells were labeled with [^{35}S]methionine for 30 min, and proteins were immunoprecipitated by using antibodies to either CFTR, hsp70, or BiP (Fig. 3*A*). No CFTR, hsp70, or BiP was detected when antibody was deleted (Fig. 3*A*, lanes 10–12). Immunoprecipitation with anti-CFTR antibody produced a mixture of bands, the most intense of which was band B of CFTR (Fig. 3*A*, lanes 2 and 3). A weak band corresponding to hsp70 was also detected in these lanes. The specificity of precipitation with CFTR antibody was confirmed by evaluation of lysates from mock-infected cells that revealed no detectable bands (Fig. 3*A*, lane 1). Lysates were also immunoprecipitated with antibodies to hsp70 and BiP. Immunoprecipitation with anti-hsp70 revealed a major band of 70 kDa in each sample (representing hsp70), as well as a band of 140 kDa in lysates of cells expressing WT CFTR (Fig. 3*A*, lane 5) and ΔF508 CFTR (Fig. 3*A*, lane 6) that was not detected in lysates of mock-infected cells (Fig. 3*A*, lane 4). The 140-kDa band precipitated by anti-hsp70 was identified as band B of CFTR by subjecting hsp70 immunoprecipitates to immunoblot analysis using anti-CFTR antibody (Fig. 3*B*). Similar analyses done with anti-BiP detected precipitation of the 78-kDa BiP protein with no coprecipitation of CFTR (Fig. 3 *A*, lanes 7–9, and *B*). These results suggest that newly synthesized CFTR complexes with hsp70 in ER.

We next sought to evaluate the role of hsp70 in the differential intracellular processing of CFTR. Cells were labeled with [^{35}S]methionine for 30 min and chased for 0, 90, and 180 min, and the lysates were prepared and subjected to immunoprecipitation with either anti-CFTR (Fig. 4*A*) or anti-hsp70 (Fig. 4*B*). Analyses of lysates from cells chased for 0 min were identical to those shown in Fig. 3: anti-hsp70 coprecipitated hsp70 with band B of both WT and ΔF508 CFTR (see Fig. 4*B*, lanes 2 and 3, respectively). ΔF508-expressing cells chased for 90 and 180 min demonstrated a diminution in band B when precipitated with anti-CFTR antibody (Fig. 4*A*, lanes 3, 6, and 9) that retained its association with hsp70 (Fig. 4*B*, lanes 3, 6, and 9). WT CFTR-expressing cells demonstrated a different pattern. As shown in Fig. 2, lysates prepared after a 90- and 180-min chase and precipitated with anti-CFTR demonstrated the conversion of most of band B to band C (Fig. 4*A*, lanes 2, 5, and 8). Incubation of these lysates with anti-hsp70 led to coprecipitation of band B but not of band C (Fig. 4*B*, lanes 2, 5, and 8). These results indicate that interaction of most of WT CFTR with hsp70 is transient. In other words, WT CFTR dissociates from hsp70 as it is transported from the ER to the Golgi, whereas ΔF508 CFTR forms a stable complex with hsp70 and is degraded in a pre-Golgi nonlysosomal compartment.

These findings raise important questions concerning the role of chaperone proteins in the selective recognition and degradation of variant proteins. The most obvious data in the literature are with BiP, a stress 70 chaperone protein localized to the lumen of the ER that facilitates assembly of secretory and plasma membrane proteins and prevents aggregation and precipitation of misfolded proteins (17–19). For CFTR, hydropathy analyses suggest that the largest extracellular domain of the protein contains only 31 amino acids. This topology predicts that BiP will probably not play major roles during folding of normal CFTR or the recognition of misfolded variant forms of the protein because most of the CFTR molecule is in a compartment separate from BiP. This prediction is consistent with data in this report indicating that CFTR does not form a detectable complex with BiP.

Other important members of the stress 70 family are the cytosolically localized chaperones hsp70. These chaperones associate with nascent polypeptides facilitating both translocation and proper folding (25, 30). In addition, cytosolic hsp70s are involved in transport of proteins to subcellular organelles and disassembly of clathrin-coated vesicles (31, 32). Potential roles hsp70 may play in the quality-control mechanisms of the cell are less well-defined than they are for BiP. Hsp70 exhibits increased binding to proteins synthesized during metabolic stress and the mutant transforming p53 protein, suggesting a role in protein stabilization (26, 33). However, to our knowledge, hsp70 has never been implicated in the biogenesis of a plasma-membrane protein or in the mislocalization of a mutant protein before this study.

Our studies indicate that hsp70 binds to both WT and ΔF508 CFTR in the ER. The complex of hsp70 with WT CFTR dissociates as CFTR is transported to the Golgi compartment. By contrast, the complex of hsp70 with ΔF508 is retained in the ER where the variant form of CFTR is degraded. The role hsp70 plays in determining the ultimate fate of WT vs. ΔF508 CFTR is a matter of speculation. It is possible that the ΔF508 mutation perturbs protein folding, which leads to increased binding of hsp70. This mutation is located in a cytoplasmic domain of CFTR, indicating that it is directly accessible to the cytosolic hsp70. This model suggests that differential binding of hsp70 to CFTR may be related to the block in transport from the ER to the Golgi compartment.

These findings begin to define the molecular events responsible for mislocalization of ΔF508 CFTR and suggest potential strategies for treating CF on the basis of disruption of the CFTR–hsp70 complex. Furthermore, the interaction between hsp70 and CFTR suggests another function for hsp70 as a chaperone that ensures quality control during the biosynthesis of plasma-membrane proteins.

We thank David Bole for the gift of the monoclonal antibody against BiP. We thank Steve Ernst, David Bole, Mike Welsh, and Francis Collins for advice. This work was supported by the Howard Hughes Medical Institute and grants from the National Institutes of Health and the Cystic Fibrosis Foundation.

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