A foldable CFTRΔF508 biogenic intermediate accumulates upon inhibition of the Hsc70–CHIP E3 ubiquitin ligase

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CFTRΔF508 exhibits a correctable protein-folding defect that leads to its misfolding and premature degradation, which is the cause of cystic fibrosis (CF). Herein we report on the characterization of the CFTRΔF508 biogenic intermediate that is selected for proteasomal degradation and identification of cellular components that polyubiquitinate CFTRΔF508. Nonubiquitinated CFTRΔF508 accumulates in a kinetically trapped, but folding competent conformation, that is maintained in a soluble state by cytosolic Hsc70. Ubiquitination of Hsc70-bound CFTRΔF508 requires CHIP, a U box containing cytosolic cochaperone. CHIP is demonstrated to function as a scaffold that nucleates the formation of a multisubunit E3 ubiquitin ligase whose reconstituted activity toward CFTR is dependent upon Hdj2, Hsc70, and the E2 UbcH5a. Inactivation of the Hsc70–CHIP E3 leads CFTRΔF508 to accumulate in a nonaggregated state, which upon lowering of cell growth temperatures, can fold and reach the cell surface. Inhibition of CFTRΔF508 ubiquitination can increase its cell surface expression and may provide an approach to treat CF.

Introduction

CFTR is a Cl^- ion channel that is localized to the apical surface of epithelial cells that line lung airways and glands (Riordan et al., 1989). Cystic fibrosis (CF) is an autosomal recessive disease and most CF patients inherit at least one CFTRΔF508 mutant allele. CFTRΔF508 lacks F508 in nucleotide binding domain (NBD) I and has a temperature-sensitive folding defect, which causes its premature degradation by the ubiquitin proteasome system (Denning et al., 1992; Jensen et al., 1995a; Ward et al., 1995). Loss of CFTR function at the cell surface leads to mortality in CF patients because of altered hydration of airway epithelia and persistent lung infections (Welsh and Smith, 1993).

An interesting feature of CFTRΔF508 is that when growth conditions are altered, it can fold, escape the endoplasmic reticulum quality control system (ERQC), and function at the cell surface (Denning et al., 1992; Brown et al., 1996). Thus, the development of agents that promote the folding or block the degradation of nascent CFTRΔF508 has the potential to provide a therapeutic avenue for the treatment of CF. Rational design of such therapeutics requires a basic understanding of the mechanism for CFTRΔF508 misfolding and the identification of the ERQC machinery that selects CFTRΔF508 for degradation.

CFTR is a 1,480 residue glycomembrane protein whose proper function requires the formation of intramolecular contacts between its two transmembrane domains, two cytoplasmic NBDs, and a regulatory domain (R-domain; Xiong et al., 1997). CFTR and CFTRΔF508 biogenesis is inefficient with 60–75% of CFTR and nearly 99% of CFTRΔF508 being degraded before reaching the cell surface (Ward and Kopito, 1994). Thus, the kinetics of CFTR and CFTRΔF508 folding are slow and nonnative biogenic intermediates of each appear to be actively selected for degradation by ERQC systems. The nature of the CFTRΔF508 biogenic intermediate that is selected for degradation is unknown, but CFTR and CFTRΔF508 appear to assume similar conformations at early stages of assembly (Zhang et al., 1998). Because F508 is located on the surface of NBD1, CFTRΔF508 assembly is proposed to go off pathway at a late stage (Lewis et al., 2004). The inability of CFTRΔF508 to fold properly makes it prone to aggregation (Qu and Thomas, 1996) and causes some of its degradation intermediates to accumulate in detergent-insoluble aggregates (Ward et al., 1995).

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Abbreviations used in this paper: ApoB48, apolipoprotein B48; BFA, brefeldin A; CF, cystic fibrosis; ERQC, endoplasmic reticulum quality control system; NBD, nucleotide binding domain; TCRα, T cell receptor α subunit; TPR, tetratricopeptide repeat motifs.

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The mechanism by which the cell monitors the conformational state of CFTRΔF508 and makes protein triage decisions that determine its fate are unclear. Current models suggest that the folded state of CFTR and CFTRΔF508 is surveyed by the cytosolic chaperone Hsc70 and Hsp90 (Yang et al., 1993; Loo et al., 1998; Zhang et al., 2001). The ER luminal lectin-binding chaperone calnexin can form a complex with the immaturely glycosylated B form of CFTRΔF508, but a direct role for calnexin in CFTR folding and/or degradation has not been demonstrated (Pind et al., 1994; Okiyoneda et al., 2004).

Cytosolic Hsc70 functions in complexes with either folding or degradatory cochaperones to mediate steps in CFTR folding and degradation (Cyr et al., 2002). The type I Hsp40 cochaperone Hdj-2 is farnesylated and localized to the cytoplasmic face of the ER where it recruits Hsc70 to bind ribosome associated CFTR to promote early stages of its assembly (Meacham et al., 1999). Hsc70 can also interact with the degradatory cochaperone CHIP to facilitate the proteasomal degradation of ER forms of CFTR and CFTRΔF508 (Meacham et al., 2001). Thus, cochaperones of Hsc70 play a central role in determining the fate of nascent CFTR.

Mechanistic insight into how CHIP functions as a degradatory cochaperone is now required to understand how the cell makes protein triage decisions for CFTRΔF508. CHIP contains three NH_2-terminal tetratricopeptide repeat motifs (TPR) that bind the COOH terminus of Hsc70 (Scheufler et al., 2000) and a noncanonical RING domain, termed the U box, which promotes interactions with E2 enzymes (Ballinger et al., 1999). The TPR repeat motifs and U box are essential for CHIP to mediate CFTR and CFTRΔF508 degradation (Meacham et al., 2001). Thus, CHIP is proposed to interact with Hsc70 to form an E3 ubiquitin ligase that targets CFTRΔF508 for proteasomal degradation. However, CHIP can function in a U box independent manner to alter the Hsc70 polypeptide binding and release cycle and negatively influence the folding of some client proteins (Ballinger et al., 1999; Dai et al., 2003). Thus, it is plausible that CHIP targets CFTR and CFTRΔF508 for degradation by acting as an antifolding factor.

Herein, we reconstituted CFTR ubiquitination with purified components and demonstrated that Hsc70 and CHIP functionally interact with the E2 UbcH5a to form a multisubunit E3 ubiquitin ligase that polyubiquitinates the cytosolic subdomains of CFTR. Inactivation of the Hsc70–CHIP E3 in cultured cells drove the accumulation of a nonaggregated and foldable CFTRΔF508 degradation intermediate. These data support a model for quality control in which aggregation of CFTRΔF508 is suppressed by Hsc70 and Hdj-2. However, when CFTRΔF508 folding intermediates become kinetically trapped in a nonnative state, CHIP attracts UbcH5a to Hsc70–CFTRΔF508 complexes and thereby facilitates CFTRΔF508 ubiquitination and degradation.

Results

Purified CHIP functions as an Hdj-2-, Hsc70-, and UbcH5a-dependent E3 ubiquitin ligase to polyubiquitinate CFTR

To investigate whether CHIP targets CFTR for degradation by acting as a U box–dependent E3 ubiquitin ligase we sought to reconstitute CFTR ubiquitination. To accomplish this goal CHIP, Hsc70, Hdj-2, and the E2 UbcH5a were overexpressed and purified from *Escherichia coli*. UbcH5a was chosen as the E2 for these experiments because it cooperates with CHIP to promote polyubiquitin chain assembly (Jiang et al., 2001). The CFTR substrate used for this ubiquitination reaction was gst–NBD1–R (Naren et al., 1999), which contains binding sites for Hdj-2 and Hsc70 (Meacham et al., 1999).

Gst–NBD1–R was incubated with different combinations of chaperones and ubiquitination enzymes and its ubiquitination was monitored by analyzing the retardation of its mobility on SDS-PAGE gels (Fig. 1 A). E1 and UbcH5a were unable to facilitate the ubiquitination of gst–NBD1–R and the addition of CHIP lead to the formation of a small quantity of ubiquitinated gst–NBD1–R. The presence of Hsc70 or Hdj-2 in combination with CHIP further stimulated the ubiquitination by UbcH5a, but the efficiency of this reaction remained low with $<3\%$ of gst–NBD1–R being detected as a mono-, di-, or triubiquitinated species. However, $>85\%$ of gst–NBD1–R was polyubiquitinated when E1, UbcH5a, Hsc70, Hdj-2, and CHIP were jointly present. Mutation of conserved residues in CHIPs U box, H260A and P269A, greatly reduced gst–NBD1–R polyubiquitination. In addition, mutation of K30A in the TPR repeat domain reduced CHIPs ubiquitination activity to levels observed when CHIP and Hdj-2 were present, but Hsc70 was omitted (Fig. 1, A and C). CHIP cooperates with UbcH5a, Hsc70, and Hdj-2 in a TPR repeat and U box–dependent manner to facilitate CFTR polyubiquitination.

U box proteins function as E3 enzymes to stimulate E2 dependent polyubiquitin chain assembly and in some cases act as E4 enzymes to elongate ubiquitin chains on monoubiquitinated proteins (Koegl et al., 1999; Cyr et al., 2002). The data presented indicates that CHIP acts in concert with Hsc70 and Hdj-2 as an E3 to stimulate UbcH5a ubiquitination activity. This conclusion is supported by the observation that UbcH5a does not monoubiquitinate, and CHIP cooperates with Hsc70 and Hdj-2 to stimulate the rate at which UbcH5a converts gst–NBD1–R into a polyubiquitinated species (Fig. S1).

To evaluate the specificity of CHIPs ubiquitination activity, its ability to cooperate with the mammalian E2s Ubc6 and Ubc7 (Tiwari and Weissman, 2000; Lenk et al., 2002) to facilitate gst–NBD1–R ubiquitination was examined (Fig. 1 B). Ubc6 and Ubc7 were used because these E2s mediate the ubiquitination of ERAD substrates in cultured cells and their purified forms function in vitro to facilitate polyubiquitin chain assembly (Tiwari and Weissman, 2001). In reaction cocktails that contained E1, UbcH5a, Hsc70, Hdj2, and CHIP, gst–NBD1–R was converted to a polyubiquitinated species in time-dependent fashion (Fig. 1 B). However, after 180 min of incubation a polyubiquitinated form of gst–NBD1–R was not detected when the purified E2 domain of Ubc6 or Ubc7 was substituted for UbcH5a in otherwise identical reaction cocktails. Thus, CHIP appears to specifically recognize UbcH5a.

Next, the ability of UbcH5a C85A to facilitate the polyubiquitination of gst–NBD1–R was determined (Fig. 1 D). UbcH5a C85A is a form of UbcH5a in which its active site

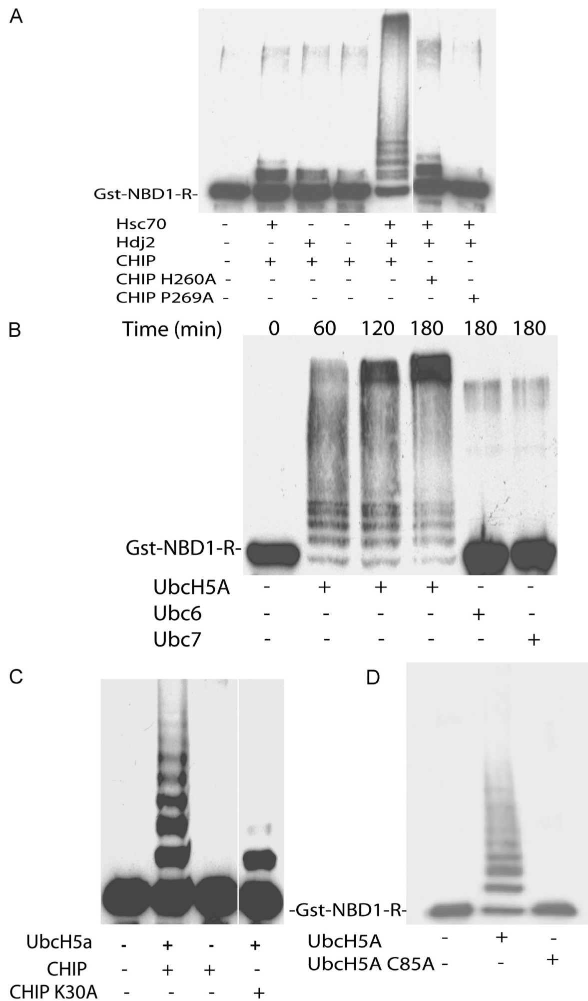

Figure 1. **Reconstitution of CFTR ubiquitination.** (A) Ubiquitination of gst–NBD1–R. (B) Ubc6 and Ubc7 cannot cooperate with Hsc70 and CHIP to ubiquitinate gst–NBD1–R. (C) CHIP K30A is defective in polyubiquitination. (D) UbcH5a C85A cannot ubiquitinate Gst–NBD–R. Ubiquitination of Gst–NBD–R (1 μM) was performed at 37°C and where indicated the following components were present: UbcH5a (4 μM), UbcH5a C85A (4 μM), Ubc6 (4 μM), Ubc7 (4 μM), CHIP (3 μM), CHIP K30A (3 μM), Hsc70 (2 μM), and Hdj2 (4 μM). In 1B-D Hsc70 and Hdj-2 were in all reactions except for lane 1. Incubations in A, C, and D were for 2 h. Ubiquitination of Gst–NBD–R was determined by Western blot with α-R domain antibody. In the x-ray films shown in A–D the band that corresponds to Gst–NBD–R is overexposed to allow for the visualization of its ubiquitinated forms. Quantitation of shorter exposures of these x-ray films indicates that >85% of Gst–NBD–R is polyubiquitinated by the joint action of UbcH5a, Hsc70, Hdj-2, and CHIP (A, lane 5). When Hsc70 or Hdj-2 were absent (A, lanes 2 and 3), UbcH5a and CHIP ubiquitinated <3% of Gst–NBD–R.

cysteine that accepts a charged ubiquitin from E1 has been mutated. UbcH5a C85A is predicted to interact with the U box on CHIP, but should not facilitate polyubiquitin chain assembly because it cannot be conjugated to ubiquitin. This supposition was found to be true as UbcH5a C85A was unable to cooperate with CHIP to promote the polyubiquitination of gst–NBD1–R. Hence, the Hsc70- and Hdj-2–dependent reconstitution of CFTR polyubiquitination requires the action of the CHIP TPR and U box domains and the active site cysteine of UbcH5a.

Overexpression of UbcH5a C85A in cultured cells inhibits CFTR and CFTRΔF508 degradation

To access whether or not UbcH5a is an in vivo component of Hsc70–CHIP E3, the effect that UbcH5a and UbcH5a C85A overexpression in HEK293 cells had on CFTR biogenesis was determined (Fig. 2 A). In addition, we compared the effect of UbcH5a or UbcH5a C85A overexpression on CFTR biogenesis to that of wild-type and dominant negative mutant forms of the human E2s Ubc6 and Ubc7 (Fig. 2 A). Elevation of UbcH5a levels caused a decrease in the accumulation of the immaturely glycosylated ER localized B form and the maturely glycosylated plasma membrane localized C form of CFTR. In contrast, overexpression of UbcH5a C85A led to a severalfold increase in the steady-state level of the B and C form of CFTR. Ubc6 and Ubc6 C91S overexpression were also observed to influence CFTR expression levels, but the effect that Ubc6 C91S had on the accumulation of the B form of CFTR was modest when compared with results obtained with UbcH5a C85A. On the other hand, Ubc7 and Ubc7 C89S overexpression did not cause a detectable change in the steady-state level of CFTR and CFTRΔF508. Because Ubc6 does not appear to interact with CHIP (Fig. 1), the effect that its overexpression has on CFTR biogenesis appears to result from its ability to function with additional quality control factors that monitor the folded state of CFTR (Gnann et al., 2004).

To explore the reason why overexpression of UbcH5a C85A drove the B form of CFTR to accumulate, its effect on the kinetics of CFTR and CFTRΔF508 degradation was determined (Fig. 2, B and C). In pulse-chase experiments, UbcH5a C85A overexpression increased the quantity of the B form of CFTR and CFTRΔF508 present at the beginning of the chase period ~1.5–3-fold (Fig. 2, legend). The UbcH5a C85A induced increase in CFTR and CFTRΔF508 levels appeared to result from the impaired degradation of nascent CFTR and CFTRΔ508 because the half-life of the B form of each was increased two to threefold (Fig. 2, D and E). In addition, the maturation efficiency of CFTR in the presence or absence of UbcH5a C85A was around 25% (Fig. 2 D). Thus, inhibition of the Hsc70–CHIP E3 complex via UbcH5a C85A overexpression inhibits CFTR degradation, but does not interfere with CFTR folding efficiency.

To gain additional support for the interpretation that CHIP and UbcH5a interact with each other to select nascent CFTR and CFTRΔF508 for degradation we demonstrated that the coexpression of CHIP and UbcH5a enhanced the effect that individual forms of each had on CFTRΔF508 degradation (Fig. 2 D). When CHIP or UbcH5a was overexpressed alone, steady-state levels of CFTRΔF508 were reduced to 30 and 41% of control levels, respectively. Yet, when CHIP and UbcH5a were coexpressed, CFTRΔF508 accumulation was reduced by >98%.

Overexpression of Ubch5a C85A does not generally inhibit ERAD

To ascertain whether or not UbcH5a C85A overexpression generally inhibited ERAD or specifically blocked CFTR degradation, its effect on the degradation of the T cell receptor α

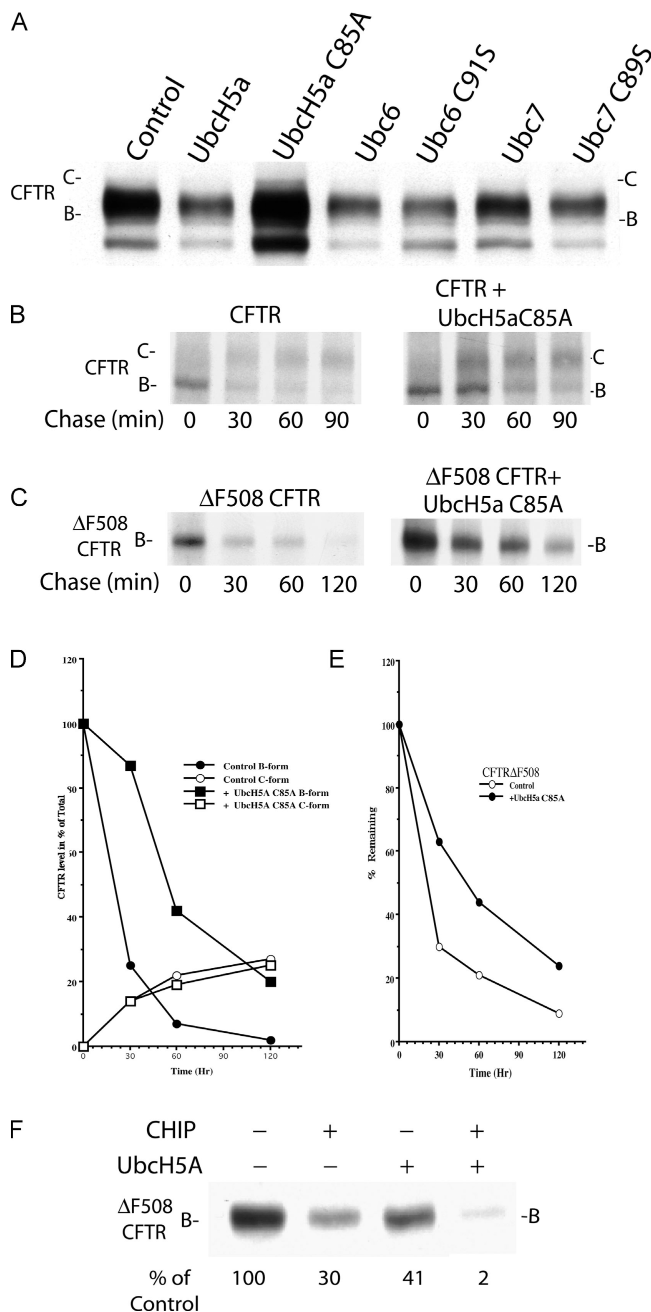

Figure 2. **Degradation of CFTR and CFTRΔF508 is inhibited by overexpression of UbcH5A C85A.** (A) Western blot analysis of CFTR levels upon overexpression of the E2s UbcH5A, Ubc6, and Ubc7. HEK293 cells were transfected transiently with expression plasmids that encode the indicated proteins. Total cell extracts were prepared 24 h after transfection in SDS-PAGE sample buffer. Nitrocellulose was probed with α-CFTR and developed. The immaturely glycosylated ER localized B form and maturely glycosylated plasma membrane associated C form of CFTR are denoted as B and C, respectively. (B and C) UbcH5A C85A overexpression slows the rate of CFTR and CFTRΔF508 degradation. Cells were labeled for 20 min with ^{35}S-translabel and a chase period was initiated by the addition of cycloheximide. At the indicated times, ^{35}S-CFTR or ^{35}S-CFTRΔF508 was immunoprecipitated from cell extracts. CFTR or CFTRΔF508 isolated in this manner was detected by SDS-PAGE and fluorography. (D and E) Graphs illustrate the processing efficiency and half-life of CFTR and CFTRΔF508. Relative CFTR and CFTRΔF508 levels were quantitated by laser densitometry of the x-ray films shown in B and C, respectively. Values were normalized to the quantity of the B form of CFTR and CFTRΔF508 present at t = 0 under the indicated experimental condition levels. t = 0 values for the B form of CFTR were 1.1 and 1.8 OD, in the absence and presence of UbcH5a C85A, respectively. t = 0 values for the B form of CFTRΔF508 were 2.3 and 7.0 OD, in the absence and presence of UbcH5a C85A, respectively. (F) CHIP and UbcH5a jointly reduce levels of CFTRΔF508 in Western blots.

subunit (TCRα) was examined (Fig. 3 A). TCRα is a transmembrane protein that exposes a large extracellular domain in the ER lumen whose unassembled form is degraded via an ERAD pathway that uses the E2s Ubc6 and Ubc7 (Tiwari and Weissman, 2001; Lenk et al., 2002). Pulse chase analysis revealed that TCRα had a half-life of around 1 h in absence or presence of UbcH5a C85A (Fig. 3, A and C). In addition, the overexpression of CHIP was not observed to influence the rate of TCRα turnover (unpublished data). In contrast, overexpression of either Ubc6 C91S or Ubc7 C89S led to an increase in the half-life of TCRα from 1 h to 1.5 and 2 h, respectively. Thus, UbcH5a C85A overexpression does not detectably hinder the turnover of a transmembrane ERAD substrate whose degradation can be blocked by interference with the action of Ubc6 and Ubc7.

Because the membrane topology of TCRα differs from that of CFTR, we also examined the sensitivity of apolipoprotein B48 (ApoB48) degradation to overexpression of UbcH5a C85A. ApoB48 is a 2,15–amino acid residue secretory protein whose nascent form has features that are similar to CFTR because it exposes surfaces in the ER lumen and cytosol, and is a substrate of cytosolic Hsp70 and Hsp90 (Gusarova et al., 2001). ApoB48 folding and exit from the ER requires its assembly into complexes with lipids, and unassembled forms are degraded via a pathway that involves the transmembrane E3 Gp78, which can interact with Ubc7 (Cyr et al., 2002; Liang et al., 2003). The overexpression of CHIP does not accelerate Apob48 degradation (Meacham et al., 2001). Likewise, the overexpression of UbcH5a C85A does not have a detectable effect on the rate of ApoB48 degradation (Fig. 3, B and C). Thus, the overexpression of UbcH5 C85A does not generally inhibit the function the cellular quality control machinery.

A detergent-soluble CFTRΔF508 degradation intermediate accumulates upon inactivation of the Hsc70-CHIP E3 complex

CFTR and CFTRΔF508 biogenic intermediates appear to be aggregation prone and therefore are selected for ubiquitination and proteasomal degradation. When the proteasome is inhibited, polyubiquitinated CFTRΔF508 accumulates in detergent-insoluble aggregates (Ward et al., 1995), but nonubiquitinated CFTRΔF508 biogenic intermediates are not well characterized. To investigate the aggregation state of nonubiquitinated CFTRΔF508, we modulated the activity of the Hsc70–CHIP E3 and determined the detergent solubility of the CFTR and CFTRΔF508 biogenic intermediates that accumulated (Fig. 4). Overexpression of UbcH5a and CHIP caused an overall decrease in the pool of CFTR and CFTRΔF508 and none of the B form was detected in the Triton X-100–insoluble fraction. When UbcH5a C85A was overexpressed, severalfold more of

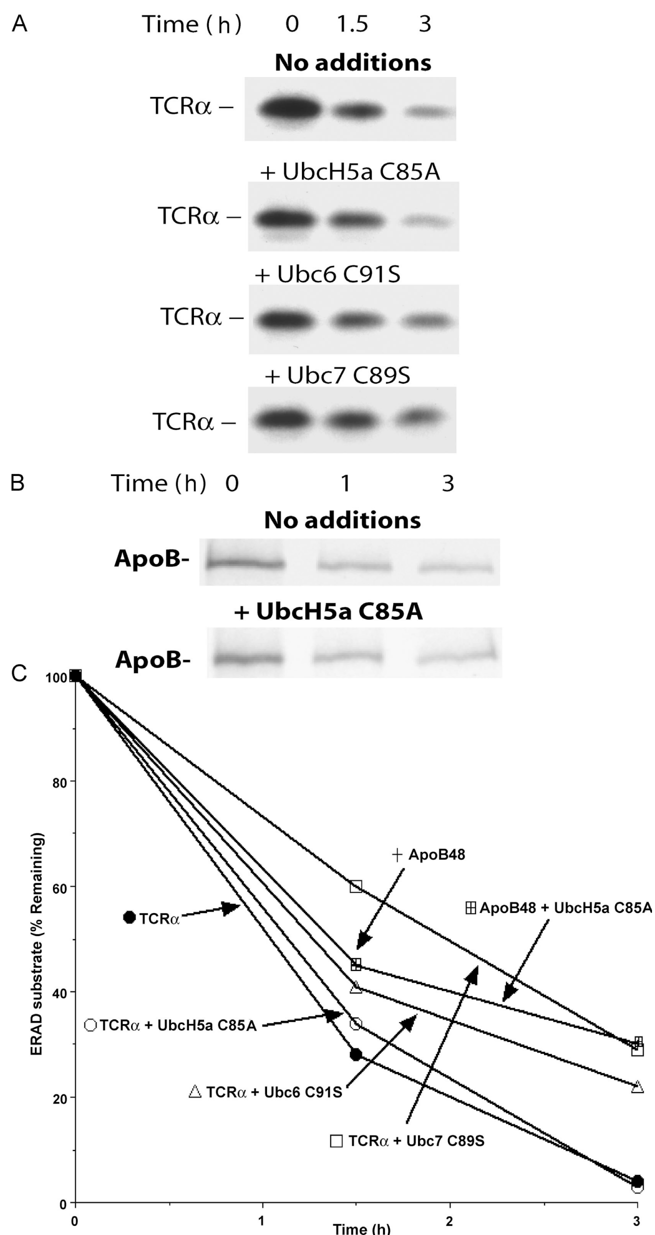

Figure 3. **Insensitivity of TCRα and ApoB48 turnover to inhibition of the Hsc70–CHIP E3.** (A) TCRα degradation. (B) ApoB48 turnover. HEK293 cells were transiently transfected with expression plasmids for the indicated proteins. 24 h after transfection, cells were labeled for 20 min with ^{35}S-translabel. A chase period was initiated by the addition of cycloheximide and, at the indicated times, cells were harvested and lysed. TCRα or ApoB48 were immunoprecipitated from cell extracts and detected by SDS-PAGE and autoradiography. (C) Quantitation of TCRα and Apob48 levels, the relative amount of each protein present at $t = 0$ is expressed as 100% of control.

the B and C form of CFTR accumulated in the Triton X-100–soluble fraction. To our surprise, we also observed UbcH5a C85A overexpression to drive a severalfold increase in the quantity of the B form of CFTRΔF508 that accumulated in a Triton X-100–soluble state. Under these experimental conditions, we also observed a small quantity of the C form of CFTR in the detergent-insoluble fraction. Because the C form of CFTR is typically soluble in Triton X-100, this material appears to represent a minor contamination of the detergent-insoluble fraction with detergent soluble material.

Paradoxically, we observed markedly different results when the action of the Hsc70–CHIP E3 was blocked via overexpression of CHIP P269A. CHIP P269A inhibited degradation of the B form of CFTR and CFTRΔF508, but the degradation intermediate that accumulated was insoluble in Triton X-100 (Fig. 4, A and B). In addition, CHIP P269A blocked the glycolytic maturation of CFTR from the B to C form. The effect that CHIP and CHIP P269A had on CFTR and CFTRΔF508 biogenesis was resultant from interactions with Hsc70 because the TPR mutant CHIP K30A had no effect on CFTR biogenesis (Fig. 4).

How do we explain the observation that the overexpression of UbcH5a C85A and CHIP P269A block CFTR degradation, but yet cause the degradation intermediates that accumulate to exhibit differential detergent solubility? CHIP P269A blocks the glycolytic maturation of CFTR, and thus retains the ability of CHIP to interact with Hsc70 to arrest CFTR folding (Meacham et al., 2001). Therefore, because CHIP P269A can hinder Hsc70s protein folding function, but can't promote CFTR degradation, it causes nonnative CFTR to aggregate. In HEK293 cells CHIP levels are normally 10-fold lower than those for Hsc70 (Meacham et al., 2001). Hence, when UbcH5a C85A is overexpressed the levels of CHIP–UbcH5a C85A complexes that form should not be more than one-tenth the level of Hsc70. Ergo, the overexpression of UbcH5a C85A can block CFTR degradation, but the levels of CHIP–UbcH5a C85A complexes are not high enough to interfere with Hsc70s ability to suppress CFTR aggregation. Therefore, UbcH5a C85A drives the accumulation of a CFTRΔF508 degradation intermediate that is stabilized in a nonaggregated state.

If the aforementioned interpretations are correct and UbcH5a C85A specifically inactivates the U box of CHIP to block CFTR degradation, then the coexpression of UbcH5a C85A and CHIP, should have the same effect on CFTR biogenesis as CHIP P269A. Indeed, we observed that the simultaneous overexpression of CHIP and UbcH5a C85A blocked the glycolytic maturation and degradation of CFTR and drove the B form of CFTR to accumulate as a detergent-insoluble aggregate (Fig. 4 D, lane 1 vs 5).

The data presented in Fig. 4 are important for the following reasons. First, these data indicate that interference with Hsc70–CHIP E3 activity drives the accumulation of a novel nonaggregated CFTRΔF508 biogenic intermediate. Second, these data demonstrate that proper chaperone function of Hsc70 is critically important for maintenance of nonnative CFTR and CFTRΔF508 in a detergent soluble state. Third, the observation that UbcH5a C85A and CHIP coexpression makes CHIP behave like CHIP P269A supports the interpretation that CHIP and UbcH5a functionally interact in vivo to ubiquitinate CFTR.

Characterization of the nonaggregated CFTRΔF508 degradation intermediate

To investigate the nature of the detergent-soluble CFTRΔF508 degradation intermediate that accumulated when UbcH5a

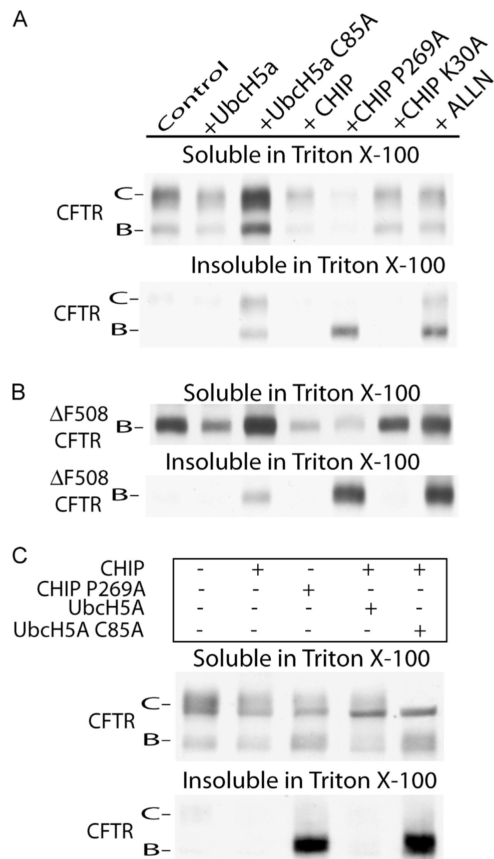

Figure 4. **Triton X-100–soluble CFTR and CFTRΔF508 degradation intermediates accumulate in response to overexpression of UbcH5a C85A.** (A and B) Analysis of the solubility of CFTR or CFTRΔF508. HEK293 were transiently tranfected with vectors that express the indicated proteins. As indicated, the proteasome inhibitor ALLN (200 μM) was added to the growth medium 4 h preceding cell lysis. (C) Coexpression of CHIP and UbcH5a C85A blocks CFTR folding and causes its biogenic intermediates to aggregate. CFTR and CFTRΔF508 were detected by Western blot. The fractionation of cell extracts into Triton X-100–soluble and –insoluble material was performed as described in the Materials and methods. The upper panel represents Triton X-100–soluble material, where as the lower part represents Triton X-100–insoluble material. The immaturely glycosylated B form of CFTR and CFTRΔF508, and maturely glycosylated C form of CFTR are denoted.

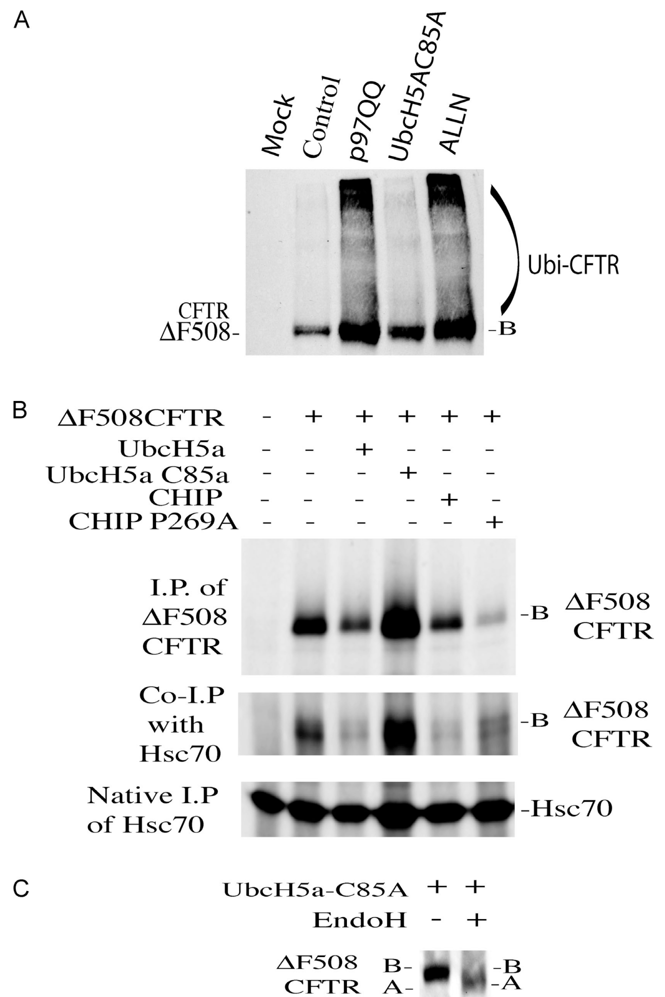

Figure 5. **Characterization of the CFTRΔF508 biogenic intermediate that accumulates when UbcH5a C85A is overexpressed.** (A) Analysis of CFTRΔ508 levels when its proteasomal degradation is inhibited by different methods. HEK293 cells were transfected with the indicated expression vectors and harvested in SDS sample buffer 24 h later. ALLN (200 μM) was added to culture media 4 h before cell harvest. CFTRΔF508 levels were determined by Western blot. Ubiquitinated CFTRΔF508 runs as a high molecular smear on SDS-PAGE gels and is denoted. (B) Complex formation between Hsc70 and CFTRΔF508. Cells were transfected with the indicated expression plasmids and 24 h after incubation they were radiolabeled for 30 min with ^{35}S-translabel and harvested. Half of each cell pellet was lysed under denaturing or native buffer conditions and immunoprecipitations were performed and analyzed by fluorography. (C) CFTRΔF508 that accumulates in the presence of UbcH5a C85A is glycosylated. Cells were harvested and half of each respective lysate was treated with endoglycosidase H whereas the other half served as the control. A and B denote the mobility of nonglycosylated and immaturely glycosylated forms of CFTRΔF508, respectively.

C85A was overexpressed, we compared its ubiquitination state to that of degradation intermediates that accumulate in response to proteasome inhibition by ALLN, or overexpression of a dominant negative form of p97 (p97 QQ; Ye et al., 2003). P97 is a cytosolic chaperone that extracts polyubiquitinated proteins from the ER and participates in CFTRΔF508 degradation (Ye et al., 2003; Dalal et al., 2004). Nonubiquitinated CFTRΔF508 migrates on SDS-PAGE gels with the same mobility as its B form, where as polyubiquitinated CFTRΔF508, which accumulated when its degradation was blocked by ALLN or p97 QQ, migrates on SDS-PAGE gels as a high molecular weight smear (Fig. 5 A). CFTRΔF508 that accumulated in response to UbcH5a C85A overexpression did not migrate as a high molecular smear and, therefore, represents a nonubiquitinated species. This result is consistent with the notion that UbcH5a C85A inhibits CFTRΔF508 ubiquitination and thereby blocks its degradation.

Next, we examined the effect that modulating Hsc70–CHIP E3 action had on complex formation between Hsc70 and CFTRΔF508 degradation intermediates (Fig. 5 B). This was accomplished by coimmunoprecipitating Hsc70–CFTRΔF508 complexes with α-Hsc70 antibody from radiolabeled cells that were transfected with the indicated form of UbcH5a or CHIP. Overexpression of UbcH5a or CHIP reduced the levels of immunoprecipitable CFTRΔF508, whereas UbcH5a C85A overexpression caused CFTRΔF508 to accumulate several fold. On

the other hand, CHIP P269A, which inhibits CFTR degradation and caused CFTRΔF508 to aggregate (Fig. 4), reduced the total amount of CFTRΔ508 that could be immunoprecipitated from cell extracts. The newly synthesized pool of Hsc70 detected by immunoprecipitation was not significantly changed when the levels of the components of the Hsc70–CHIP–UbcH5a E3 complex were altered. Yet, we did observe that the levels of Hsc70–CFTRΔF508 complexes were lower when UbcH5a and CHIP were overexpressed, and were elevated when UbcH5a C85A was overexpressed. Nonetheless, under all of the aforementioned experimental conditions tested, the changes in the levels of Hsc70–CFTRΔF508 complexes appeared proportional to changes in the total amount of immunoprecipitable CFTRΔF508 present in the cell extracts. The major conclusion drawn from these results is that the detergent soluble CFTRΔF508 degradation intermediate that accumulates upon UbcH5a C85A overexpression is associated with Hsc70.

Data presented thus far suggest that inhibition of Hsc70–CHIP E3 activity drives the accumulation of an ER membrane inserted CFTRΔF508 biogenic intermediate that is arrested at a biogenic stage where it has the potential to either fold or be degraded. If this is the case then the CFTRΔF508 that accumulates in response to UbcH5a C85A overexpression should be glycosylated. Indeed, we observed that the gel mobility of the CFTRΔF508 that accumulates in response to UbcH5a C85A overexpression was increased when total cell extracts were treated with endoglycosidase H, which removes ASN-linked glycans from glycoproteins (Fig. 5 C). Thus, inhibition of the Hsc70–CHIP E3 activity promotes the accumulation of an immaturely glycosylated and detergent soluble form of CFTRΔF508 that is bound to Hsc70.

CFTRΔF508 that accumulates in the ER when Hsc70-CHIP action is blocked can fold to the native state

To ascertain whether or not the CFTRΔF508 biogenic intermediate that accumulates in response to inhibition of Hsc70–CHIP E3 function is capable of folding we determined if it could be chased to its maturely glycosylated C form (Fig. 6 A). Transiently transfected HEK293 cells were grown for 24 h after transfection and then treated with cycloheximide to inhibit new protein synthesis. The fate of the accumulated CFTRΔF508 was then monitored by Western blot after the indicated chase incubation at 37° or 26°C (Fig. 6 A). The low-temperature chase incubation was incorporated into the design of this experiment because nascent CFTRΔF508 exhibits a temperature-sensitive folding defect and can fold to the native state and accumulate in its maturely glycosylated C form when cells are cultured at 26°C (Denning et al., 1992).

When control cells were allowed to synthesize CFTRΔF508 at 37°C and were then incubated at either 37° or 26°C, >90% of the total protein present at $t = 0$ was degraded during the 8-h chase period. When UbcH5a C85A was coexpressed with CFTRΔF508 and a chase incubation was performed at 37°C, 40 to 50% of the total CFTRΔF508 present at $t = 0$ remained in the cell for up to 24 h. However, even though UbcH5a C85A stabilized the B form of CFTRΔF508, its conversion to the C form was not detected during the chase reaction at 37°C. In contrast, when chase incubations were performed at 26°C, a significant portion of the CFTRΔF508 that was stabilized in the B form by UbcH5a C85A, was converted to the maturely glycosylated C form. The formation of the maturely glycosylated C form of CFTRΔF508 was proportional to the loss of B form and could be detected in cells after 8 h of chase time and appeared to be complete after 16 h. The glycolytic maturation of CFTRΔF508 observed in the presence of UbcH5a C85A during the chase incubation at 26°C was inhib-

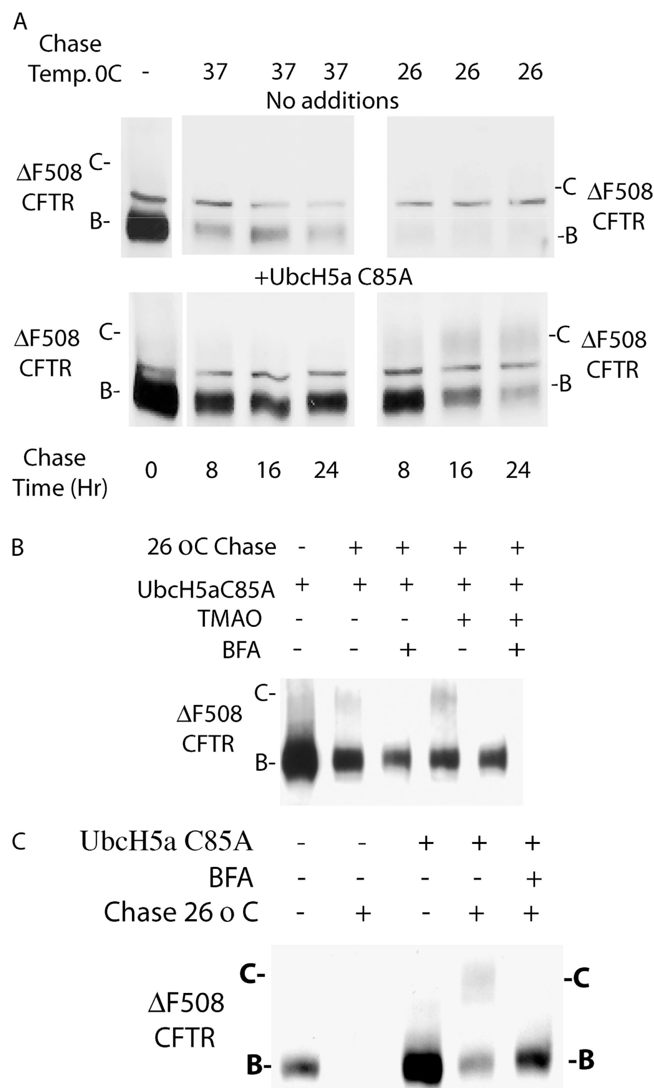

Figure 6. **Glycolytic maturation of CFTRΔF508 degradation intermediates that accumulate in response to UbcH5a C85A overexpression.** (A) The CFTRΔF508 that accumulates when UbcH5a C85A is overexpressed becomes maturely glycosylated when cell growth temperatures are reduced to 26°C. HEK293 cells were transiently transfected with the indicated plasmids and were treated with cycloheximide (25 μg/ml) 24 h later. Cells were then either shifted to 26°C or maintained at 37°C for the indicated time.(B) Glycolytic processing of CFTRΔF508 in cells incubated at 26°C for 16 h is blocked by BFA and enhanced by the chemical chaperone TMAO (75 mM). (C) COS-7 cells are capable of maintaining CFTRΔF508 degradation intermediates in a folding competent state. This experiment was performed as described for panel A except that the chase time was for 24 h at 26°C. The immaturely glycosylated and maturely glycosylated forms of CFTRΔF508 are denoted as B and C, respectively.

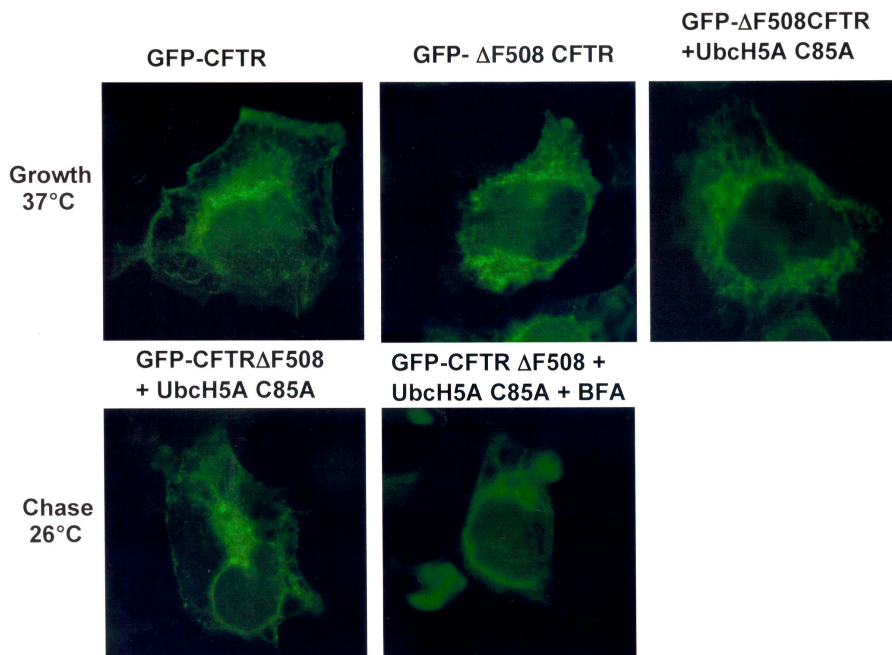

Figure 7. **Localization of GFP–CFTRΔF508 in HEK293 cells.** Cells grown on glass coverslips were transiently transfected with GFP–CFTR or GFP–CFTRΔF508 with or without UbcH5a C85A and cultured for 24 h at 37°C. Where indicated 25 μg/ml cyclohexamide and/or brefeldin A (10 μM) was added to culture media. After 4 h of culture at 26°C these cells were fixed and the coverslips were mounted on glass slides. Images were collected and processed as described in the Materials and methods section.

ited by brefeldin A (BFA) and appears to result from trafficking of CFTRΔF508 out of the ER (Fig. 6 B). To determine if we could further increase the folding efficiency of the CFTRΔF508 that accumulated in the presence of UbcH5a C85A, cells were treated with the chemical chaperone TMAO just before the initiation of the chase reaction (Brown et al., 1996). TMAO treatment of cells increased the quantity of UbcH5a C85A stabilized CFTRΔF508 that could be processed to a maturely glycosylated C form around twofold. Thus, a portion of CFTRΔF508 that accumulated in response to inhibition of its ubiquitination remains in a foldable state that can be brought back on pathway by alteration of cell growth temperatures or chemical chaperones.

To probe whether inhibition of the Hsc70–CHIP complex stabilizes CFTRΔF508 in a folding competent conformation in more than one cell type, we examined the effect that UbcH5a C85A overexpression had on CFTRΔF508 expression and folding in COS7 cells (Fig. 6 C). UbcH5a C85A overexpression was again observed to drive the accumulation of the B form of CFTRΔF508. In addition, a significant portion of the B form that accumulated in the presence of Ubc5Ha C85A could be chased at 26°C, in a BFA-sensitive manner, to the maturely glycosylated C form. Hence, the Hsc70–CHIP complex can regulate biogenesis of CFTRΔF508 in more than one cell type.

To demonstrate that the CFTRΔF508 that was converted to the C form during the 26°C chase incubation was trafficked to the cell surface the localization of GFP–CFTRΔF508 was examined under these experimental conditions and compared with that of GFP–CFTR (Fig. 7). At 37°C, GFP–CFTR was detected both at the cell surface and in a perinuclear location that corresponds to the ER (Moyer et al., 1998). At 37°C, in the presence or absence of UbcH5a C85A, GFP–CFTRΔF508 was only detected in its soluble ER form. However, when UbcH5a C85A-transfected cells were cultured for 24 h at 37°C, treated with cycloheximide, and then incubated at 26°C for 4 h, a pool of GFP–CFTRΔF508 accumulated, in a BFA-sensitive fashion, at the cell surface.

The collective data presented in Figs. 5–7 demonstrate that when the activity of the Hsc70–CHIP ubiquitin ligase is reduced, CFTRΔF508 accumulates as an immaturely glycosylated species that is not a dead-end folding intermediate. Instead, the cell can maintain a pool of kinetically trapped CFTRΔF508 folding intermediates in a detergent soluble and foldable state.

Discussion

Herein we provide new insights into how CFTR and CFTRΔF508 biogenic intermediates are partitioned between folding and degradation pathways. The data presented suggest a model for quality control in which newly synthesized CFTR and CFTRΔF508 initiate folding, but intermediates of each accumulate in a kinetically trapped conformation that is maintained in a soluble state by Hsc70. CHIP then interacts with Hsc70 and functions via a two-step mechanism to attract UbcH5a into Hsc70–CFTR complexes. Then the Hsc70–CHIP–UbcH5a E3 formed acts to polyubiquitinate CFTR.

Interestingly, inhibition of Hsc70–CHIP E3 activity caused the accumulation of a nonaggregated and ER localized CFTRΔF508 biogenic intermediate that was bound by Hsc70. Addition of chemical chaperones to growth media and reduction of cell growth temperatures permitted this nonubiquitinated CFTRΔF508 degradation intermediate to fold, exit the ER, and accumulate on the cell surface. These are the first data that describe a nonubiquitinated CFTRΔF508 biogenic intermediate and they demonstrate that it can be maintained in a nonaggregated and foldable state. This new information suggests that the development of drug cocktails that contain ubiquitination blockers and chemical chaperones could increase the cell surface expression of CFTRΔF508 and provide a treatment for CF.

The nature of the folding defect that arrests the progression of CFTRΔF508 through its folding cascade and what causes it to be selected for proteasomal degradation is not entirely clear. One school of thought is that CFTRΔF508 is highly prone to misfolding and aggregation and is therefore selected for ERAD. Such a notion is supported by the observation that inhibition of the proteasome blocks CFTRΔF508 degradation and drives the accumulation of ubiquitinated forms of CFTRΔF508 in Triton X-100–insoluble aggregates (Ward and Kopito, 1998). However, because the inactivation of the Hsc70–CHIP E3 ligase leads to the accumulation of a soluble ER localized CFTRΔF508 biogenic intermediate, the data we present support a different view. It appears that inhibition of the proteasome leads polyubiquitinated CFTRΔF508 to aggregate because it can be extracted from the ER membrane by the p97–UFD1–NPL4 complex (Ye et al., 2003), and because it cannot be degraded, polyubiquitinated CFTRΔF508 accumulates in aggresomes (Ward and Kopito, 1998). On the other hand, the nonubiquitinated CFTRΔF508 that accumulates in response to inhibition of the Hsc70–CHIP E3 does not aggregate because it is inserted into the ER membrane and is bound by cytosolic Hsc70. Thus, while CFTRΔF508 has a folding defect that prevents it from passing quality control and escaping the ER, it does not appear to be overly aggregation prone and cellular chaperones can maintain it in a foldable state.

Because the cellular activity of CHIP and UbcH5a influence the partitioning of CFTR biogenic intermediates between folding and degradation pathways, we were interested in investigating whether inhibition of the Hsc70–CHIP E3 would influence the processing efficiency of CFTR and CFTRΔF508. In pulse-chase experiments UbcH5a C85A overexpression increased the half-life of the B form of CFTR and CFTRΔF508 from two- to threefold. Therefore, UbcH5a C85A overexpression increased the steady-state levels of CFTR and CFTRΔF508 severalfold. However, the processing efficiency of CFTR from its B form to its C form remained at around 25% whether or not the Hscp70–CHIP E3 complex was active. Thus, while the elevation of cellular Hsc70–CHIP E3 activity can divert the B form of CFTR away from its folding pathway, the ability of full-length CFTR to stay on pathway and collapse to the native state appears to be limited by its intrinsic folding pathway and/or additional quality control factors.

We conclude that the E2 UbcH5a is a cytosolic factor that functions with Hsc70 and CHIP to mediate CFTR ubiquitination. This conclusion is supported by three lines of experimental evidence. First, purified CHIP and UbcH5a cooperated to facilitate the polyubiquitination of CFTR. Second, when CHIP and UbcH5a were coexpressed together they appeared to act synergistically to reduce the steady-state levels of CFTRΔF508. Third, the coexpression of UbcH5a C85A with CHIP, blocked CHIPs ability to degrade CFTR and converted it into a protein that behaved like the CHIP U box mutant P269A.

UbcH5a is a member of a family of conserved E2 proteins that include UbcH5b and UbcH5c that are nearly 90% identical to each other (Scheffner et al., 1994; Jensen et al., 1995b). In addition to UbcH5a, purified CHIP can interact with UbcH5b and UbcH5c, and mRNAs for each of these E2 proteins is present in all tissues tested (Jiang et al., 2001; Jensen et al., 1995b). Thus, we propose that CHIP functions with an UbcH5 E2 family member to ubiquitinate CFTR and other Hsc70 substrates, but we are not able to state whether it prefers one family member to the other. At this point, it is interesting to note that UbcH5 proteins are related to the yeast Ubc4/5 proteins that function to target misfolded proteins for degradation and protect cells from protein denaturing physiological stress (Seufert and Jentsch, 1990). In fact, one member of the Ubc4/5 family, Ubc1, has been shown to function on the ER surface to ubiquitinate ERAD substrates (Bays et al., 2001). Thus, it is logical that UbcH5 is a component of an E3 complex, which contains molecular chaperones, that serves to prevent the accumulation of toxic protein aggregates.

A potential caveat to the interpretation that Hsc70 and CHIP interact with a UbcH5 family member to select CFTR for degradation is that the overexpression of UbcH5a C85A may nonspecifically inhibit the action of other cytosolic quality control factors that function on the ER surface to mediate ERAD. Though possible, data from the control studies with the ERAD substrates TCRα and ApoB48, whose degradation relies on cytsolic E2s, demonstrate that their degradation was not delayed by overexpression of UbcH5a C85A. Thus, it appears that the reduced rates of CFTR degradation caused by UbcH5a C85A overexpression are due to specific inactivation of the Hsc70–CHIP–UbcH5 E3 complex.

The E2s Ubc6 and Ubc7 function with E3s such as gp78 and Doa10 on the cytoplasmic face of the ER to ubiquitinate a variety of substrates (Cyr et al., 2002). Hence, it is plausible that E2–E3 complexes that contain Ubc6 and/or Ubc7 function to select CFTR and CFTRΔF508 for degradation. Sommer and colleagues have explored this concept and found that overexpression of Ubc6, but not Ubc7, modulates the rate of CFTRΔF508 degradation (Lenk et al., 2002). When we compared the effect that dominant negative forms of Ubc6, Ubc7, and UbcH5a had on CFTR and CFTRΔF508 expression, UbcH5a C85A and Ubc6 C91S drove the accumulation of the B form of CFTR, whereas Ubc7 had no apparent effect. The influence that UbcH5a C85A had on the accumulation of the B form of CFTR and CFTRΔF508, was markedly more dramatic than that of Ubc6 C91S, yet Ubc6 clearly plays a role in CFTR quality control. Studies in yeast demonstrate that Ubc6 cooperates with the transmembrane E3 Doa10 to degrade membrane and cytosolic proteins (Swanson et al., 2001) and Doa10 is required for efficient CFTR turnover in yeast (Gnann et al., 2004). Thus, the Doa10–Ubc6 E3 may function alongside the Hsc70–CHIP–UbcH5 E3 to mediate quality control of CFTR. The Hsc70–CHIP–UbcH5 E3 recognizes cytosolic regions of CFTR, whereas the Doa10/Ubc6 E3 may recognize unassembled transmembrane regions. This scenario would explain why turnover of the B form of CFTR and CFTRΔF508 is delayed, but not completely blocked, by the inactivation of the Hsc70–CHIP E3 complex. A critical question pertaining to the function of CHIP as a quality control factor is related to the mechanism by which it regulates Hsc70 polypeptide binding and protein folding activity. The data presented suggest that CHIP functions via a two-step mechanism to determine the fate of

Hsc70 clients such as CFTR. The first step involves the binding of CHIP to the COOH-terminal EEVD motif in the lid domain of Hsc70 (Ballinger et al., 1999; Scheufler et al., 2000). This event alters the Hsc70 polypeptide binding and release cycle to arrest CFTR folding and may involve the transient stabilization of Hsc70–CFTR complexes. This putative event would give the U box on CHIP the time required to attract UbcH5 to Hsc70–CFTR complexes and facilitate CFTR ubiquitination.

Interestingly, the ability of CHIP to ubiquitinate Hsc70 clients can be modified by other cochaperones. Data presented herein demonstrate that Hdj-2 cooperates with Hsc70 and CHIP to mediate CFTR ubiquitination. However, the cochaperone HspBP1, which is a member of a family of nucleotide exchange factors that promote substrate release from Hsc70, blocks the ability of CHIP to ubiquitinate CFTR (Alberti et al., 2004). Thus, the fate of proteins that are bound to Hsc70 is regulated by its interactions with multiple cochaperones. To understand this process, the temporal relationship and driving force for interactions between Hsc70 and its folding or degradatory cochaperones needs to be determined.

Materials and methods

Plasmids and antibodies

The plasmids used for cell transfection were: pCDNA3.1CFTR and pCDNA3.1CFTRΔF508 (Meacham et al., 2001); pEGFPC2-CFTR and pEGFPC2-CFTRΔF508 (Moyer et al., 1998); pCDM8 2B4 TCRα (Bonifacino et al., 1989); pCDNA-ApoB48 (H. Ginsberg, Columbia Presbyterian Medical Center, New York, NY); pCAGGS His_6UBCH5A C85A and pCAGGS His_6UBCH5A (Jiang et al., 2001); pCMVPLDmyc-UBC6 and pCMVPLDmyc-UBC6 C91S (Lenk et al., 2002); pCDNA myc-UBC7 and pCDNA myc-UBC7 C89S (Tiwari and Weissman, 2001); pCDNA3.1CHIP, pCDNA3.1CHIP K30A, and pCDNA3.1CHIP P269A (Jiang et al., 2001; Meacham et al., 2001). pGEXCFTR 371–855 was termed pGSTNBD1-R (Naren et al., 1999).

The following plasmids were used for overexpression of the indicated proteins in *E. coli*; pET9d Hdj2 and pET11a Hsc70 (Meacham et al., 1999). Plasmids prepared for this study were pET11a His_6UbcH5a, pET11a-His_6UbcH5a C85A, pET11d His_6Ubc7, pET11a His_6Ubc6 1–243, pET30His_6CHIP, pET30His_6CHIPK30A, pET30His_6CHIPH260A, and pET30 His_6CHIPP269A. E1 was purchased from Calbiochem.

The antibodies used for Western blots and/or immunoprecipitations were αCFTR clone MM13-4 from Upstate Biotechnology and αCFTR R-domain antibody from R&D Systems. αTCR was from BD Biosciences and αHsc70 was from Stressgen Biotechnologies.

Protein purification

The proteins used in in vitro ubiquitination assays were purified after overexpression in *E. coli*. Hsc70 was purified by a combination of ATP–agarose and anion exchange chromatography (Cyr et al., 1992). Hdj-2 was purified by anion exchange and hydroxyapatite chromatography (Meacham et al., 1999). His_6-tagged proteins were purified by metal chelate chromatography (Lu and Cyr, 1998).

GstNBD1-R was expressed in *E. coli* strain BL21 (DE3) and cells from a 600-ml culture were harvested after a 16-h induction at 30°C with 0.2 mM IPTG. Cell pellets were resuspended and incubated for 30 min on ice in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethyl sulfonyl fluoride, 1 mM DTT, and lysozyme 0.1 μg/ml. The extract was supplemented with 1% sarkosyl and sonicated. Gst–NBD1–R was then purified with glutathione–agarose beads and had a final concentration near 2 mg/ml.

Reconstitution of CFTR ubiquitination

The experimental conditions for the reconstitution of gst–NBD1–R ubiquitination were described previously (Koegl et al., 1999). Ubiquitination assays were performed in a reaction buffer composed of 20 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 2.5 mM ATP, 2 mM DTT, 10 μM bovine ubiquitin, 0.1 μM rabbit E1 and 1 μM gst–NBD1–R. The indicated E2 protein was added at 4 μM and the other factors were included at the concentration indicated in Fig. 1. Incubations were performed at 37°C for the indicated time and terminated by the addition of 20 μl of SDS sample buffer to 25 μl reaction cocktails. Proteins were resolved on 7% SDS-PAGE gels and then transferred to nitrocellulose membranes that were decorated with αR domain antibody and developed.

Assays for CFTR biogenesis

HEK293 and Cos-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum and a mixture of 1% penicillin and streptomycin at 37°C and transfected with CFTR and CFTRΔF508 expression plasmids. Steady-state levels of CFTR and CFTRΔF508 levels were determined by Western blot and CFTR processing efficiency was measured by pulse chase analysis (Meacham et al., 2001). Details of the protocols for direct immunoprecipitations or coimmunoprecipitations were as described previously (Meacham et al., 2001). To verify the identity of CFTR isolated from chaperone complexes CFTR was reimmunoprecipitated from coimmunoprecipitates with α-CFTR (Meacham et al., 1999). Treatment of cell extracts with endoglycosidase H was conducted as described previously (Meacham et al., 1999).

Fluorescence microscopy

HEK293 cells were cultured on glass coverslips and transfected transiently with 1μg pEGFPC2–CFTR or pEGFPC2–CFTRΔF508 either alone or in combination with 3 μg pCAGGS His_6UBCH5A C85A. 24 h later, cells were washed twice for 5 min with 2 mls of PBS and fixed with 4% paraformaldehyde at room temperature. As indicated, cells were grown for 24 h after transfection and then treated with 25 μg/ml cyclohexamide and incubated for an additional 4 h at 26°C. Coverslips were mounted on glass slides with the preservative Fluoromount-G. Images were collected using a Nikon E600 microscope and a Princeton Instruments CCD camera. Images were processed with Metamorph (Universal Imaging Corp.) and Adobe Photoshop software.

Online supplemental material

Data presented in Fig. S1 demonstrate that the joint presence of Hsc70, Hdj-2, and CHIP stimulates dramatically the rate at which UbcH5a conjugates gst–NBD1–R with ubiquitin. When Hsc70 or Hdj-2 were omitted from reactions mono-, di-, and triubiquitination of gst–NBD1–R was observed, but this reaction was slow and inefficient. Thus, Hsc70, Hdj-2, and CHIP act jointly to stimulate the ubiquitination activity of UbcH5a and therefore exhibit an activity that fits the definition of a multisubunit E3 ubiquitin ligase. Fig. S1 is available at http://www.jcb.org/cgi/content/full/jcb.200410065/DC1.

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