The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation

Geoffrey C. Meacham*, Cam Patterson†, Wenyue Zhang*, J. Michael Younger* and Douglas M. Cyr*‡

*Department of Cell Biology and Cystic Fibrosis Center, University of Alabama Medical Center, 1918 University Boulevard, Birmingham, Alabama 35294-0005, USA
†Program in Molecular Cardiology and the Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599-7075, USA
‡e-mail: dcyr@cellbio.bhs.uab.edu

The folding of both wild-type and mutant forms of the cystic-fibrosis transmembrane-conductance regulator (CFTR), a plasma-membrane chloride-ion channel, is inefficient[1–4]. Most nascent CFTR is retained in the endoplasmic reticulum and degraded by the ubiquitin proteasome pathway[5–7]. Aberrant folding and defective trafficking of CFTRΔF508 is the principal cause of cystic fibrosis[3,8,9], but how the endoplasmic-reticulum quality-control system targets CFTR for degradation remains unknown. CHIP is a cytosolic U-box protein that interacts with Hsc70 through a set of tetratricorepeat motifs[10]. The U-box represents a modified form of the ring-finger motif that is found in ubiquitin ligases[11] and that defines the E4 family of polyubiquitination factors[12,13]. Here we show that CHIP functions with Hsc70 to sense the folded state of CFTR and targets aberrant forms for proteasomal degradation by promoting their ubiquitination. The U-box appeared essential for this process because overexpresion of CHIPΔU-box inhibited the action of endogenous CHIP and blocked CFTR ubiquitination and degradation. CHIP is a co-chaperone that converts Hsc70 from a protein-folding machine into a degradation factor that functions in endoplasmic-reticulum quality control.

The function of CHIP in membrane protein biogenesis was first suggested by localization studies that showed that a portion of this co-chaperone colocalizes with CFTR and Hsc70 at the endoplasmic reticulum (ER; Fig. 1). Furthermore, we have shown that elevated CHIP levels, through the expression of exogenous Myc–CHIP, prevent the cell-surface localization of CFTR and cause green fluorescent protein (GFP)–CFTR[14] to accumulate in a perinuclear location. When cells are treated with the proteasome inhibitor ALLN, degradation of misfolded CFTR is inhibited and it accumulates in intracellular inclusions termed aggresomes[15–17]. In ALLN-treated cells, the localization of CHIP is altered, and it can be colocalized in the aggresome with proteasomes, Hsc70 and CFTR. Thus, CHIP has the potential to interact with CFTR in the ER or in aggresomes. It is important to note, however, that CFTR degradation initiates in the ER and that CFTR is not normally detected in aggresomes. Thus, CHIP probably functions at the ER to influence GFP–CFTR localization.

To determine how CHIP alters the localization of GFP–CFTR, we examined its influence on the biosynthetic processing of CFTR and CFTRΔF508. Western blots show that elevating CHIP activity reduces the steady-state levels of both the maturely glycosylated, plasma-membrane-localized C form, but did not cause the accumulation of the immaturely glycosylated, ER-localized B form (Fig. 2a). CHIP could also reduce the accumulation of the CFTRΔF508 B form. Reduction of CFTR levels did not seem to result from CHIP-dependent alterations in the expression of the cellular protein-folding or quality-control machinery[18–21], because the elevation of its levels did not cause detectable changes in the steady-state concentrations of Hsc70, Hdj-2, Hsp90 or calnexin (Fig. 2b). Instead, the elevation of CHIP activity relative to that of other co-chaperones of Hsc70 seemed to alter the fate of immature CFTR.

Figure 2c indicates that CHIP may function to target CFTR for degradation through a pathway that involves the proteasome. CHIP-dependent CFTR degradation was blocked by pretreating the cells with the proteasome inhibitor ALLN for 4 h. In ALLN-treated cells that expressed excess CHIP, CFTR maturation was blocked, but the B form was not degraded and it accumulated as a soluble degradation intermediate. Thus, when the proteasome is inhibited CHIP can divert CFTR from the biosynthetic pathway, but CFTR is not degraded. These data indicate that CHIP may help target CFTR for degradation, but that it does not seem to be involved in its proteolysis.

Results from pulse-chase experiments shown in Fig. 2d and e show that CHIP reduces the steady-state levels of CFTR and CFTRΔF508 by promoting the degradation of newly synthesized forms of these proteins. At $t = 0$, similar quantities of newly synthesized immature CFTR were present in controls and in cells that overexpressed CHIP. In control cells a significant portion of the B form was converted to the C form. In contrast, CHIP expression blocked the glycolytic maturation of CFTR and caused° almost all of the B form to be degraded within the first 1 h of the chase period.

To determine the domains in CHIP that are required for it to promote CFTR degradation, we carried out deletion analysis and quantified the results (Fig. 2f). These studies showed that both the tetratricorepeat (TPR) motif and U-box are necessary, but neither is sufficient for CHIP to accelerate the rate of CFTR degradation. A requirement for a TPR motif indicates that CHIP may need to interact with Hsc70 to promote CFTR degradation. A functional requirement for the U-box – a domain known to facilitate protein polyubiquitination[13] – implies that CHIP regulates CFTR ubiquitination.

An alternative, but less likely mechanism of action, is that CHIP blocks CFTR maturation by causing a general blockade of the secretory pathway. To exclude this possibility, we examined the maturation of the transferrin receptor and found that it proceeds with normal kinetics when CHIP is overexpressed (Fig. 2g). In related control studies, CHIP did not influence the tunicamycin-sensitive modification of CFTR with *N*-linked oligosaccharides (data not shown). Thus, CHIP does not appear to promote CFTR degradation by interfering with insertion of its transmembrane domains into the ER.

To evaluate whether increasing the cellular activity of other Hsc70 co-chaperones would have the same effect as CHIP has on membrane protein biogenesis, we co-expressed CFTR with Hdj-2 (ref. 19), Bag-1 (refs 22, 23) and HIP[24]. None of these co-chaperones

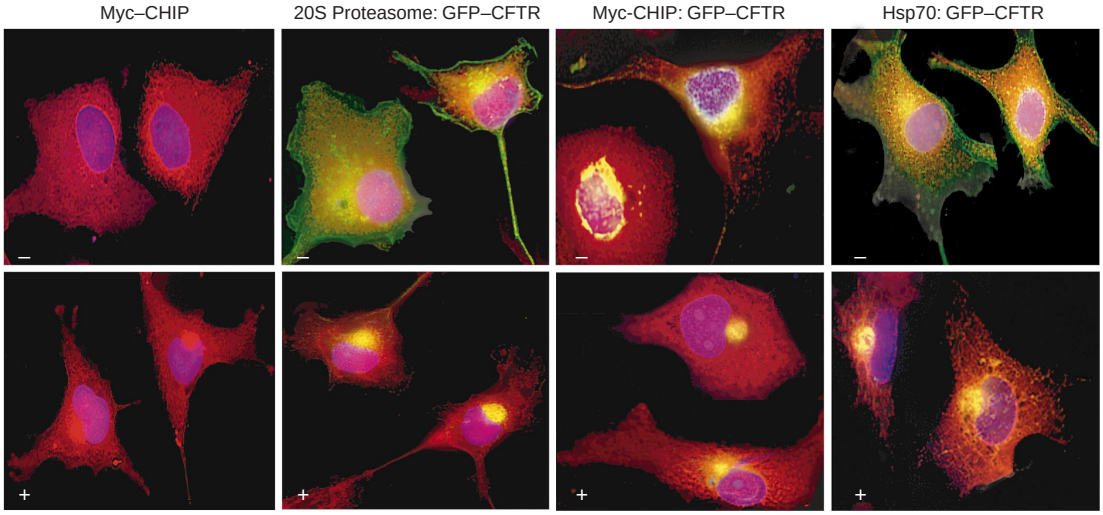

Figure 1 **The effect of CHIP expression on GFP–CFTR localization.** Cos-7 cells were transiently transfected with GFP–CFTR alone or in combination with Myc-tagged CHIP. Twenty-four hours after transfection, cells grown on cover slips were fixed and analysed by indirect immunoflourescence. '–' denotes untreated cells; '+' denotes cells that were incubated for 12 h with 100 μM ALLN, an inhibitor of the 20S proteasome. Treatment of cells with proteasome inhibitors leads to the accumulation of misfolded protein in the cytosol and the accumulation of centers for proteolysis termed cytosolic aggresomes[15,16]. In the merged images shown, the green channel corresponds to GFP–CFTR, the blue channel to nuclear staining by the Hoechst dye and the red channel to either Myc–CHIP, the 20S proteasome or Hsp70 as indicated. The yellow patterns reflect colocalization of the GFP–CFTR and the indicated proteins.

had a detectable influence on CFTR or CFTRΔF508 biogenesis (data not shown). Thus, the ability of CHIP to control the fate of CFTR biogenic intermediates appears to be unique amongst the co-chaperones of Hsc70 that we tested.

We examined the specificity of CHIP action in ER quality control by determining its influence on the fate of newly synthesized apolipoprotein B48. Apolipoprotein B48 has 2,152 amino-acid residues, is degraded at the ER by the proteasome, and has been proposed as a substrate of Hsc70 (refs 25, 26). Unexpectedly, CHIP had no detectable influence on the kinetics of apolipoprotein B48 degradation (Fig. 2h). CFTR is a member of the ABC transporter family and is structurally and functionally related to the multidrug resistance protein (MDR)[1]. MDR is processed efficiently and, unlike CFTR, has rapid folding kinetics and would therefore not be expected to be a substrate of Hsc70 or other chaperones. Consistent with this theory, CHIP did not influence the glycolytic processing or turnover of MDR (data not shown). These data show that CHIP does not generally block the folding or trafficking of newly synthesized membrane proteins. In addition, these data suggest that CHIP and Hsc70 exhibit specificity in the selection substrates that they target for degradation through the proteasome.

If CHIP is directly involved in the selection of CFTR biogenic intermediates for degradation, then it should be able to form specific complexes with ER forms of this ABC-transporter protein. Indeed, we observed that endogenous CHIP could form co-immunoprecipitable complexes with the B form of CFTR and CFTRΔF508 (Fig. 3a). In addition, recognition of CFTR by CHIP and Hsc70 seemed to occur in a conformation-specific manner because the C form was not co-immunoprecipitated with Hsc70 or CHIP. These data support the interpretation from localization studies that suggest that CHIP and Hsc70 function at the level of the ER to regulate the fate of immature CFTR.

It is possible that CHIP indirectly interferes with the binding of Hsc70 to CFTR or promotes the release of CFTR from Hsc70 (ref. 27), thereby causing it to misfold and be degraded. To discount this mechanism, we examined the influence of CHIP overexpression on complex formation between Hsc70 and CFTR (Fig. 3b). CHIP had little detectable influence on the total quantity of the immature B form of CFTR that could be co-immunoprecipitated with Hsc70. Therefore, CHIP probably acts on CFTR while it is bound to Hsc70.

In addition to Hsc70 and CHIP, Hdj-2 and Hsp90 are cytosolic chaperones that interact with the B form of CFTR[19,21] and therefore have the potential to function in CFTR degradation. Furthermore, CHIP has also been shown to interact with Hsp90 and influence the fate of its substrates[27]. We therefore evaluated whether CHIP influences CFTR biogenesis through Hsc70 alone or in combination with these other chaperones. We compared the levels of co-immunoprecipitated complexes formed between the B form of CFTR and endogenous forms of Hsc70, CHIP, Hdj-2 and Hsp90 (Fig. 3c). Levels of CHIP–CFTR and Hsc70–CFTR complexes were similar and contained more than 40% of the CFTR present in the cell. In contrast, CFTR–Hsp90 complexes were not detected above background levels and CFTR–Hdj-2 complexes were markedly lower than those observed with Hsc70 and CHIP. These results may reflect differences in the stability of complexes formed between CFTR different chaperones, but they suggest that CHIP and Hsc70 function independently of other chaperones to influence the fate of CFTR.

If CHIP targets substrates of Hsc70 to the ubiquitin-proteasome pathway for degradation, then it might be expected to interact with components of this system. We therefore examined whether CHIP could be isolated in a complex with components of the ubiquitin-proteasome system. When Myc–CHIP was immunoprecipitated from cells that did not express CFTR and that were lysed under native buffer conditions, it was found in complexes that contained Hsc70 and the C8 subunit of the proteasome. We were unable to determine whether the interactions between CHIP and the C8 subunit of the proteasome observed were direct, but these interactions seemed to be specific as they were not observed when SDS was included in cell extracts to disrupt native protein–protein interactions. Consistent with these finding, the Patterson group[27] has shown that CHIP forms complexes with the S5A subunit of 19S cap of the proteasome. Thus, CHIP interacts with components of the ubiquitin-proteasome system, and these interactions may be important for CFTR degradation.

We next used a permeabilized cell system to monitor the influence of purified CHIP on the incorportation of ^{125}I-labelled ubiquitin into CFTR expressed by HEK293 cells (Fig. 4a). Under conditions

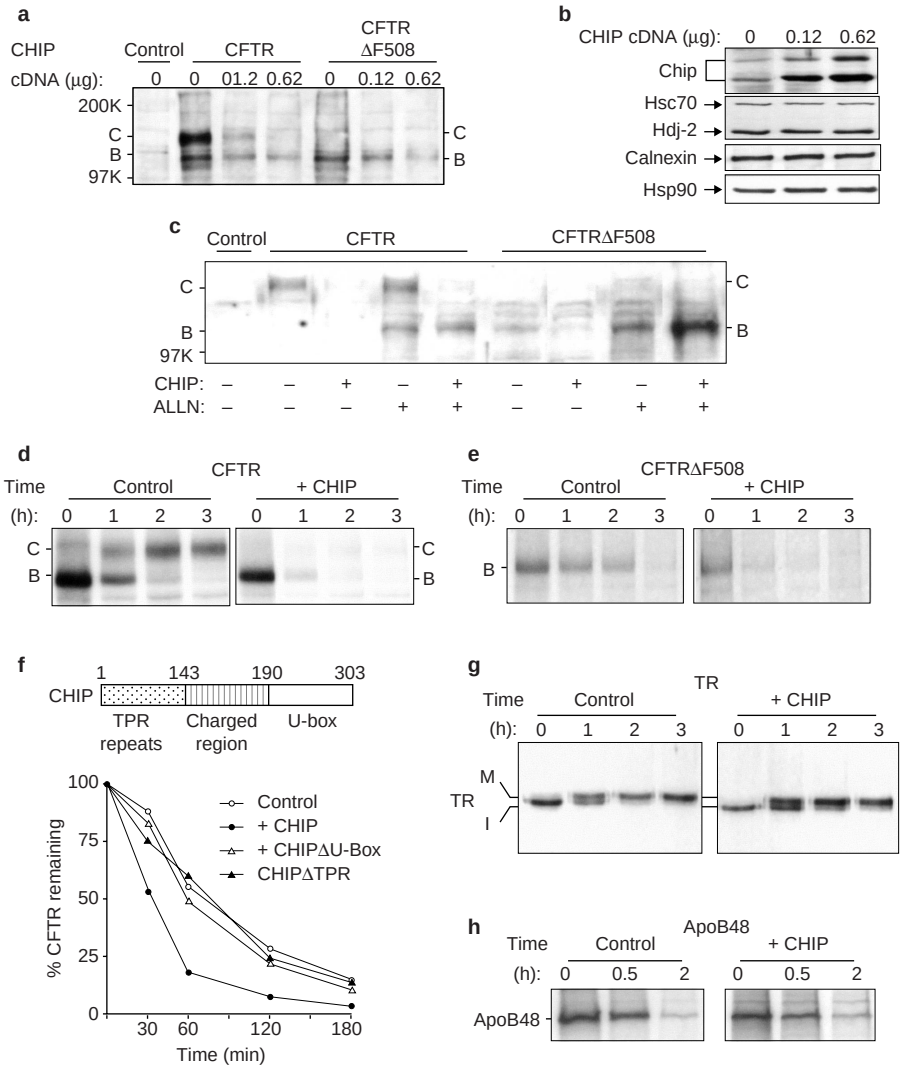

Figure 2 **The influence of CHIP on CFTR biogenesis. a**, Effect of CHIP expression on steady-state levels of CFTR and CFTRΔF508 in transiently transfected HEK293 cells. Cells were transfected with CFTR or CFTRΔF508 expression plasmid and pCDNA3.1–CHIP. All transfections contained 1.5 μg of pCDNA3.1 in total. Cell extracts were prepared for western blots under denaturing conditions 24 h after transfection. B and C denote the immaturely glycosylated ER and maturely glycosylated plasma-membrane forms of CFTR, respectively. **b**, Steady-state levels of CHIP and other molecular chaperone proteins in cells transfected with pCDNA3.1–CHIP. Western blots were probed with antibodies specific for the indicated proteins. CHIP antibody binds to two bands: the bottom band migrates to a position corresponding to purified CHIP (data not shown). The presence of the upper band is variable, it may correspond to a post-translationally modified form of CHIP[10]. HEK293 cells are transfected at ~90% efficiency; when 0.12 and 0.62 μg of pCDNA3.1–CHIP were used, CHIP levels increased by an estimated 4- and 9-fold, respectively. **c**, Effect of ALLN on CHIP-dependent CFTR degradation. HEK293 cells that transiently expressed CFTR or CFTRΔF508 alone or in combination with CHIP were treated for 4 h with 100 μM ALLN as indicated. Cell extracts were prepared, and the steady-state levels of CFTR and CFTRΔF508 determined by western blot. **d**, Pulse-chase analysis of CFTR maturation in ^{35}S-labelled HEK293 cells. Cells were transfected with 0.25 μg of pCDNA–CFTR, and where indicated 0.62 μg of pCDNA3.1–CHIP. Labelling was carried out for 20 min, and radiolabelled CFTR was isolated from cell extracts after the indicated chase period by immunoprecipitation. Immunoprecipitation reactions were analysed by SDS–PAGE and fluorography. **e**, Effect of CHIP on CFTRΔF508 degradation. **f**, Domain requirements for CHIP function in CFTR degradation. Top, putative domain boundaries in CHIP. CHIPΔTPR is residues 143–303; CHIPΔU-box is residues 1–190. Bottom, pulse-chase analysis of the effect of CHIPΔU-box and CHIPΔTPR on CFTR maturation. Cells were transfected with 0.25 μg of pCDNA3.1–CFTR and 0.62 μg of either pCDNA3.1–CHIPΔU-box or CHIPΔTPR. In these conditions, levels of CHIPΔU-box and CHIPΔTPR expression were equivalent to those of CHIP exhibited in Fig. 2b. Cell extracts were treated with PNGaseF for 1 h to cleave CFTR into a species that migrates as a single band. CFTR was immunoprecipitated with anti-CFTR, and analysed by SDS–PAGE and fluorography. The intensity of the single CFTR-specific band was then quantified and is expressed as a percentage of the total material present at $t = 0$. Each time point represents an average of three independent experiments. **g**, Pulse-chase analysis of transferrin receptor (TR) maturation in CHIP expressing cells. I and M denote the maturely and immaturely glycosylated forms of TR[36]. **h**, The effect of CHIP expression on the degradation kinetics of apolipoprotein B48 (ApoB48). For **g** and **h** the experimental conditions were as in **d** except TR and ApoB48 expression plasmids were substituted for CFTR.

in which the proteasome was inhibited by MG132 (ref. 6), CHIP stimulated the incorporation of ^{125}I-labelled ubiquitin into CFTR and CFTRΔF508 by about twofold. Conversely, purified CHIPΔU-box acted in a dominant-negative manner and blocked CFTR ubiquitination by about 60%. These data are similar to those from the Patterson group[27] showing that CHIP can enhance ubiquitination of the glucocorticoid receptor[27]. Thus, CHIP can function as a ubiquitination factor, and the U-box seems to be essential for this function.

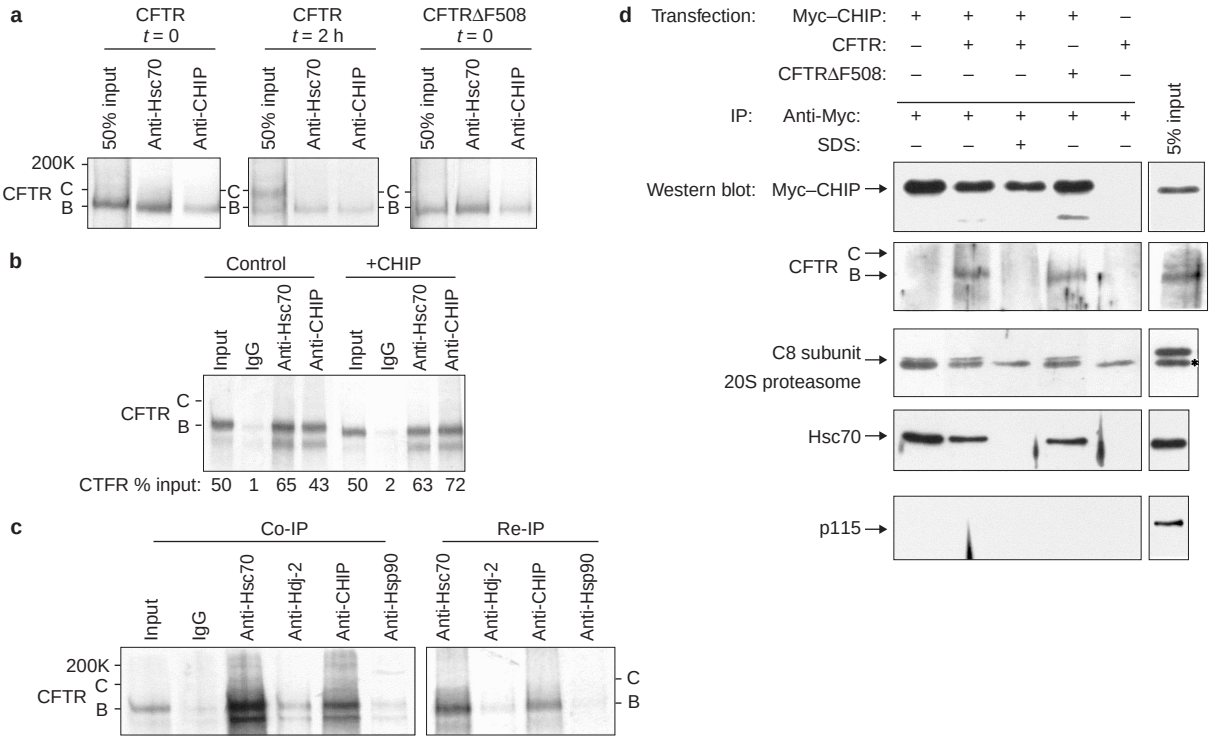

Figure 3 **Complex formation between CHIP and CFTR. a**, The B form but not the C form of CFTR can be co-immunoprecipitated with CHIP. HEK293 cells were transfected with CFTR or CFTRΔF508 expression plasmids and incubated for 24 h. Cells were labelled for 20 min, and cell extracts were prepared before or after a 2-h chase period under native conditions. Products of the initial co-immunoprecipitation reaction with the indicated chaperone were then re-immunoprecipitated under denaturing conditions with anti-CFTR and analysed[19]. Input lane contains 50% of the total CFTR that could be immunoprecipitated with anti-CFTR under non-native conditions with anti-CFTR. **b,** Influence of CHIP on Hsp70–CFTR complex formation. This experiment was carried out as in **a** except that CFTR was expressed in HeLa cells. HeLa cells were used for this and subsequent experiments presented below because CFTR could be expressed to higher levels and Hsp70–CFTR complexes were more abundant and isolated with greater efficiency. **c**, Examination of complex formation between CFTR and different cytosolic chaperones. Co-immunoprecipitations of CFTR from radiolabeled cell extracts were carried out as in **a**, using the indicated antibodies. Immunoglobulin-γ (IgG) indicates the quantity of CFTR that is precipitated using non-immune sera. Re-IP represents the products of a co-immunoprecipitation reaction that could be re-immunoprecipitated under non-native conditions with anti-CFTR. **d**, Western blot of proteins that can be co-immunoprecipitated with CHIP. HeLa cells were transiently transfected with expression plasmids encoding Myc-tagged CHIP, CFTR or CFTRΔF508 as indicated. Cell extracts were made under non-denaturing conditions except the lane '+SDS'. Where indicated, anti-Myc was used to immunoprecipitate Myc–CHIP and associated proteins. The components of Myc–CHIP immune complexes were then probed by western blot using anti-Myc, anti-CFTR, anti-C8 subunit of the 20S proteasome, anti-Hsc70 or anti-p115. p115 is an ER/golgi localized protein involved in transport vesicle tethering[17]. The 5% input lane represents the signal generated by the indicated sera when 5% of the whole-cell extract that was used for the co-immunoprecipitation was blotted. Asterisk denotes a background band known to be recognized by anti-C8 that does not specifically associate with CHIP.

If the U-box is required for CHIP to facilitate CFTR ubiquitination, then high-level overexpression of CHIPΔU-box should inhibit the action of endogenous CHIP and block CFTR degradation. Indeed, we observed that when CHIPΔU-box was expressed to levels that were 20-fold higher than those for endogenous CHIP, it acted in a dominant-negative fashion to block CFTR degradation. This led to the accumulation of the B form of CFTR and CFTRΔF508, but did not enhance the maturation of either. Data shown in Fig. 4a and b strongly suggest that CHIP actively participates in the ubiquitination of CFTR, and that the U-box has a principal role in this function.

Two control experiments verify that CHIPΔU-box acts by a mechanism that involves its interaction with Hsc70. First, the Patterson group[10] showed that CHIP and CHIPΔU-box can bind Hsp70 in the same manner. Second, the binding of CHIP to Hsc70 reduced the extent to which Hsp40 proteins stimulate the ATPase activity of Hsp70 (ref. 10). Thus, we tested whether purified CHIP and CHIPΔU-box could function similarly to reduce the degree by which Hdj-2 stimulates the ATPase activity of Hsc70. The results shown in Fig. 4c show this to be the case. Thus, with regard to it ability to interact with Hsc70, CHIPΔU-box retains the same function as CHIP, yet it cannot promote CFTR degradation. These data indicate that the influence of CHIP on CFTR biogenesis is not simply caused by nonspecific inhibition of the protein-folding function of Hsc70.

We have shown that CHIP, a co-chaperone in the TPR motif family[10,24,28], can convert Hsc70 from a protein-folding machine into a degradation factor. Data presented here and elsewhere[27], coupled with the sequence homology between the U-box of CHIP and the ring-finger domain of E3s (ref. 11), suggest that the CHIP–Hsc70 complex targets misfolded CFTR to ubiquitin-conjugating enzymes, which in turn facilitate its ubiquitination. In yeast, the ubiquitin-conjugating enzymes UBC6 and UBC7 help degrade membrane proteins[29,30]; thus CHIP may interact with human homologues of these proteins[31,32] to facilitate CFTR ubiquitination, but this remains to be elucidated.

Our data indicate that the Hsc70/CHIP complex may function in ER quality control to sense the folded state of membrane proteins that have large cytosolic domains. However, turnover of apolipoprotein B48, a protein that is proposed to expose a loop of relative molecular mass 70,000–90,000 in the cytosol[33], is not effected by CHIP expression, which suggests that there are several mechanisms by which the cell targets misfolded membrane proteins for degradation. This specificity maybe dictated by the sequences or structures of

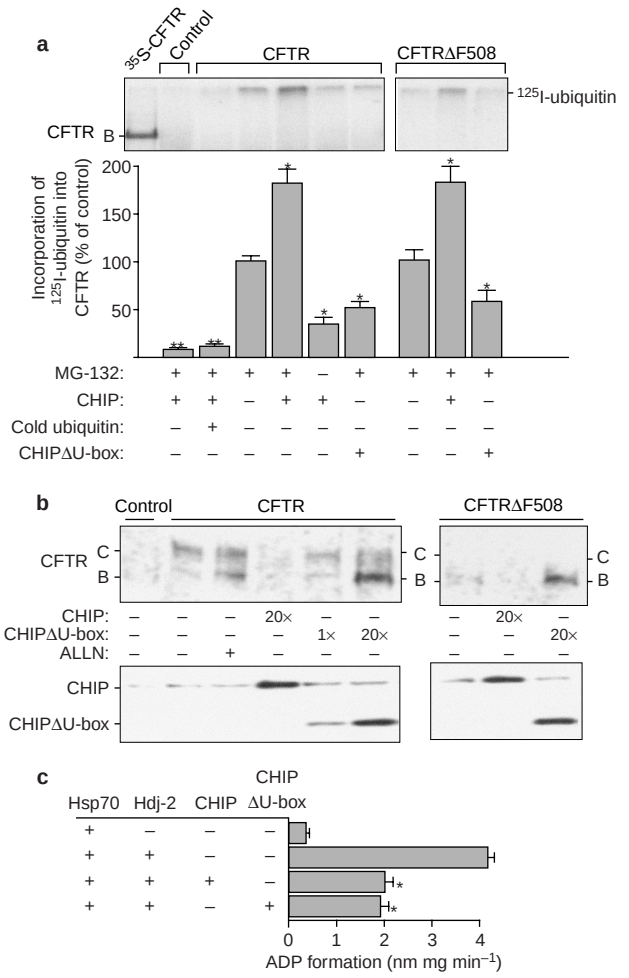

Figure 4 **The U-box is required for CHIP to promote CFTR ubiquitination. a**, Cell-free assay for CFTR ubiquitination. HEK293 cells were transiently transfected with pCDNA3.1 CFTR or CFTRΔF508 and incubated for 24 h. Cells were then collected, permeabilized with digitonin and incubated for 10 min in conjugation buffer containing ^{125}I-labelled ubiquitin. Reactions were supplemented with the indicated materials; CHIP (15μM) or CHIPΔU-box (15 μM), unlabelled ubiquitin (3 mM) and MG-132 (100 μM). CHIP was purified as described[10]. CFTR/^{125}I-labelled ubiquitin conjugates were solubilized in SDS sample buffer and isolated by immunoprecipitation with anti-CFTR. Top, migration of CFTR/^{125}I-labelled ubiquitin conjugates; bottom, quantitation of data (mean ± s.d.) from four different experiments. Data are exhibited as a percentage of the total incorporation of ^{125}I-labelled ubiquitin into CFTR observed when just MG132 was added to cell extracts. **b**, High-level overexpression of CHIPΔU-box blocks CFTR degradation. HEK293 cells were transiently transfected with expression plasmids encoding CFTR or CFTRΔF508 alone or in combination with CHIP or CHIPΔU-box expression plasmids. Cells were treated as in Fig. 2c were prepared for western blot using anti-CFTR (top) or anti-CHIP (bottom). B and C denote the immaturely and maturely glycosylated forms of CFTR. **c**, Effect of purified CHIP and CHIPΔU-box on the Hdj-2-stimulated ATPase activity of Hsp70. Conversion of ^{32}P-ATP to ^{32}P-ADP was monitored by thin layer chromatography[38]. Rates of Hsc70 ATPase activity shown are the means ± s.d. of three experiments. Asterisk, significantly different from the control ($P < 0.05$, Student's *t*-test); two asterisks, $P < 0.005$.

the cytosolic domains on different membrane proteins that are presented to the Hsc70–CHIP complex. Thus, it will be important to identify additional substrates of the Hsc70–CHIP complex and to determine how these proteins are selected.

Our data suggest that relative levels of the cellular protein-folding and surveillance machinery dictate the time given to CFTR biogenic intermediates to reach the native state and thus influence the overall efficiency of its maturation. We estimate that Hsc70, Hdj-2 and CHIP represent 1.3, 0.23 and 0.02% of total protein in HEK293 cells. Thus, the levels of Hdj-2 are about 10 times greater than those of CHIP, which suggests the cell adjusts conditions to favour folding of CFTR. When CHIP levels are elevated as little as fourfold, the balance between CFTR folding and degradation is shifted markedly towards degradation. This is likely to occur because kinetics for CFTR folding are slow and because elevation of CHIP activity shortens the time given for triage decisions[34]. Thus, the cellular levels of the co-chaperones that specify Hsc70 function seem to influence directly the folding efficiency of CFTR. If this is the case, then cells that express different levels of CHIP[10] might process CFTR and CFTRΔF508 with different efficiencies. Indeed, the processing efficiency of CFTRΔF508 has been observed to vary in different tissues[35], and it will be interesting to determine whether increased processing efficiency correlates with reduced levels of CHIP expression. □

Methods

Antibodies.

Preparation of anti-CFTR, anti-Hsc70, anti-Hdj-2, anti-CHIP and anti-TR has been described[10,19,36]. The following antibodies were also used: mouse monoclonal anti-Hsp90 (H38220; Transduction Labs), rabbit polyclonal anti-calnexin (SPA860; Stressgen), mouse monoclonal anti-CFTR (MAB25031; R&D Systems), sheep polyclonal anti-ApoB (726494; Roche), mouse monoclonal anti-Myc (OP-10; Calbiochem), rabbit polyclonal anti-MDR (PC03; Calbiochem) and mouse monoclonal anti-20S Proteasome (PW8110; Affiniti Research). Anti-P115 was a gift from E. Stzul.

Cell transfection.

HEK293 cells (Microbix; PD0201), HeLa cells (ATCC; CCL-2) and Cos-7 (ATCC; CRL1651) cells were grown in six-well (35 mm per well) trays and transiently transfected using the Effectene (Qiagen) transfection reagent with the indicated amounts of pCDNA3.1 expression plasmids. Transfection conditions were optimized so that the efficiency was around 90, 60 and ~40% for the HEK293, HeLa and Cos-7 cells, respectively. HEK 293 cells (7.5×10^5 per well) were transfected for 3 h with 0.25 μg of CFTR or CFTR ΔF508 cDNA alone or in combination with cDNAs encoding CHIP or CHIP mutants. Transfected HEK293 cells were then incubated for 24 h in growth medium (DMEM + 10% fetal bovine serum + 1% penicillin and streptomycin) and used for further analysis. Cos-7 cells were transfected for 3 h with 1 μg of pEGFPC2-CFTR cDNA alone or in combination with 0.5 μg of pCDNA–Myc–CHIP followed by a 24-h post-transfection period in OptiMEM. Transfected HeLa cells were then infected with vaccinia virus expressing T7 RNA polymerase (vTF7.3) and cells were used 8 h after infection[19].

Indirect immunofluorescence.

Transfected Cos-7 cells were treated with 100 μm ALLN (Calbiochem) for 12 h before fixation to promote the formation of aggresomes[15]. Cells were washed twice with 1 ml of PBS and fixed with ice-cold absolute methanol for 10 min at –20 °C. Fixed cells were incubated with anti-Myc (1/100), anti-20S proteasome (1/100) or anti-Hsc70 (1/250) and then incubated with Texas red goat antimouse IgG (Molecular Probes). GFP–CFTR was imaged directly as indicated[14]. Images were collected and analysed using an Olympus IX70 epifluorescence microscope and processed with IP lab spectrum software.

Metabolic labelling and immunoprecipitation of CFTR.

Transfected HEK293 cells were starved of methionine in methionine-free DMEM (Life Technologies) for 1 h at 37 °C/5% CO_2 and metabolically labelled for the indicated time with Tran^{35}S-label (100 μCi per well; 1,200 Ci mmol^{-1}; ICN Radiochemicals). Cells were washed twice with 1 ml of PBS and lysed immediately or supplemented with methionine and cycloheximide (25 μg ml^{-1}) and incubated in DMEM for a chase period. Cell extracts for western blots or non-native immunoprecipitations were prepared in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.2% SDS, 50 mM HEPES pH 7.4). Cell extracts for co-immunoprecipitations were made under native conditions with PBS + 0.1% Triton X-100 (PBST). Before initiation of immunoprecipitation reactions, all lysates were precleared with Pansorbin (2% final; Calbiochem) for 10 min at 4 °C. Direct immunoprecipitations or co-immunoprecipitations were carried out as described[19]. Where indicated, re-immunoprecipitation of CFTR was carried out using anti-CFTR sera to verify the identity of CFTR isolated from chaperone complexes[19]. To determine whether expression of CHIP or CHIP truncations influenced the glycosylation of CFTR, immunoprecipitates were treated with PNGaseF to isolate a single species of CFTR. Deglycosylation was carried out a 25 °C for 1 h with 250 U PNGaseF in digestion buffer (50 mM sodium phosphate, pH 7.5, 1% NP-40, 10% glycerol, 0.5% SDS and 100 mM β-mercaptoethanol). Digestion reactions were stopped by the addition of SDS sample buffer and aliquots were analysed by SDS–PAGE and visualized by autoradiography.

Cell-free assay for CFTR ubiquitination.

Conditions for this assay have been described[37]. Briefly, 24 h after transfection, HEK293 cells were collected and resuspended in PB buffer (115 mM KOAc, 25 mM HEPES, pH 7.4, 5 mM $NaOAc_2$ and 0.5 mM EGTA) containing 0.025% digitonin (Waco), an ATP-regenerating system composed of 5 mM Mg-ATP, 80 mM creatine phosphate and 0.5 mg ml^{-1} creatine phosphokinase and 10 mM dithiothreitol. ^{125}I-labelled ubiquitin (300 μm; 13,000 c.p.m. per pM) was prepared using IODO-beads (Pierce). CHIP and CHIPΔUbox were purified as described[10]. Where indicated, buffer, purified CHIP (15 μm final), purified CHIP ΔU-box (15 μm final), 100 μm MG-132 or cold ubiquitin (3 mM final) was added to the reaction and incubated at 37 °C for 10 min. Conjugation reactions were stopped by the addition of SDS sample buffer. Solubilized CFTR-ubiquitin conjugates were diluted in RIPA buffer and isolated by immunoprecipitation as described above and then analysed by SDS–PAGE and fluorography.

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Correspondence and requests for materials should be addressed to D.M.C.