Endoplasmic Reticulum Protein Quality Control is Determined by Cooperative Interactions Between Hsp/c70 and the CHIP E3 Ligase

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Running title: Mechanism of CHIP/Hsc70-mediated CFTR degradation

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Background: The CHIP E3-ligase regulates Hsp70 pro-degradation activities.

Results: The P269A CHIP U-box mutation induces CHIP oligomerization and modulates nucleotide- and substrate-dependent interactions between the TPR domain and Hsp70 C-terminus.

Conclusions: The U-box domain plays a key role in CHIP recruitment to Hsp70-client complexes, possibly by controlling oligomerization.

Significance: Hsp70-CHIP substrate triage is governed by complex allosteric interactions between multiple domains in both proteins.

SUMMARY

The C-terminus of Hsp70 Interacting Protein (CHIP) E3 ligase functions as a key regulator of protein quality control by binding the C-terminal M/IEEVD peptide motif of Hsp/c70(90) with its N-terminal tetratricopeptide repeat (TPR) domain and facilitating poly ubiquitination of misfolded client proteins via its C-terminal catalytic U-box. Using CFTR as a model client, we recently showed that the duration of the Hsc70-client binding cycle is a primary determinant of stability. However, molecular features that control CHIP recruitment to Hsp/c70, and hence fate of the Hsp/c70 client, remain unknown. To understand how CHIP recognizes Hsp/c70, we utilized a dominant negative mutant in which loss of a conserved proline in the U-box domain (P269A) eliminates E3 ligase activity. In a cell-free reconstituted ERAD system, P269A CHIP inhibited Hsc70-dependent CFTR ubiquitination and degradation in a dose dependent manner. Optimal inhibition required both the TPR and U-box indicating cooperativity between the two domains. Neither wild-type nor the P269A mutant changed the extent of Hsc70 association with CFTR nor the dissociation rate of the Hsc70-CFTR complex. However, the U-box mutation stimulated CHIP binding to Hsc70 while promoting CHIP oligomerization. CHIP binding to Hsc70 binding was also stimulated by presence of an Hsc70 client with a preference for the ADP bound state. Thus, the Hsp/c70 M/IEEVD motif is not a simple anchor for the TPR domain. Rather CHIP recruitment involves reciprocal allosteric interactions between its TPR and U-box domains and the substrate binding- and C-terminal domains of Hsp/c70.

INTRODUCTION

In eukaryotic cells, protein synthesis, folding, and assembly is subject to a stringent quality control (QC) system charged with the role of degrading proteins that fail to achieve a native conformation (1-7). Such a system requires a selective mechanism to discriminate misfolded from properly folded substrates, tag those substrates that are misfolded, and deliver them to cellular degradation machinery.
Substrate recognition is mediated in part, by a network of cellular chaperones that includes the Hsp40, Hsp70 and Hsp90 heat shock protein families. In addition to facilitating folding, these chaperones recruit ubiquitin ligases (E3) and conjugating enzymes (E2) that covalently attach polyubiquitin chains and ultimately target the substrate for degradation by the 26S proteasome (8-10). Thus, the Hsp40-Hsp/c70 network directly facilitates both productive folding as well as substrate degradation. While the precise mechanisms that control these opposing roles remain poorly understood, the ultimate fate of client proteins involves selective and temporal recruitment of accessory co-chaperones that regulate the Hsp/c70 binding cycle (5, 11-15).

The C-terminus of Hsp70 Interacting Protein (CHIP) is a key component in Hsp/c70-mediated protein triage (16-18). CHIP contains three distinct domains: i) an N-terminal tetratricopeptide repeat (TPR) domain that binds the EEVD motif on the C-terminus of Hsp/c70, ii) a linker region involved in homodimerization, and iii) a C-terminal U-box domain with E3 ligase activity (16, 17, 19, 20). As such, CHIP provides a direct bridge between the Hsp40/Hsp/c70 chaperone network and the ubiquitin proteasome system (18). In combination with the E2 enzymes, UbcH5 and Ubc13 (21-24), CHIP is responsible for ubiquitinating proteins implicated in a variety of diseases that include cystic fibrosis, neurodegeneration, and cancer (17, 25, 26).

Despite its importance in protein triage, molecular interactions between CHIP and Hsp/c70 that regulate client binding, release and ubiquitination remain poorly understood (16, 22, 27-30). CHIP was originally reported to decrease Hsp70 substrate binding and Hsp70-mediated substrate refolding, as well as Hsp40 (Hdj-1 and Hdj-2)-stimulated ATPase activity (17, 19). In contrast, CHIP overexpression in heat stressed cells was subsequently shown to increase Hsp/c70-dependent protein refolding (31) and Hsp/c70-substrate binding (32). Thus, under different circumstances, CHIP appears to stabilize Hsp/c70-client complexes to either promote a productive folding outcome or recruit E2 ubiquitin conjugating enzymes. More recently, when examined in vitro using purified proteins, CHIP did not change either ADP dissociation from Hsp70, ATP binding to Hsp70, or the half-life of Hsp70-client complex (33). The latter findings suggested that CHIP acts in a relatively passive manner by stochastically ubiquitinating substrates as they cycle on and off Hsp/c70 during attempted folding. Consistent with this interpretation, we previously showed that CHIP-mediated ubiquitination was primarily dependent on the duration of Hsp/c70-client binding cycle and its regulation by nucleotide exchange factors such as Bag1 (15). Thus, multiple mechanisms appear to control formation and outcome of the ternary CHIP-Hsp/c70-client complex. Understanding how components of this complex are recruited and thereby control client fate remains a major challenge.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a well-studied substrate for ER quality control (34-38). CFTR is a member of the ATP Binding Cassette (ABC) transporter superfamily (ABCC7) and functions as a protein kinase A-regulated and ATP-dependent chloride channel at the apical membrane of epithelial cells. In most cell types, CFTR folding is intrinsically inefficient. Approximately 70% of wild-type CFTR and more than 99% of disease related trafficking mutants (the most common being ΔF508) is ubiquitinated, exported to the cytosol, and degraded by the UPS via ER-associated degradation (ERAD) (2, 5, 7, 34, 38). CHIP is among several E3 ligases including RMA1, Nedd4-2 and gp78 that facilitate ubiquitination of misfolded CFTR molecules (17, 22, 39-41).

To better understand how CHIP facilitates degradation, we examined a dominant negative mutant (P269A), which lacks a conserved proline in the U-box domain that is required for E3 ligase activity (22, 32, 42). Our approach was to use an in vitro translation and degradation system that recapitulates ERAD in a native-like cellular environment. Importantly, this system is readily amenable to biochemical manipulation and lacks compensatory transcriptional and translational mechanisms. It is thus possible to perturb chaperone levels and activities without secondary consequences that typically complicate similar maneuvers in intact cells (43-46). Moreover, because CFTR is the only radiolabeled client protein, it is relatively simple to characterize CHIP, Hsp/c70, and CFTR interactions that are responsible for ubiquitination and degradation. Our results show that CHIP binding to Hsp/c70 is influenced by allosteric interactions between the i) TPR and U-box domains of CHIP and ii) nucleotide/client-binding and C-terminal domains of Hsp/c70. These results indicate that CHIP-mediated triage of Hsp/c70 clients involves a complex interplay between multiple domains that
govern affinity of the TPR for the EEVD motif.

EXPERIMENTAL PROCEDURES

Plasmids

Bacteria expression plasmids pET-P269A-CHIP, pET-CHIP-TPR (amino acids 1–197), pET-CHIP-Ubox (amino acids 197–303), and pET-CHIP-P269A-Ubox were generated from wild-type (WT) pET30-CHIP (provided by D.M. Cyr; 22) by PCR amplification and ligation into NcoI and XhoI sites of the pET30 plasmid (22). A BamHI and XhoI fragment of CBag (47, 48) was generated by PCR and ligated into the same sites of pGEX4T.1 to obtain pGEX4T.1-CBag. All PCR-amplified sequences were confirmed by DNA sequencing. Others plasmids used were described previously (15).

His and GST-tag Protein Expression and Purification

Recombinant proteins were expressed in E. coli BL21(DE3) transformed with corresponding pET30 and pGEX4T.1 plasmids by induction with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (at A_{260} = 0.6) and incubation for 6 hours at 24 °C as described (15). Recombinant proteins were purified by TALON metal affinity or glutathione-uniflow resin (BD Biosciences, San Jose, CA) according to manufacturer’s instructions, concentrated using 10 kDa or 30 kDa cutoff Centricon filters (Millipore, Billerica, MA) with buffer replacement (protein storage buffer; 50 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, and 1 mM DTT), flash frozen, and stored at −80 °C. UbcH5α and Hdj-2 (49) were stored in the presence of 300 mM and 500 mM NaCl, respectively. Proteins were at least 90–95% pure as confirmed by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining (Fig. 1A). ATPase activity of purified His-Hsc70 was 0.36 (min⁻¹) in the absence of Hdj-2 and 0.96 (min⁻¹) in the presence of both Hdj-2 and CBag, in good agreement with previous reports (15, 48).

In vitro CFTR degradation

Microsomal membranes containing newly synthesized radiolabeled CFTR were added to RRL lacking endogenous hemin (60–70% v/v, prepared precisely as described, 15, 44, 50) and incubated at 37 °C. Recombinant proteins were added at concentration indicated. At times indicated, aliquots were precipitated in 20% trichloroacetic acid (TCA), centrifuged at 16,000g for 10 min and [35S]methionine in the supernatant (TCA sol) was counted in a Beckmann LS6500 scintillation counter (15). Total [35S] in each sample was determined by counting an aliquot of the degradation reaction. Mock reactions were used to correct for nonspecific association of [35S] and translation of endogenous mRNA remnants. The percent of protein degraded into TCA-soluble peptide fragments at each time point was determined by:

\[
\text{% TCA soluble} = \frac{(\text{CFTR}(T_n - T_0) - \text{Mock}(T_n - T_0))}{(\text{CFTR}(total - T_0) - \text{Mock}(total - T_0))} \times 100
\]

where \( T_n \) and \( T_0 \) were TCA-soluble counts at \( T = n \) and \( T = 0 \) min, respectively. Percent of degradation restored by addition of recombinant Hsc70, WT CHIP, and/or UbcH5α was determined at \( T = 60 \) min using the following formula:

\[
\text{% restoration} = \left[ \frac{(\% \text{TCA sol}_\text{restore} - \% \text{TCA sol}_\text{p269a})}{(\% \text{TCA sol} - \% \text{TCA sol}_\text{p269a})} \right] \times 100
\]

where \( \% \text{TCA sol} \) is control reaction without P269A CHIP, % TCA sol_p269a is reaction with P269A CHIP, and % TCA sol_restore is reaction with P269A CHIP and Hsc70, WT CHIP, and/or UbcH5α. Values represent mean ± s.e.m. of three or more experiments.

In vitro CFTR transcription and translation

CFTR RNA was transcribed from pSP-CFTR plasmid (15, 37, 50) at 40 °C for 2 hours as described previously (15), precipitated by LiCl, rinsed three times with 70% ethanol, and dissolved into ddH₂O. CFTR in vitro translation was performed at 24 °C for 2 hours in a reaction containing 50 ng/μl CFTR RNA, 40% (v/v) nuclease-treated rabbit reticulocyte lysate (RRL), canine pancreas microsomes (3 to 4 A_{260}) precisely as described (15). Following translation, microsomes were pelleted at 180,000g for 10 min, through 0.5 M sucrose in buffer A (50 mM HEPES-NaOH, pH 7.5, 100 mM KCl, 5 mM MgCl₂, and 1 mM DTT). The membrane pellet was rinsed once with 0.1 M sucrose in buffer A and resuspended in the same buffer at half volume of original translation reaction.

IC₅₀ of CFTR conversion into peptides for P269A CHIP and TPR domain

Apparent IC₅₀ values of CFTR conversion into peptides for P269A CHIP and TPR domain were obtained from a graphical fit of the data using the equation \( I = C/(IC₅₀ + C) \) where \( C \) is the concentration of CHIP (μM) and \( I \) is the inhibition fraction. \( I = 1 - \frac{[\% \text{TCA solCHIP}]}{[\% \text{TCA sol}]} \) where % TCA sol is obtained from the control reaction without CHIP and % TCA solCHIP is obtained from the parallel reaction containing P269A CHIP or CHIP (15).
Ubiquitination assay and immunoprecipitation.
Degradation reactions (10 µl) were diluted into 250 µl of buffer B (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) containing protease inhibitor (PI) mixture (Roche Applied Science, Indianapolis, IN) as described (15). For co-immunoprecipitation of Hsc70-CFTR complex, translation reaction or microsomes containing newly synthesized radiolabeled CFTR were diluted into 500 µl of buffer C (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing PI. For CFTR release from Hsc70, microsomes were incubated with 10 µM WT CHIP, P269A CHIP, or CBag in buffer A containing 0.1 M sucrose and 1 mM ATP at 24 °C. After incubation, 500 µl of ice cold buffer C containing 10 mM EDTA was added, and lysate was clarified at 16,000g at 4 °C for 20 min. Mouse anti-(mono-/poly-)ubiquitin antibody (FK2, Biomol International, Plymouth Meeting, PA) or rabbit anti-Hsp/c70 antisera (gift of Dr. William J. Welch, 51) was added. Samples were rotated for 1 hour at 4 °C, and 5 µl of ImmunoPure-immobilized Protein G (Pierce Biotechnology, Rockford, IL) or Affi-Gel Protein A (BioRad Laboratories, Hercules, CA) was added and rotated overnight. Samples were washed 5 times with buffer B or C and three times with Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, and 137 mM NaCl) and eluted with SDS sample buffer. Eluates were separated on an SDS-PAGE gel and analyzed by phosphorimaging as described (15).

Immunoblotting
Immunoblotting was performed as described (15, 52). Briefly, proteins were transferred to a PVDF membrane (BioRad Laboratories, Hercules, CA) after SDS-PAGE and blocked with 5% (w/v) skim milk in TTBS (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20) for 1 hour prior to incubation with mouse anti-Hsp/c70 antibody (N-27, gift of Dr. W. J. Welch, 51), 1:5,000 or rabbit anti-CHIP (PC-711, EMD Chemicals, Gibbstown, NJ), 1:5,000. Membrane was washed five times with TTBS and was incubated for 1 hour with goat antimouse IgG-HRP (BioRad Laboratories, Hercules, CA), 1:5,000 or goat anti-rabbit IgG-HRP (BioRad Laboratories, Hercules, CA), 1:10,000. Membranes were washed again with TTBS, and proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA) and Fuji Film (Light Labs, Dallas, Texas).

His-CHIP pull down
His-CHIP or His-P269A CHIP (25 µg) was loaded onto 10 µl of Ni-NTA beads (Qiagen, Valencia, CA) in buffer D (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 0.1% Triton X-100). Samples were rotated at 4 °C for 1 hour and washed 4 times with buffer D. Translation reaction or lystate microsomes (as above) were then added to the beads in buffer C (final volume 250 µl) and rotated at 4 °C for 2 hours. Beads were washed and eluted as described above, and eluates were separated on an SDS-PAGE gel and analyzed by CBB staining (His-CHIP) or phosphorimaging (CFTR). For interaction of His-CHIP with RRL Hsc70, 50 µl of RRL or desalted RRL (using PD-10 Desalting Column, GE Healthcare Biosciences, Pittsburgh, PA, pre-equilibrated in 10 mM HEPES-NaOH, pH7.5) was added to CHIP-containing beads in buffer D with 5 mM MgCl$_2$ (final volume 60 µl) and mixed in the presence or absence of 3 mM ATP or ADP at 4 °C for 4 hours. Beads were washed 6 times with buffer D containing 20 mM imidazole and eluted with 500 mM imidazole in TBS. Eluates were separated on an SDS-PAGE gel and subjected to CBB staining (His-CHIP) or immunoblotting (Hsc70).

CHIP binding to client-bound Hsc70
Two nmol of biotinylated G17A peptide (GenScript, Piscataway, NJ), corresponding to residues Gly545 to Ala561 in human CFTR, (53) were loaded on 10 µl of NeutrAvidin agarose (Thermo Scientific, Waltham, MA) in TBS and rotated at 24 °C for 1 hour. Beads were washed three times with TBS and incubated with 500 pmol of GST-Hsc70 in 50 µl of buffer E (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 5 mM MgCl$_2$, 1 mM ATP, 0.1% Triton X-100) at 24 °C for 10 min. ATP was depleted by addition of hexokinase (20 U/ml) and 2-deoxyglucose (20 mM) to stabilize Hsc70 binding. Beads were then washed three times with buffer D containing 10 mM EDTA prior to addition of 12.5 pmol of His-CHIP or His-P269A CHIP (final volume 50 µl) and incubation at 4 °C for 1 hour. Beads were washed 5 times with buffer D, three times with TBS, prior to elution, SDS-PAGE and immunoblotting.

GST-Hsc70 pull down
GST or GST-Hsc70 (200 pmol) were loaded on 10 µl of glutathione-unicflow resin (BD Biosciences, San Jose, CA) in buffer E containing 1 mM ATP, rotated at 4 °C for 1 hour and washed 4 times with wash
buffer E containing 1 mM ATP. His-CIP or His-P269A CHIP (200 pmol) was added (final volume 50 µl) and beads rotated at 4 °C for 1 hour, were washed 5 times with buffer D, 3 times with TBS, eluted with SDS sample buffer and analyzed by SDS-PAGE and immunoblotting.

Analysis of protein conformation
Glutaraldehyde Crosslinking - WT or P269A CHIP (2 µg) was incubated with 0.025% (v/v) glutaraldehyde in 10 µl of protein storage buffer for 10 min at 30 °C (20, 32). The cross-linking reaction was stopped by the addition of SDS sample buffer and analyzed by 10% SDS-PAGE followed by CBB staining.

Blue Native PAGE - WT or P269A CHIP (5 µg) was mixed with Blue-Native sample buffer (0.5% (w/v) CBB-G250, 10 mM Bis Tris-HCl, pH 7.0, 50 mM aminocaproic acid, 1% (w/v) n-Dodecyl β-D-maltoside, 5% (w/v) sucrose, final volume 20 µl) and applied on a NativePAGE Novex 4-16% Bis-Tris gel (Life Technologies, Grand Island, NY). NativeMark unstained protein standard (Life Technologies, Grand Island, NY) was used for molecular weight estimation. Electrophoresis was carried out at 4°C at 150 V for 45 min (cathode buffer = 50 mM Tricine, 15 mM BisTris-HCl, pH 7.0, and 0.02% CBB-G250, anode buffer = 50 mM Bis Tris-HCl, pH 7.0 and then at 250 V for 45 min after reducing CBB-G250 dye concentration to 0.002% (9). After running, the gel was stained with CBB.

Limited proteolysis - WT or P269A CHIP (4 µg) was incubated with indicated concentration of proteinase K in 10 µl of protein storage buffer for 10 min on ice. Reaction was stopped by the addition of 2 mM PMSF and boiled with SDS sample buffer. Aliquots were analyzed by 12% SDS-PAGE and CBB staining.

RESULTS
P269A CHIP Inhibits Hsc70-dependent CFTR Ubiquitination and Degradation
To investigate the mechanism of CHIP-mediated client recognition, CFTR was expressed in a cell-free rabbit reticulocyte lysate (RRL) translation/degradation system in the presence of ER microsomes and [35S]methionine (15, 37, 50). In this system, CFTR is primarily generated as a full length, ~160 kDa radiolabeled protein that is membrane integrated and contains both non-glycosylated and core-glycosylated forms (37 and data not shown). Extensive studies in our laboratory have shown that CFTR ubiquitination is inefficient during translation at 24°C, which allows us to temporally separate protein synthesis from subsequent degradation-related events (15, 37, 50, 54). However, when ER microsomes containing newly synthesized CFTR are isolated and incubated at 37 °C in RRL lacking exogenous hemin, CFTR is rapidly converted into a high molecular weight ubiquitinated species and subsequently degraded into TCA soluble peptide fragments by the 26S proteasome (Fig. 1B, C and 15, 37). Addition of recombinant P269A CHIP (Fig. 1A) stabilized full-length CFTR (Fig. 1B, lanes 5-12) and inhibited CFTR cleavage in a dose dependent manner (Fig. 1D). In contrast, addition of wild type (WT) CHIP had only a marginal effect in increasing CFTR degradation (1.25-fold) (Fig. 1C, E), which likely reflects the robust degradation activity of RRL where CHIP is not a limiting factor. The IC50 of P269A CHIP (2.5 µM) is similar to the Hsc70 concentration in RRL (2 µM) (Fig. 1D, F, and 15), although actual stoichiometry depends on protein oligomerization status (see Fig 3).

CFTR immunoprecipitation using anti-ubiquitin antibody demonstrated that P269A CHIP also decreased substrate ubiquitination both in the presence and absence of the proteasome inhibitor MG132 (Fig. 1G). The inhibitory effect P269A CHIP was restored to 77% of control level by re-addition of purified recombinant Hsp70, WT CHIP, and UbcH5a proteins (Fig. 1H and I). Note that the Hsc70 is the dominant Hsp70 family member in RRL and is referred to as such in these experiments. Thus, consistent with cell-based studies, P269A CHIP acts in a dominant negative manner in vitro to inhibit Hsc70-dependent CFTR ubiquitination and degradation.

Both the U-box and TPR Domain of P269A CHIP are Required for Optimal inhibition of CFTR Degradation.

We next used recombinant U-box and TPR domains from WT and P269A CHIP to determine which domain(s) were responsible for inhibiting degradation (17, 20, 22, Fig. 2A). As expected, the isolated U-box domain from either WT or P269A CHIP failed to inhibit CFTR degradation (Fig. 2B, C). In contrast, addition of the TPR domain decreased CFTR degradation (Fig. 2D) as has been shown previously in intact cells (17). Surprisingly though, the TPR construct was nearly 20 fold less potent than full length P269A CHIP (apparent IC50 of 36 µM, Fig. 1F and 2E). Note that this effect is unlikely to be due to TPR degradation in RRL as the recombinant
protein is present in approximately 1000 fold excess of our typical degradation substrates (e.g. CFTR). These results indicate that the TPR domain of P269A CHIP does not compete for Hsc70 binding in a simple manner with the TPR from WT CHIP. Rather, inhibition appears to involve a more complex interaction with the mutant U-box domain.

**The P269A Mutation Alters CHIP Conformation and Stimulates Oligomerization**

How might P269A exert its effect on other CHIP domains? Several studies have shown the functional importance of CHIP oligomerization. CHIP is natively dimeric, and dimerization of CHIP is essential for the E3 ubiquitin ligase activity (20). Heat treatment induces CHIP oligomerization and promotes chaperone function (32). We therefore investigated the oligomeric state of WT and P269A CHIP by chemical crosslinking (20, 32) and Blue Native (BN)-PAGE. Prior to crosslinking, both proteins migrated as ~40 kDa monomers on SDS-PAGE (Fig. 3A). After crosslinking WT CHIP migrated primarily as an ~80 kDa dimer with some larger oligomeric forms (~250 kDa) as reported (20, 32). P269A CHIP was also oligomeric, but yielded slightly less crosslinked dimer than WT (Fig. 3A, lane 4). BN-PAGE (in the absence of crosslinker) further indicated that both proteins are oligomeric in solution, although P269A CHIP was predominantly found to reside in a larger complex (Form B) than WT CHIP (Form A) (Fig. 3B). Accounting for theoretical molecular weight of CHIP dimer (80 kDa), it is likely that CHIP migrates aberrantly on Blue-Native PAGE, similar to its reported behavior during gel filtration (20), thereby making it difficult to determine the precise stoichiometry. However, P269A appears to stimulate formation of larger (and/or more stable) oligomers. Finally, P269A CHIP was more susceptible to limited Proteinase K digestion as demonstrated by early cleavage of a major 35 kDa proteolytic fragment generated from WT protein (Fig. 3C). Thus, in addition stimulating formation of large oligomeric complexes, P269A alters CHIP structure by inducing a more protease accessible (open conformation).

**P269A CHIP Does Not Affect CFTR Association with Hsc70 or Kinetics of Hsc70-CFTR Release**

CHIP has been reported to affect Hsp/c70-client binding, Hsp/c70 mediated ATP hydrolysis, and client refolding (19, 32). We therefore tested whether the P269A mutation interfered with Hsc70 binding to CFTR by co-immunoprecipitation from RRL using anti Hsp/c70 antisera. Results, shown in Figure 4 (panels A and B) demonstrate that addition of either WT or P269A CHIP at levels that inhibit degradation (10 µM) had no detectable effect on the amount of CFTR bound to Hsc70 under basal conditions. In contrast, addition of the C-terminal domain of Bag-1 (termed CBag, 47, 55), which stimulates Hsc70 ADP-ATP exchange, markedly reduced CFTR-Hsc70 binding as shown previously (Fig. 4A and B, 15).

CHIP was originally shown to suppress Hsp40-stimulated Hsp70 ATPase activity and to stimulate client binding to Hsp70 (17, 19). These results suggested that it might increase stability of the Hsc70-client complex by reducing turnover of the Hsp70 binding cycle. We therefore tested whether CHIP might alter the kinetics of CFTR release from Hsc70, which is triggered by ADP-ATP exchange. When ER microsomes containing Hsc70-CFTR complexes were isolated from RRL in the absence of ATP, CFTR remained stably bound to Hsc70. In contrast, ATP addition resulted in dissociation of Hsc70, which was further stimulated by addition of CBag (Fig. 4C and D) consistent with previous results (15). Importantly, addition of WT or P269A CHIP (plus ATP) had no effect on either the amount of CFTR bound, or the rate of Hsc70-CFTR dissociation above that observed for ATP alone (Fig. 4C and D). Similarly, the steady state ATPase activity of Hsc70 (in the presence or absence of Hdj-2) was also not affected by addition of equimolar WT or P269A mutant CHIP (data not shown). These results indicate that P269A CHIP inhibits CFTR degradation by a mechanism other than altering stability of Hsc70-CFTR interactions or duration of Hsc70-CFTR binding cycle.

**P269A CHIP Recognizes CFTR-Hsc70 Complex More Efficiently than WT CHIP**

Because TPR and U-box domains are both required to optimally inhibit CFTR degradation, we next tested whether the P269A mutation might allosterically alter affinity of the TPR for the Hsc70-CFTR complex. Microsomes containing newly synthesized, radiolabeled CFTR were isolated from RRL either after ATP depletion (to stabilize Hsc70 binding) or after incubation with ATP and CBag (to release Hsc70 from CFTR, Fig. 5A, lanes 2 and 4, respectively). Following solubilization, samples were then incubated with excess His-tagged WT CHIP or P269A CHIP, and bound CFTR was isolated using nickel-NTA affinity resin. In the absence of ATP,
approximately 4 times more CFTR was recovered with P269A CHIP than WT CHIP under identical conditions (Fig. 5B lane 1-3 and Fig. 5C). Addition of ATP and CBag released most CFTR from Hsc70, although a small amount of CFTR was still pulled down by P269A CHIP but not WT CHIP (Fig. 5B lanes 5, 6).

To confirm that CHIP does not bind CFTR directly, microsomes containing Hsc70-CFTR complexes were isolated and incubated for 3 min in either the absence of ATP or presence of CBag plus ATP, and samples were affinity purified with His-CHIP as described above (Fig. 5D). Again, several fold more CFTR was recovered by P269A CHIP than WT CHIP in the ATP-depleted samples (Fig. 5D, lanes 2 and 3), whereas Hsc70 release prior to addition of CHIP eliminated CFTR recovery by WT CHIP and markedly decreased CFTR pull-down for the P269A mutant (Fig. 5D lanes 5 and 6). Thus, CHIP binding to CFTR occurs indirectly via ATP-sensitive interactions, likely with the Hsc70-CFTR complex. This interaction is strongly increased by the P269A U-box mutation.

**The P269A Mutation Increases Hsc70 Binding in a Nucleotide-Dependent Manner**

Results of Figure 5 suggest that the dominant negative phenotype exhibited by P269A CHIP is due at least in part to an increased affinity for Hsc70. We therefore immobilized recombinant WT and mutant His-CHIP as bait to pull down endogenous Hsc70 from RRL. Unexpectedly, P269A CHIP exhibited only a modest increase (1.5-fold) in Hsc70 binding compared to WT CHIP (Fig. 6A and B). However, RRL likely contains a mixture of client-bound and unbound Hsc70 in various states of nucleotide occupancy. RRL was therefore desalted by gel filtration to remove endogenous nucleotides and then supplemented with ATP (to promote client release), ADP, or no nucleotide to stabilize client binding. In the presence of excess ATP, P269A CHIP bound Hsc70 1.5 fold better than WT CHIP (Fig. 6C lanes 1 to 3 and Fig. 6D). In the absence of nucleotide, there was an increase in baseline Hsc70 recovery for WT CHIP and this effect was further accentuated for mutant CHIP (Fig. 6C, lanes 4 to 6 and Fig. 6D). Interestingly, addition of ADP, which is expected to stabilize Hsc70-client complexes, resulted in nearly a five fold increase in Hsc70 binding to mutant over WT CHIP (Fig. 6C, lanes 7 to 9 and Fig. 6D). It is not clear why Hsc70 binding to WT CHIP was reduced in the presence of ADP, but this effect may reflect heterogeneity of client complexes or Hsc70 regulatory co-factors. Taken together, these results provide evidence that nucleotide occupancy of the N-terminal domain of Hsc70 allosterically influences the affinity of its C-terminal EEVD motif for CHIP, and this effect is more pronounced for U-box mutant, which strongly favors the ADP bound conformation.

**Hsc70 Client loading Stimulates P269A CHIP binding**

Given that Hsc70 client binding is stabilized by ADP, one possibility is that affinity of the CHIP TPR domain for Hsc70 is influenced by occupancy of the substrate binding cleft. Because it is difficult to determine the fraction of Hsc70 that is bound to clients in cytosolic extracts, we tested this hypothesis using recombinant proteins and a known peptide substrate derived from NBD1 of CFTR (Gly545 to Ala561, G17A peptide) (53). Biotinylated G17A peptide was immobilized on NeutraAvidin agarose, and beads were incubated with purified recombinant GST-Hsc70 protein (Fig. 7A). To facilitate client loading, binding was initiated in the presence of ATP for 10 min and then treated with hexokinase to convert remaining nucleotide to ADP (ATP depletion) or ATP-containing buffer (Fig. 7B). Hsc70 binding to beads was undetectable in the absence of peptide, whereas binding was readily observed in following ATP depletion as expected (Fig. 7B). This observation allowed us to compare the interaction of WT and P269A CHIP specifically with client-bound Hsc70 (Fig. 7C). Neither form of CHIP bound to peptide in the absence of Hsc70. However, both forms were recovered from the Hsc70-client complex, with three fold more mutant CHIP was recovered than WT CHIP (Fig. 7C), which is in good agreement with the increase in CFTR recovery by mutant CHIP (Fig. 6B and C).

To further confirm that the U-box mutation selectively increases affinity of CHIP to the client-bound form of Hsc70, GST-Hsc70 was immobilized on glutathione resin in the presence of ATP to remove residual client proteins. Beads were then incubated with WT or P269A CHIP. Results reveal that in the absence of an Hsc70 client P269A CHIP binding was only slightly increased (~1.5 fold) over WT CHIP (Fig. 7D), which is similar to the difference observed for CHIP binding to Hsc70 in RRL (Fig. 6C and D).

**DISCUSSION**

In this study, we used a reconstituted cell free system to better understand how CHIP-Hsp/c7o-
client complex formation contributes to quality control of a prototypical ERAD substrate. Our results demonstrate that dominant negative P269A CHIP, which lacks E3 ligase activity (22, 32, 42), strongly inhibits Hsc70-dependent CFTR ubiquitination and degradation. Interestingly, inhibition does not occur via a simple competition with the WT CHIP TPR domain (56, 57). Rather, the U-box mutation increases the efficiency of CHIP binding to Hsc70 (presumably to the C-terminal EEVD motif) over that of WT CHIP without affecting the rate of Hsc70-mediated ATP hydrolysis or the kinetics of ATP-mediated client release. This effect was also observed using recombinant components in the absence of ubiquitin and E2 enzymes. Thus, CHIP binding to Hsc70 is subject to allosteric interactions between the TPR and U-box domains independent of substrate ubiquitination. These functional findings raise the possibility that the U-box contributes to induced unfolding of the highly flexible TPR domain during binding to the EEVD peptide motif (57). Consistent with this notion, limited proteolysis revealed that P269A does not simply disrupt catalytic activity, but also induces structural changes that result in a more open (protease sensitive) conformation. Surprisingly, this structural change was also associated with formation of large oligomers with increased affinity for Hsc70.

A second finding was that the preferential interaction of Hsc70 with P269A CHIP is dependent upon occupancy of the Hsc70 client-binding cleft. This was observed both by manipulating ADP and ATP availability in RRL, which mimics native cytosolic conditions, and with a defined peptide substrate using recombinant proteins. Given the dynamic nature of the Hsp/c70 binding cycle, our findings impose an additional layer of control on CHIP recruitment that depends not only on the interplay between TPR and U-box domains of CHIP, but also on cross talk between client binding domain, the C-terminus, and possibly the ATPase domain of Hsc70. Thus formation and stability of CHIP-Hsc70-client complexes does not necessarily result from non-specific binding as has been suggested (58), but rather involves multiple domains in both proteins, and potentially CHIP oligomerization, that regulates TPR-EEVD affinity.

CHIP was originally identified as a co-chaperone and negative regulator of Hsp/c70 (19) and later shown to function as a U-box ubiquitin E3 ligase for a variety of Hsp/c70 client proteins (16, 17). For the majority of substrates, CHIP-mediated ubiquitination is obligatorily coupled to Hsp70 and thus is dependent on the Hsp/c70 binding cycle (22, 27-30). In this cycle, Hsp/c70 recruits clients in its ATP-bound state and then forms a stable complex upon ATP hydrolysis. Substrate release is stimulated by spontaneous or nucleotide exchange factor (NEF)-mediated ATP/ADP exchange (5, 55). Although early studies suggested that CHIP suppresses Hsp40-stimulated Hsp/c70 ATPase activity and inhibits Hsp/c70 client binding (17, 19), our results show that for CFTR, it does not affect either Hsc70 association (Fig. 4A and B) or duration of Hsc70-CFTR binding in a single release cycle (Fig. 4C and D). These findings are consistent with recent studies from the Mayer group which concluded that recruitment of CHIP to Hsp70-client complexes is a decisive factor in triaging pro-folding from pro-degradation outcomes (33). However, our results provide evidence that CHIP recruitment to Hsc70 is not solely a passive process, but one that can be actively modulated by client binding (shown here) in addition to posttranslational modification (e.g. phosphorylation) (59). Thus, it would follow logically that increased affinity for the Hsp70-client complex, over Hsc70 alone, would enable CHIP to more efficiently interact with the cellular pool of Hsp/c70 that is actively engaged with unfolded potential ubiquitination targets (33).

An important objective in protein folding diseases such as cystic fibrosis is to devise strategies in which misfolded, but potentially functional, proteins can be rescued from ERAD and delivered to their cellular site of function. Because of its central role in both folding and degradation, the Hsp40/70 network provides a potential target for such a manipulation (14). Indeed, evidence suggests that blocking Hsp/c70 function (60-63) or manipulating Hsp40 cochaperones (11, 12, 64, 65) can protect a subpopulation of newly synthesized CFTR from degradation. Consistent with this, we recently showed that the duration of Hsc70-client binding cycle is a major determinant of CFTR ubiquitination and degradation (15). It is difficult however, to manipulate Hsp/c70 function in intact cells due to the high expression levels and compensatory effects on the proteostatic network. Moreover, while Hsp/c70 inhibition might decrease degradation, it would also likely have broad and potentially deleterious effects on the cellular folding environment. In this respect selectively targeting the degradation arm of Hsp/c70 through CHIP could potentially accomplish this goal.
while leaving pro-folding activities intact. Such a strategy might be selective block CHIP interactions, while preserving interactions with other co-chaperones that compete for the EEVD binding (e.g. Hop) and recruitment of the Hsp90 maturation complex (57, 66). Although CHIP and Hop bind the EEVD motif with similar affinity (57, 58), the finding that substrate and nucleotide may modulate this process raises the possibility that specific substrate properties might impact which co-chaperones are ultimately recruited and hence, the fate of the ternary complex.

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ABREVIATIONS USED: CBag, C-terminal domain of Bag-1; CFTR, cystic fibrosis transmembrane conductance regulator; CHIP, C-terminal Hsc70-interacting protein; ER, endoplasmic reticulum, ERAD; endoplasmic reticulum-associated degradation; RRL, rabbit reticulocyte lysate; TCA, trichloroacetic acid; TPR, tetratricopeptide repeat.

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FIGURE LEGENDS

Figure 1. P269A CHIP inhibits CFTR ubiquitination and degradation in vitro. (A) Recombinant His-tagged proteins. Each His-tagged protein was purified, and 1 µg was separated on SDS-PAGE and stained with Coomassie Bryant Blue (CBB). (B) CFTR was translated in RRL in the presence of canine pancreas rough microsomes and [35S]Met. Pelleted microsomes were incubated in an in vitro degradation reaction in the absence (left) or presence of 10 µM WT (middle) or P269A CHIP (right). Aliquots were analyzed by SDS-PAGE and phosphorimaging at times indicated. (C, D) CFTR conversion to TCA soluble fragments in the presence and absence of WT CHIP (C) or P269A CHIP (D). (Mean ± s.e.m. n = 3-4). (E) CFTR conversion into TCA soluble fragments at 60 min in the presence of indicated concentration of WT CHIP normalized to control reaction without CHIP. (F) Apparent IC50 Values of P269A CHIP. Data shows the fraction of CFTR converted into TCA-soluble peptides that was inhibited by addition of P269A CHIP as determined in EXPERIMENTAL PROCEDURES. (G) Phosphorimage of in vitro synthesized CFTR subjected to in vitro degradation for 1 hr in the presence of 10 µM P269A CHIP and/or 100 µM MG132. Samples were immunoprecipitated with anti-ubiquitin antibody (FK2) or non immune sera (NIS) prior to SDS-PAGE. Right gel shows 20% input for immunoprecipitation. (H) CFTR degradation in the absence (control) or presence of 3 µM P269A CHIP and combination of 10 µM Hsc70, WT CHIP, and/or UbcH5a as indicated. Graph shows percent of CFTR converted to TCA-soluble fragments at each point (mean ± s.e.m. n = 3-5). (I) Quantification of data as in panel E showing the extent of degradation inhibition by P269A CHIP that was restored by addition of recombinant protein (mean ± s.e.m. n = 3-5).
Figure 2. U-box and TPR domains are both required for optimal inhibition of CFTR degradation. (A) Diagram of CHIP, U-box, and TPR constructs showing location of P269A mutation. (B-D) CFTR degradation (as in Figure 1) in the absence (control) and presence of recombinant WT U-box (B), or P269A U-box proteins (C), or TPR (D) (mean ± s.e.m. n = 3). (E) Apparent IC\textsubscript{50} Values of TPR. Data shows the fraction of CFTR converted into TCA-soluble peptides that was inhibited by addition of TPR domain.

Figure 3. P269A CHIP forms higher order oligomers than wild-type CHIP
(A) His-tagged WT or P269A CHIP (5 μM) was incubated with glutaraldehyde for 10 min at 30 °C. The cross-linking reaction was stopped by the addition of SDS sample buffer and analyzed by 12% SDS-PAGE followed by CBB staining. (B) WT or P269A CHIP was analyzed by 4-16% Blue-Native PAGE followed by CBB staining. (C) WT or P269A CHIP (10 μM) was incubated with indicated concentration of proteinase K for 10 min on ice. Reaction was stopped by the addition of PMSF and boiled with SDS sample buffer. Aliquots were analyzed by 12% SDS-PAGE and CBB staining.

Figure 4. CHIP does not affect Hsc70-CFTR binding or kinetics of dissociation. (A) Following CFTR synthesis, WT CHIP, P269A CHIP, or CBag was added to the translation reaction, and microsomes were collected, solubilized, and immunoprecipitated with non immune sera (NIS) or anti-Hsc70 antisera prior to SDS-PAGE and phosphorimaging. (B) Quantification of CFTR-Hsc70 co-immunoprecipitation as shown in panel A (mean ± s.e.m. n = 3-4). (C) Microsomes containing newly synthesized CFTR were solubilized in Triton X-100 and immunoprecipitated with anti-Hsc70 antisera 0, 1 or 3 min after addition of ATP, WT CHIP, P269A CHIP, or CBag as indicated. (D) Quantification of co-immunoprecipitation as shown in panel C (mean ± s.e.m. n = 3-4).

Figure 5. P269A increases CHIP binding to Hsc70-CFTR complex. (A) CFTR was synthesized in RRL and treated with hexokinase/2-deoxy glucose (-ATP) or ATP and/or GST-CBag for 10 min. Microsomes were then isolated, solubilized, and immunoprecipitated with non immune sera (NIS) or anti-Hsc70 antisera. (B) Following synthesis, the translation reaction was incubated with hexokinase/2-deoxyglucose or GST-CBag plus ATP as in panel A. Microsomes were collected, solubilized, and CFTR was affinity purified with immobilized His-tagged WT CHIP, P269A CHIP, or Ni-NTA (Ni, control). (C) Quantification of experiments as shown in panels B (mean ± s.e.m. n = 3). (D) Microsomes were isolated after ATP depletion as in panel A, and further incubated for 3 min in buffer (without ATP) or in the presence of CBag plus ATP to remove bound Hsc70, prior to affinity pull-down with His-tagged WT or P269A CHIP.

Figure 6. P269A CHIP preferentially binds ADP-Hsc70. (A) Hsc70 was recovered from RRL using immobilized His-tagged WT or P269A CHIP, or Ni-NTA (Ni). (B) Quantification of experiments as shown in panel A (mean ± s.e.m. n = 4). (C) Hsc70 was affinity isolated as in panel A, but using desalted RRL supplemented with ATP, ADP, or no nucleotide as indicated. Recovered Hsc70 was detected by immunoblotting. (D) Quantification of experiments as shown in panel C (mean ± s.e.m. n = 4).

Figure 7. P269A CHIP preferentially binds client-bound Hsc70. (A) Schematic of strategy to isolate client-bound Hsc70. (B) Recombinant GST-Hsc70 was incubated with biotinylated G17A peptide immobilized on NeutrAvidin agarose in the presence of ATP. After 10 min incubation, ATP was depleted or sample was diluted with buffer containing ATP to release client. Bound GST-Hsc70 was eluted with SDS and immunoblotted with anti-Hsc70 antisera. (C) In vitro binding assay between G17A-bound GST-Hsc70 and CHIP. His-tagged WT CHIP or P269A CHIP was incubated with immobilized G17A peptide-bound GST-Hsc70. After washing, His-CHIP and Hsc70 were eluted with SDS and analyzed by immunoblotting. (D) Binding assay between client-free GST-Hsc70 and WT or P269A CHIP. His-tagged WT or P269A CHIP was incubated with GST (control) or GST-Hsc70 immobilized on glutathione resin. After washing, His-CHIP was eluted with SDS and analyzed immunoblotting. Parallel immunoblotting with anti-Hsc70 confirmed equal loading of GST-Hsc70. Graphs show mean +/- s.e.m. (n ≥3)
Figure 1 Matsumura and Skach

A. His-tagged proteins

B. CFTR degradation

C. WT CHIP

D. P269A CHIP

E. WT CHIP

F. P269A CHIP

G. Input (Short exposure)

H. TCA-soluble (% total)

I. Restoration (%)
Figure 3 Matsumura and Skach

A

B

C

Figure legend:


B: BN-PAGE analysis showing WT and P269A mutants in different forms.

C: Western blot analysis of WT and P269A mutants at various concentrations (μg/ml) of GA.
Figure 4 Matsumura and Skach

A

IP: Hsc70
- + - + -
WT CHIP
- P269A CHIP
- CBag
NIS
kDa
220 - 97 - 66 -
1 2 3 4 5

CFTR

B

WT CHIP
P269A CHIP
CBag

CFTR CoIP with Hsc70

C

IP: Hsc70
0 1 3 min
-ATP
+ATP
WT CHIP + ATP
P269A CHIP + ATP
CBag + ATP

CFTR

D

WT CHIP
P269A CHIP
CBag

CFTR CoIP with Hsc70

-ATP
+ATP

Release (min)
0 1 2 3
0 0.25 0.5 0.75 1
1.25
0.75
0.5
0.25

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Figure 5 Matsumura and Skach

A - CoIP: Hsc70
- + + ATP
- + GST-CBag

B - Cytosol + microsomes
His-CHIP pull down
- ATP
+ ATP + CBag

C - CFTR pull-down with CHIP
CBB staining

D - Microsomes
His-CHIP pull down
- ATP
+ ATP + CBag

CFTR pull-down with CHIP
CBB staining

Microsomes
Figure 6 Matsumura and Skach

A

C

D

His-CHIP pull down

IB: Hsc70
CBB staining

1 2 3

WT P269A

Hsc70 binding (%)

WT P269A

His-CHIP pull down

IB: Hsc70
CBB staining

1 2 3 4 5 6 7 8 9

WT P269A

Hsc70 binding (%)

WT P269A

WT P269A

- +ADP

+ATP

- +ATP

- +ADP

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Figure 7 Matsumura and Skach

A

G17A

ATP
deflection

G17A

ATP

ADP

Biotin

GST-Hsc70

B

ATP addition

ATP depletion

C

G17A

Biotin

ADP

C

CHIP

D

GST

ATP

C

CHIP

CHIP binding (%)

IB: Hsc70

IB: CHIP

WT

P269A

WT

P269A

WT

P269A
Endoplasmic reticulum protein quality control is determined by cooperative interactions between Hsp/c70 and the CHIP E3 ligase

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