Cell

Sequential Quality-Control Checkpoints Triage Misfolded Cystic Fibrosis Transmembrane Conductance Regulator

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DOI 10.1016/j.cell.2006.06.041

SUMMARY

Cystic fibrosis arises from the misfolding and premature degradation of CFTRΔF508, a Cl^- ion channel with a single amino acid deletion. Yet, the quality-control machinery that selects CFTRΔF508 for degradation and the mechanism for its misfolding are not well defined. We identified an ER membrane-associated ubiquitin ligase complex containing the E3 RMA1, the E2 Ubc6e, and Derlin-1 that cooperates with the cytosolic Hsc70/CHIP E3 complex to triage CFTR and CFTRΔF508. Derlin-1 serves to retain CFTR in the ER membrane and interacts with RMA1 and Ubc6e to promote CFTR's proteasomal degradation. RMA1 is capable of recognizing folding defects in CFTRΔF508 coincident with translation, whereas the CHIP E3 appears to act posttranslationally. A folding defect in CFTRΔF508 detected by RMA1 involves the inability of CFTR's second membrane-spanning domain to productively interact with amino-terminal domains. Thus, the RMA1 and CHIP E3 ubiquitin ligases act sequentially in ER membrane and cytosol to monitor the folding status of CFTR and CFTRΔF508.

INTRODUCTION

CFTR is a Cl^- ion channel that is localized to the apical surface of epithelial cells that line ducts and lung airways (Riordan et al., 1989). Around 90% of cystic fibrosis (CF) patients inherit at least one copy of the CFTRΔF508 allele, which is missing F508 from nucleotide binding domain (NBD) I. CFTRΔF508 is synthesized and properly inserted into the membrane of the endoplasmic reticulum (ER), but it fails to reach the native state and accumulates in a kinetically trapped, but foldable, conformation (Cyr, 2005; Younger et al., 2004). Kinetically trapped CFTRΔF508 is recognized by the ER quality-control machinery (ERQC), polyubiquitinated, retrotranslocated, and degraded by the proteasome (Cheng et al., 1990; Denning et al., 1992; Jensen et al., 1995; Kreda et al., 2005; Meacham et al., 2001; Ward et al., 1995). The network of quality-control (QC) factors that triages nascent CFTR and other ER proteins is not well defined, but is generally known as the ER-associated degradation pathway (ERAD) (McCracken and Brodsky, 1996). Loss of CFTR function at the cell surface is the cause for mortality in CF patients because it alters the hydration of the mucosal layer that lines airway epithelia and gives rise to persistent microbial infections with resultant lung fibrosis and failure (Rowe et al., 2005).

Folding defects exhibited by CFTRΔF508 are conditional, and treatment of cells with chemical or pharmacological chaperones promotes its proper folding and channel function at the plasma membrane (Brown et al., 1996; Denning et al., 1992). Corrected CFTRΔF508 has a markedly shorter half-life at the cell surface than CFTR (Sharma et al., 2004), yet patients that express low levels of functional CFTR display a mild disease phenotype (Welsh and Ostedgaard, 1998). Therefore, agents that promote CFTRΔF508 folding or block the degradation of folded CFTRΔF508 have potential as CF therapeutics (Pedemonte et al., 2005; Van Goor et al., 2006). Guidance for the development of CF therapeutics requires a basic understanding of the mechanisms for CFTRΔF508 misfolding and the identification of the ERQC machinery that selects CFTRΔF508 for ERAD.

CFTR folding is complex because it is a 1480 residue glycomembrane protein, and its two membrane-spanning domains (MSDs), two cytoplasmic NBDs, and a regulatory domain (R domain) must interact with each other to form a Cl^- ion channel (Ostedgaard et al., 1997; Xiong et al., 1997). In most cell types tested, the biosynthesis of CFTR takes ~10 min, and the assembly of CFTR and CFTRΔF508 into a channel is inefficient, with ~60%–75% of CFTR

and nearly 99% of newly synthesized CFTRΔF508 being degraded (Ward and Kopito, 1994). The folding efficiency of CFTR can vary in different cell types (Varga et al., 2004), but it is clear that CFTRΔF508 does not reach the native state and is degraded prematurely in human airway cells (Kreda et al., 2005). CFTR and CFTRΔF508 appear to assume similar conformations at early stages of assembly (Zhang et al., 1998), but CFTRΔF508 folding becomes arrested at a poorly defined step.

Since F508 is located in a surface-exposed loop on NBD1 that makes contacts with MSD1 in bacterial ABC transporters, the loss of the F508 side chain may prevent proper contact formation between NBD1 and MSD1 and lead to misassembly of multiple regions in CFTR (Chang and Roth, 2001). In addition, loss of F508 from the NBD1 backbone causes purified NBD1 to exhibit a temperature-sensitive folding defect (Thibodeau et al., 2005). Thus, misfolding and misassembly of ΔF508 NBDI appear to cause CFTRΔF508 to be targeted for degradation, but how ERQC detects such folding defects is not clear.

Defects in the folding pathway of CFTRΔF508 described above suggest that it accumulates in a conformation that exposes nonnative surfaces in the cytosol as well as in the ER membrane. Cytosolic Hsc70 interacts with ER membrane localized Hdj2, the U-box protein CHIP, and the E2 UbcH5 to form a multisubunit E3 complex that ubiquitinates cytosolic regions of CFTRΔF508 (Meacham et al., 1999, 2001; Younger et al., 2004). Yet, upon inactivation of the Hsc70/CHIP E3 complex, CFTRΔF508 is still unable to escape the ER membrane and is degraded (Younger et al., 2004). Thus, it is possible that additional E2/E3 ubiquitin ligase complexes, which might monitor the folded state of CFTR's transmembrane domains, function in conjunction with the cytosolic Hsc70/CHIP E3 complex to triage CFTR and CFTRΔF508.

Studies in mammalian cells are yet to identify an ER-localized E3 ubiquitin ligase that functions in ERAD of CFTR. Nonetheless, there is evidence to suggest that a tail-anchored and ER-localized E2 ubiquitin-conjugating (Ubc) enzyme, Ubc6e, facilitates CFTR degradation (Lenk et al., 2002). Ubc6e does not appear to interact with the E3 CHIP to ubiquitinate CFTR (Younger et al., 2004). Therefore, we sought to identify the cognate E3 ubiquitin ligase that functions with Ubc6e and then define the role that this E2/E3 complex plays in QC of CFTR.

We identified the tail-anchored RING domain protein RMA1/RNF5 (Didier et al., 2003; Matsuda et al., 2001) as a component of an ER-associated E3 ubiquitin ligase complex that contains Ubc6e and the transmembrane quality-control factor Derlin-1 (Lilley and Ploegh, 2004; Ye et al., 2004). The RMA1 E3 complex appears to sense the assembly status of CFTR's amino-terminal regions at a folding step that occurs prior to NBDII synthesis and is defective in CFTRΔF508. The CHIP E3 appears to act after NBDII synthesis and detects folding defects that involve terminal steps in CFTR assembly.

RESULTS

RMA1 Cooperates with Ubc6e to Target CFTRΔF508 for Degradation

To identify new QC checkpoints that function to triage CFTR and CFTRΔF508, we sought to identify factors that function in ERQC with Ubc6e. We chose to investigate whether the human homolog of *Arabidopsis* RMA1 (Didier et al., 2003; Matsuda et al., 2001) interacts with Ubc6e to target CFTR and CFTRΔF508 for proteasomal degradation. RMA1 is a 180 amino acid residue tail-anchored ER membrane protein that contains a cytoplasmic RING domain (Figure 1A). RMA1 colocalizes with GFP-CFTRΔF508 in the ER membrane (Figure 1B) and cofractionates with the ER marker calnexin (Figure 1C). In addition, the B form of CFTRΔF508 (Figure 1D) and CFTR (data not shown) coimmunoprecipitate in a complex that contains *myc*-RMA1. When the RING domain mutant *myc*-RMA1 C42S was substituted for *myc*-RMA1 in transfections, several-fold more CFTRΔF508 coprecipitated with the RMA1 mutant. Thus, RMA1 is an ER-localized E3 protein that enters into complexes with CFTRΔF508 (Figure 1D).

To determine whether RMA1 can divert CFTR from its folding pathway and target it for degradation, the effect that RMA1 overexpression had on the accumulation of the immaturely glycosylated, nonnative B and maturely glycosylated, folded C form of CFTR was determined (Figure 2A). A 2-fold increase in RMA1 levels dramatically reduced the steady-state level of the C form of CFTR, and this action was not accompanied by the accumulation of the B form (Figure 2A). Treatment of cells with the proteasome inhibitor ALLN prevented RMA1 from reducing CFTRΔF508 levels and caused a large quantity of CFTRΔF508, which would normally have been degraded, to accumulate as an ubiquitin conjugate (Figure 2B).

Pulse-chase analysis showed that elevation of RMA1 caused a 60% reduction in the accumulation of ^{35}S-CFTR and ^{35}S-CFTRΔF508 during the 20 min pulse-labeling period (Figure 2C). Then, in the chase period, RMA1 blocked the glycolytic maturation of CFTR and accelerated degradation of the CFTRΔF508 B form around 2-fold. The ability of RMA1 to divert the B form of CFTR and CFTRΔF508 from its folding pathway was lost when RMA1 C42S or RMA1ΔTM was substituted for RMA1 in cotransfections (Figure 2D).

When RMA1 and Ubc6e levels were reduced by treatment of cells with siRNA oligonucleotides, CFTRΔF508 levels increased nearly 4-fold, and this effect correlated with reduced expression of RMA1 and Ubc6e (Figure 2E). Thus, RMA1 is an ER-localized RING E3 that can be isolated in coprecipitates with the B form of CFTR, and the modulation of its activity influences the fate of nascent CFTR and CFTRΔF508.

Data generated thus far indicate that RMA1 is a component of an ERQC complex that degrades CFTR and CFTRΔF508 equally well. However, it remained possible that the deletion of ΔF508 causes more nascent

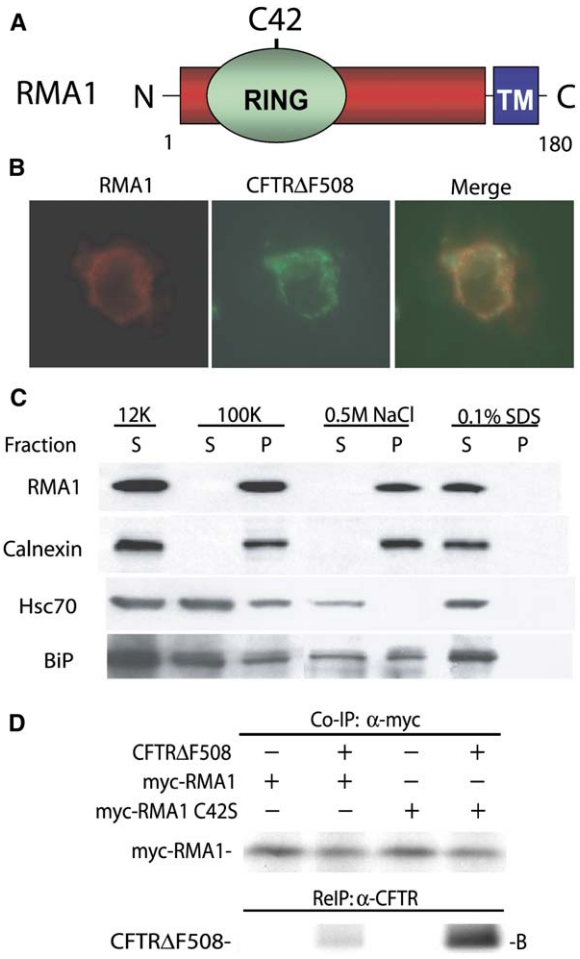

Figure 1. RMA1 Is an ER Membrane-Associated E3 Ubiquitin Ligase that Coprecipitates with CFTRΔF508

(A) The predicted domain structure of RMA1. C42 is a residue in the RING domain that is required for RMA1 ubiquitin ligase activity. TM denotes the transmembrane region, and N- and C- identify the amino- and carboxy-terminal ends of RMA1, respectively.

(B) Colocalization of *myc*-RMA1 and GFP-CFTRΔF508 in transiently transfected HEK293 cells by fluorescence microscopy.

(C) Fractionation of RMA1 with membranes that contain the ER marker calnexin. Cell extracts were prepared and subjected to differential centrifugation and probed for the presence of *myc*-RMA, calnexin, Hsc70, and BiP by Western blot. Where indicated, membranes that pelleted when extracts were spun at 100,000 × g were washed with 0.5 M NaCl or resuspended in 0.1% SDS and then reisolated. S and P denote the supernatant and pellets of centrifuged samples.

(D) RMA1 and CFTRΔF508 can be coprecipitated with each other. CFTRΔF508 was coexpressed with *myc*-RMA1 or *myc*-RMA1C42S, and extracts from ^{35}S-labeled HEK293 cells were prepared in PBS supplemented with 0.1% Triton X-100. Coimmunoprecipitations were carried out with α-*myc*, and then CFTRΔF508 was reimmunoprecipitated with α-CFTR. Autoradiographs of bands corresponding to RMA1 and CFTRΔF508 are shown.

CFTRΔF508 than CFTR to accumulate in a conformation that is recognized by RMA1 and/or its interacting partners. To explore this possibility, we compared the sensitivity of CFTR and CFTRΔF508 to elevation of RMA1 again, but reduced the level of RMA1 by utilizing 0.5 μg of RMA1 expression plasmid instead of 1.0 μg (Figure 3A). Under these conditions, RMA1 overexpression only reduced CFTR levels by ~30%, but CFTRΔF508 accumulation was reduced by ~90%. Thus, triage of CFTRΔF508 is more sensitive to elevation of RMA1 activity than CFTR. Yet, since RMA1 promotes the degradation of both CFTR and CFTRΔF508, it is not likely that the RMA1 E3 can sense a true difference in the conformation of these proteins. Instead, it is likely that the folding of a larger portion of nascent CFTRΔF508 than nascent CFTR arrests at a stage where its nonnative conformation can be recognized by components of the RMA1 E3 complex.

To evaluate the range of proteins that are selected for ERAD by RMA1, the sensitivities of CFTRΔF508, TCRα, and ApolipoB48 to RMA1 overexpression were compared (Figure 3B–C). The steady-state level of the B form of CFTRΔF508 was reduced by ~85% when cells were transfected with 1 μg of pcDNA-RMA1. Under these same conditions, the accumulation of TCRα and ApoB48 was largely unaffected. Yet, when cells were transfected with 3 μg of pcDNA-RMA1, a decrease in the steady-state levels of TCRα and ApoB48 was observed (Figure 3A). Decreased steady-state levels of TCRα and ApoB48 resultant from RMA1 overexpression correlated with a 2-fold increase in the degradation rates of these proteins (Figures 3C and 3D). The observed differences in the sensitivities of CFTRΔF508, TCRα, and ApoB48 to alterations in RMA1 levels indicates that RMA1 overexpression does not cause the pleiotropic degradation of ERAD substrates. However, it is difficult to judge the relative concentrations of these respective ERAD substrates in the cell, so it is premature to conclude that the RMA1 E3 can selectively recognize different classes of ERAD substrates.

To determine whether RMA1 and Ubc6e functionally interact, we analyzed whether they could act jointly to enhance CFTRΔF508 degradation (Figure 4). Individual levels of Ubc6e and RMA1 were adjusted to reduce CFTRΔF508 accumulation by 50%–60%. When RMA1 and Ubc6e were coexpressed at this same level, there was a dramatic reduction in CFTRΔF508 accumulation (Figures 4A and 4C). The RING domain mutant RMA1 C42S and the Ubc domain mutant Ubc6e C91S were unable to substitute for their wild-type counterparts and reduce the steady-state level of CFTRΔF508. Thus, RMA1 and Ubc6e appear to cooperate in a RING domain- and Ubc domain-dependent manner to degrade CFTR.

The purified RING domain of RMA1 can interact with Ubc5 to promote polyubiquitin chain assembly (Matsuda et al., 2001). Therefore, we determined whether or not RMA1 could cooperate with UbcH5a to modulate CFTRΔF508 levels in cultured cells (Figure 4B). The combined overexpression of RMA1 and UbcH5a did not result in a synergistic reduction in the steady-state levels of CFTRΔF508 (Figures 4B and 4D). In addition, pulse-chase analysis revealed that the combined expression of RMA1 and UbcH5a did not reduce the accumulation of CFTR beyond that observed when only RMA1 activity was elevated

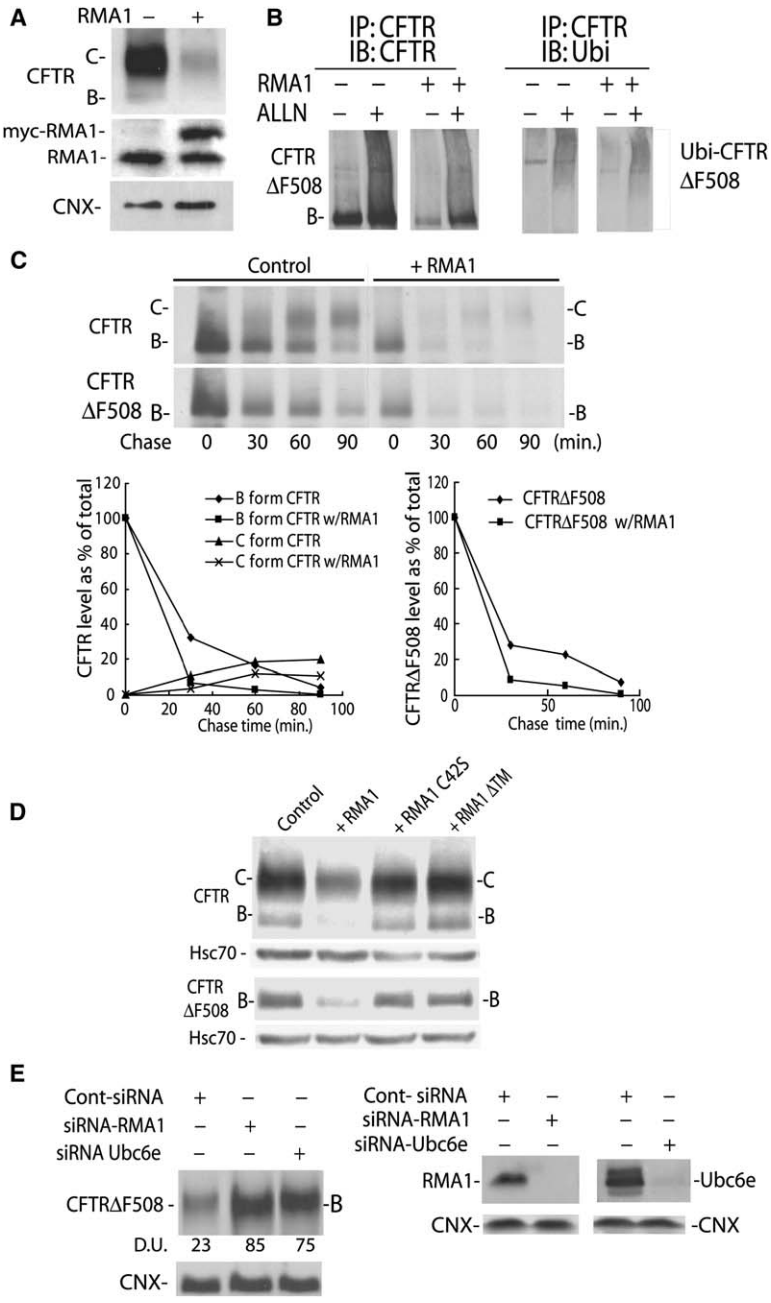

Figure 2. RMA1 Promotes the Degradation of CFTR and CFTRΔF508

(A) Overexpression of RMA1 reduces the steady-state level of CFTR. Western blots of extracts prepared from cells in which *myc*-RMA1 (pCDNA3.0-*myc*RMA1, 1.0 μg) was coexpressed with CFTR.

(B) Reduction of CFTRΔF508 levels by RMA1 is blocked by ALLN. CFTRΔF508 was coexpressed with RMA1, and ALLN (200 μM) was added to cells 4 hr prior to lysis. Extracts were prepared in lysis buffer supplemented with 5 mM N-ethyl maleimide, and the protein concentration of extracts was determined with a detergent-compatible Bradford assay. CFTRΔ F508 was then immunoprecipitated from 30 μg of protein extract. Immunoprecipitates were then probed for the presence of CFTRΔF508 or ubiquitin by Western blot.

(C) The effect of RMA1 on the kinetics of CFTR and CFTRΔF508 biogenesis. Cells were labeled with ^{35}S-translabel for 20 min, and after a given chase period, CFTR or CFTRΔF508 was immunoprecipitated and processed for autoradiography. Values from quantitation of CFTR and CFTRΔF508 represent the percentage of the B form of CFTR and CFTRΔF508 that was present at t = 0. Values are representative of two experiments, and the quantitation displayed is from the data shown in the above panels.

(D) Steady-state levels of CFTR and CFTRΔF508 when coexpressed with wild-type or mutant forms of RMA1.

(E) Steady-state levels of CFTRΔF508 were determined 24 hr after cotransfection with siRNA duplexes (50 nM) against a nonspecific mRNA, RMA1, or Ubc6e. As a control, the ability of these siRNAs to block the expression of RMA1 or Ubc6e when cells were transfected with 0.25 μg of pcDNA3.1(+)-RMA1 or Ubc6e was determined. Western blots of calnexin (CNX) or Hsc70 serve as gel loading controls.

(Figure 4E). In contrast, the coexpression of RMA1 and Ubc6e synergistically reduced the quantity of ^{35}S-labeled CFTRΔF508 that accumulated during a 20 min labeling period (Figure 4F). When similar experiments were conducted with CFTR, nearly identical results were observed (data not shown). These collective data support the conclusion that RMA1 and Ubc6e form a specific E2/E3 pair that selects nascent CFTR and CFTRΔF508 for degradation.

The pulse-chase analysis of CFTR and CFTRΔF508 biogenesis (Figures 2 and 4E) shows that significantly less of the B form of these proteins accumulates when RMA1 activity is increased. Therefore, it is possible that RMA1 and Ubc6e recognize folding defects in CFTRΔF508 during or soon after translation. Alternatively, the coexpression of RMA1 and Ubc6e with CFTRΔF508 might simply reduce its expression level. To address these questions, we examined the effect of RMA1 and Ubc6e on CFTRΔF508 biosynthesis in the absence and presence of the proteasome inhibitor ALLN (Figure 4F). ^{35}S-CFTRΔF508 accumulated with time over the course of a 20 min labeling period, and ALLN was observed to increase the accumulation of CFTRΔF508 around 2-fold at every time point. Yet, in cells where RMA1 or RMA1 and Ubc6e were overexpressed, the accumulation of CFTRΔF508 was reduced

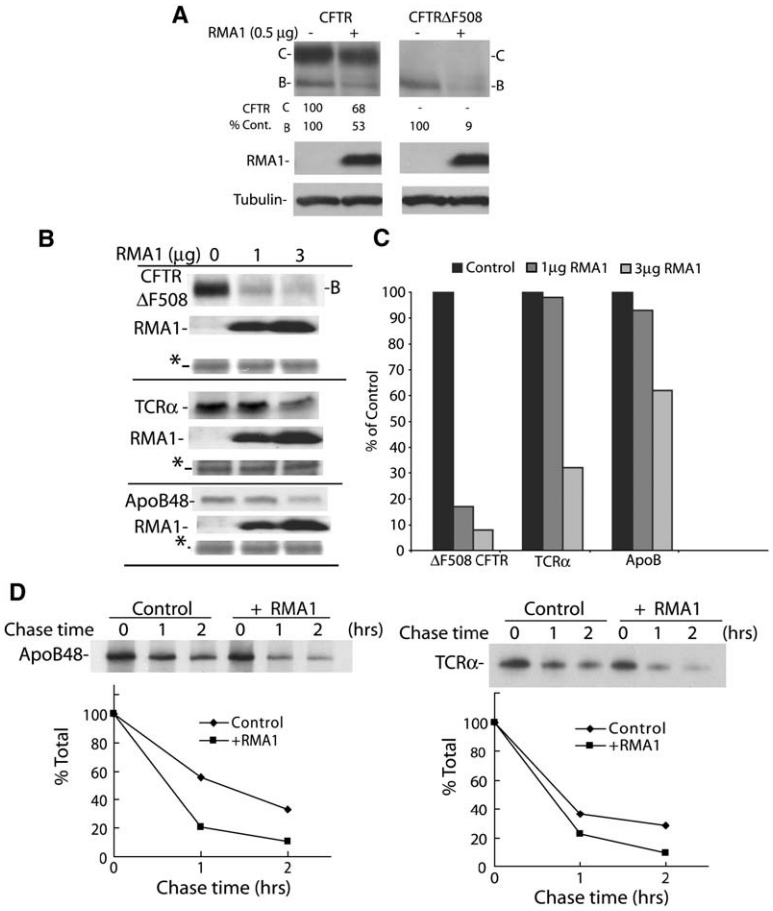

Figure 3. Comparison of the Sensitivity of Different ERAD Substrates to Elevation of RMA1 Activity

(A) A side-by-side comparison of the sensitivity of CFTR and CFTRΔF508 to a lower level of RMA1 overexpression. Instead of cotransfecting cells CFTR and CFTRΔF508 expression plasmids and 1.0 μg of pCDNA3.0-*myc*-RMA1 as in Figures 1 and 2, cells were transfected with CFTR expression plasmids and 0.5 μg of pCDNA3.0-*myc*-RMA1.

(B) The influence of RMA1 overexpression on CFTRΔF508, TCRα, and Apolipoprotein B48 levels. The indicated ERAD substrates were expressed alone or with pcDNA-RMA1 (1.0 or 3.0 μg). The steady-state level of the indicated protein was determined 18 hr posttransfection by Western blot.

(C) Quantitation of the steady-state levels of CFTRΔF508, TCRα, and Apolipoprotein B48 from autoradiographs shown in panel (A).

(D) Kinetics of Apolipoprotein B48 and TCRα degradation. Where indicated, cells were cotransfected with 3.0 μg of pcDNA3.1(+)-RMA1. Cells were labeled with ^{35}S-translabel for 20 min and chased for the indicated times. Levels of immunoprecipitated Apolipoprotein B48 or TCRα were expressed as a percentage of the total amount present at t = 0.

at all time points. However, the coexpression of RMA1 and Ubc6e did not hinder synthesis of CFTRΔF508, because treatment of cells with ALLN restored the labeling of CFTRΔF508 to a level that was greater than that observed in the control. Thus, a large portion of CFTRΔF508 appears to be degraded cotranslationally, and the RMA1/Ubc6e E3 complex appears capable of mediating this process. Our interpretations of the data presented in Figure 4 are consistent with the finding that CFTR can be ubiquitinated cotranslationally (Sato et al., 1998). Yet, whether RMA1 and Ubc6e truly act cotranslationally and ubiquitinate CFTR and CFTRΔF508 while it is attached to the ribosome requires further study.

Derlin-1 Interacts with RMA1 and Ubc6e to Facilitate CFTR Degradation

RING domain E3 ubiquitin ligases such as RMA1 are typically members of multisubunit complexes in which they serve as scaffolds that link the ubiquitin-conjugating activity of an E2 to the substrate-binding activity of a specific effecter molecule (Hershko and Ciechanover, 1998). Thus, it is plausible that RMA1 promotes CFTR degradation by functioning in a complex that contains a membrane-inserted substrate selector that senses the folded state of CFTR (Figure 5). The ERQC factor Derlin-1 contains four transmembrane-spanning domains and is known to form complexes with different transmembrane E3s and a number of other ERQC factors and appears to have multiple functions in ERAD (Katiyar et al., 2005; Lilley and Ploegh, 2005; Schuberth and Buchberger, 2005; Ye et al., 2005). Therefore, we tested whether Derlin-1 might be involved in the selection of CFTR for degradation by modulating Derlin-1 activity and determining the effect this had on CFTR biogenesis.

Elevation of Derlin-1 levels by ~3-fold led to the retention of the B form of CFTR in the ER (Figures 5A and 5B). HA-Derlin-1 formed coimmunoprecipitable complexes with the newly synthesized B form of CFTR (Figure 5C). Derlin-1 was also found to remain associated with the pool of B form that was retained in the ER when its activity was elevated to the point where it blocked CFTR folding by ~50% (Figure 5C). Under these same experimental conditions, complex formation between HA-Derlin-1 and the C form of CFTR was not observed. In addition, HA-Derlin-1 was also demonstrated to enter into coimmunoprecipitable complexes with CFTR370X, which represents MSDI of CFTR (Figure 5D). Eight percent of the total immunoprecipitable CFTRΔF508 and 12% of the total immunoprecipitable CFTR370X present in cells was coprecipitated with HA-Derlin-1 (see the legend to

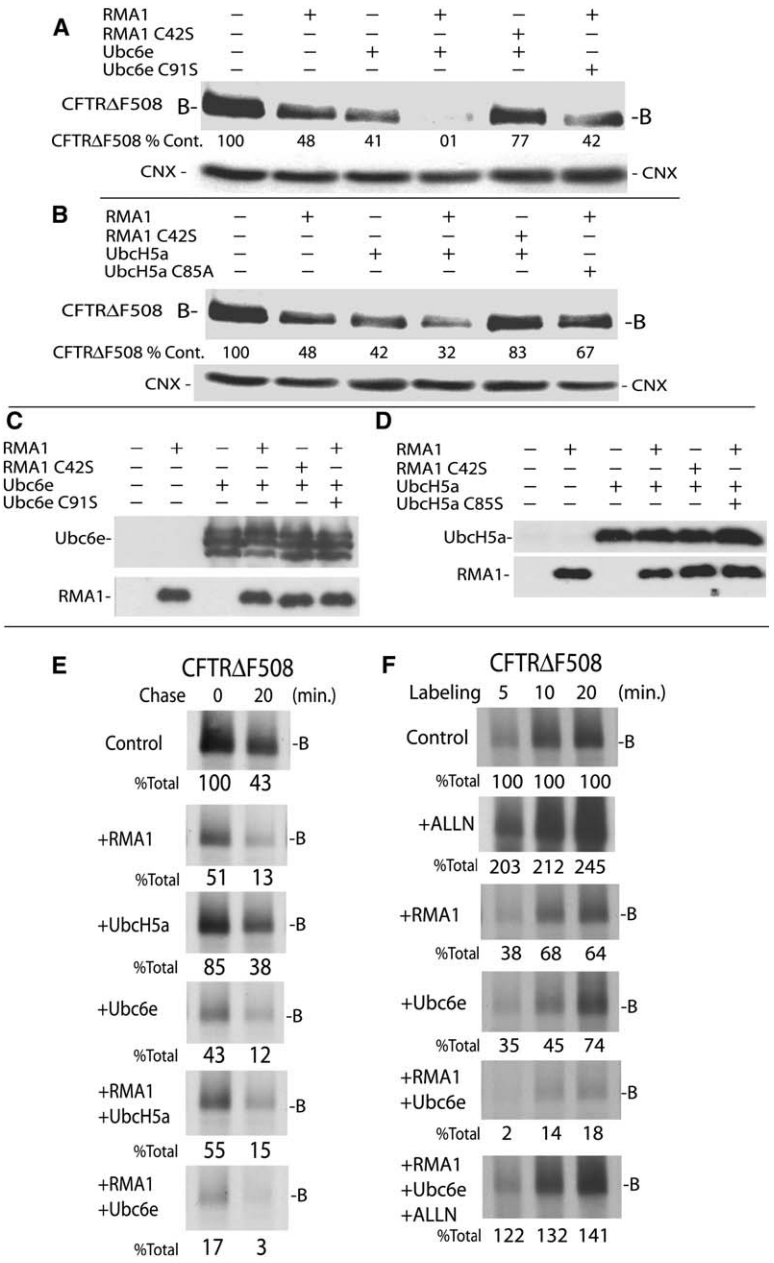

Figure 4. RMA1 Cooperates with Ubc6e to Promote Degradation of CFTRΔF508

(A and B) Western blots for CFTRΔF508 that was expressed alone or in combination with the indicated Ubc and RMA1 (similar results were observed when CFTR was utilized as a substrate). Proteins in cell extracts were resolved on 8% SDS-PAGE gels, and Western blots were decorated with the indicated antibody.

(C and D) Western blots of cell extracts that show the expression level of different forms of FLAG-RMA1 and *myc*-Ubc6e or *myc*-UbcH5a when they were coexpressed with CFTRΔF508. Proteins in cell extracts were resolved on 12.5% SDS-PAGE gels, and Western blots were decorated with the indicated antibody against Myc or FLAG epitopes.

(E) RMA1 and Ubc6e cooperate to promote CFTRΔF508 degradation. CFTRΔF508 was immunoprecipitated from ^{35}S-labeled cells at the beginning and end of a 20 min chase period and detected by autoradiography.

(F) Synthesis of ^{35}S-CFTRΔF508 under the indicated conditions. When present, ALLN (200 μM) was added to cell culture media 1 hr before the addition of ^{35}S-translabel. Bands were quantitated by laser densitometry and are expressed as a percentage of total CFTRΔF508 present at t = 0 in the control.

Figure 5). The level of complex formation detected between Derlin-1 and either CFTR or CFTR370X is similar to the quantity of CFTR that coprecipitated with Hsc70 (Meacham et al., 1999). It should also be noted that the level of CFTR:Derlin-1 complexes detected is likely to represent an underestimation of the total level of complex formation that occurs in the cell because Derlin-1:substrate complexes are expected to dissociate upon dilution during isolation protocols.

The retrotranslocation factor p97 is known to be present in complexes that contain Derlin-1 and E3 ubiquitin ligases (Ye et al., 2005; Lilley and Ploegh, 2005), and we observed it to coprecipitate with Derlin-1 and CFTR370X (Figure 5D). The presence of p97 in a complex with Derlin-1 and a CFTR biogenic intermediate implies that the retention of CFTR in the ER is coupled to its ubiquitination by RMA1 and Ubc6e and retrotranslocation.

If Derlin-1 plays an active role in the retention of nonnative CFTR in the ER, then the reduction of its cellular activity should allow more CFTR to escape the ER and accumulate in its C form. Indeed, the treatment of cells with Derlin-1 siRNAs enabled ~2-fold more of the folded C form of CFTR and the nonnative B form of CFTRΔF508 to accumulate (Figure 5E). These collective data demonstrate that the positive and negative modulation of Derlin-1 activity reciprocally modulates the partitioning of

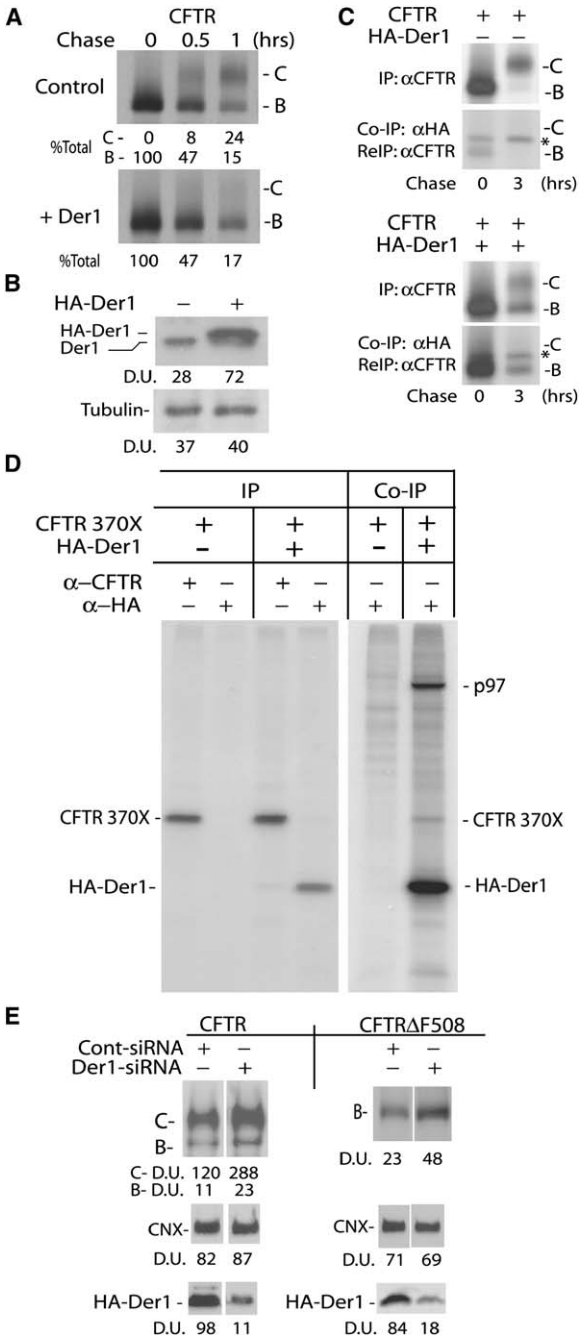

Figure 5. Derlin-1 Retains the B Form of CFTR in the ER

(A) Pulse-chase analysis of CFTR biogenesis in the presence or absence of transiently expressed Derlin-1 [pcDNA3.1(+)-HA-Derlin-1, 0.05 μg]. Cells were labeled for 20 min with ^{35}S-translabel and then lysed under nonnative conditions. The protein concentration in cell extracts was determined, and CFTR was immunoprecipitated from 30 μg of total extract protein.

(B) Western blot showing the expression level of endogenous Derlin-1 relative to overexpressed HA-Derlin-1.

(C) The B form of CFTR coimmunoprecipitates with HA-Derlin-1. CFTR was expressed alone or with HA-Derlin-1 [pcDNA3.1(+)-HA-Derlin-1, 0.025 μg], and extracts of ^{35}S-labeled cells were prepared and split after the indicated chase time. CFTR was immunoprecipitated under denaturing conditions from one portion of the cell extract with α-CFTR. HA-Derlin-1 was immunoprecipitated from the other portion under native buffer conditions, and the quantity of CFTR that coprecipitated was determined by reimmunoprecipitating CFTR from HA-Derlin-1 immunoprecipitates. The band marked with a * denotes a background band that precipitates with protein-G beads. Direct immunoprecipitations of CFTR were carried out with 30 μg of cell extract protein as the starting material, and HA-Derlin-1 coimmunoprecipitations were conducted with 120 μg of cell extract. Gels shown in the panels were exposed to X-ray film for the same time. The quantity of the B form of CFTR observed to nonspecifically precipitate with α-HA represents less than 1% of the total immunoprecipitable CFTR, and the quantity of the B form of CFTR that coprecipitated with Derlin-1-HA at t = 0 and t = 3.0 of the chase period represents around 12% of total immunoprecipitable CFTR at each respective time point.

(D) CFTR370X can be coimmunoprecipitated with HA-Derlin-1. Cells were transfected with CFTR370X and HA-Derlin-1 and radiolabeled for 30 min. Extracts were prepared under native conditions and split. In panels denoted IP, HA-Derlin-1 and CFTR370X were immunoprecipitated under nonnative conditions. In panels labeled Co-IP, HA-Derlin-1 was precipitated with α-HA antibody, and p97 and CFTR370X present in the coprecipitates were identified by reimmunoprecipitation (data not shown). Direct immunoprecipitations were conducted from 30 μg of cell extract, and coimmunoprecipitations utilized 120 μg of extract protein as starting material. Panels were exposed to X-Ray film for the same time period, and CFTR370X that coprecipitated with HA-Derlin-1 was calculated to be equivalent to 8% of total CFTR370X.

(E) Treatment of cells for 24 hr with siRNAi (50 nM) against Derlin-1 leads to increased expression of CFTR and CFTRΔF508. Demonstration of the ability of siRNAs to block synthesis of HA-Derlin-1 expressed from pcDNA3.1(+)-HA-Derlin-1 was utilized as a control. Where indicated, Western blots of tubulin and calnexin (CNX) levels in cell extracts were used as load controls. Quantitation of bands on gels is expressed either in raw densitometer units (panels [B] and [D]) or as a percentage of total B form of CFTR present at t = 0 (panel [A]).

CFTR between folding and degradation pathways. The exact mechanism for Derlin-1 function in retaining CFTR in the ER is not clear, but it appears to involve complex formation with the membrane-spanning domains of CFTR. Thus, Derlin-1 appears capable of functioning as a component of the substrate selector for the RMA1/Ubc6e E3 ubiquitin ligase complex.

To determine whether Derlin-1 cooperates with RMA1 and Ubc6e to mediate QC of CFTR, the ability of these different ER membrane proteins to physically and functionally interact was examined (Figure 6). Extracts of cells were prepared from ^{35}S-labeled cells that coexpressed FLAG-RMA1 with *myc*-Ubc6e, HA-Derlin-1, and CFTRΔF508 under native buffer conditions. Then FLAG-RMA1 was immunoprecipitated and found to specifically associate with a number of different radiolabeled proteins. Ubc6e, Derlin-1, and CFTRΔF508 were identified as interaction partners of RMA1 via their reimmunoprecipitation from the RMA1 coprecipitate (Figure 6A). Complex formation between these proteins and RMA1 appeared specific, as we were unable to detect Sec61, Erdj4, or calnexin in products of RMA1 or Derlin-1 coprecipitation reactions (data not shown). Ubc6e and Derlin-1 also appeared to functionally interact to promote degradation of CFTRΔF508, because their combined expression

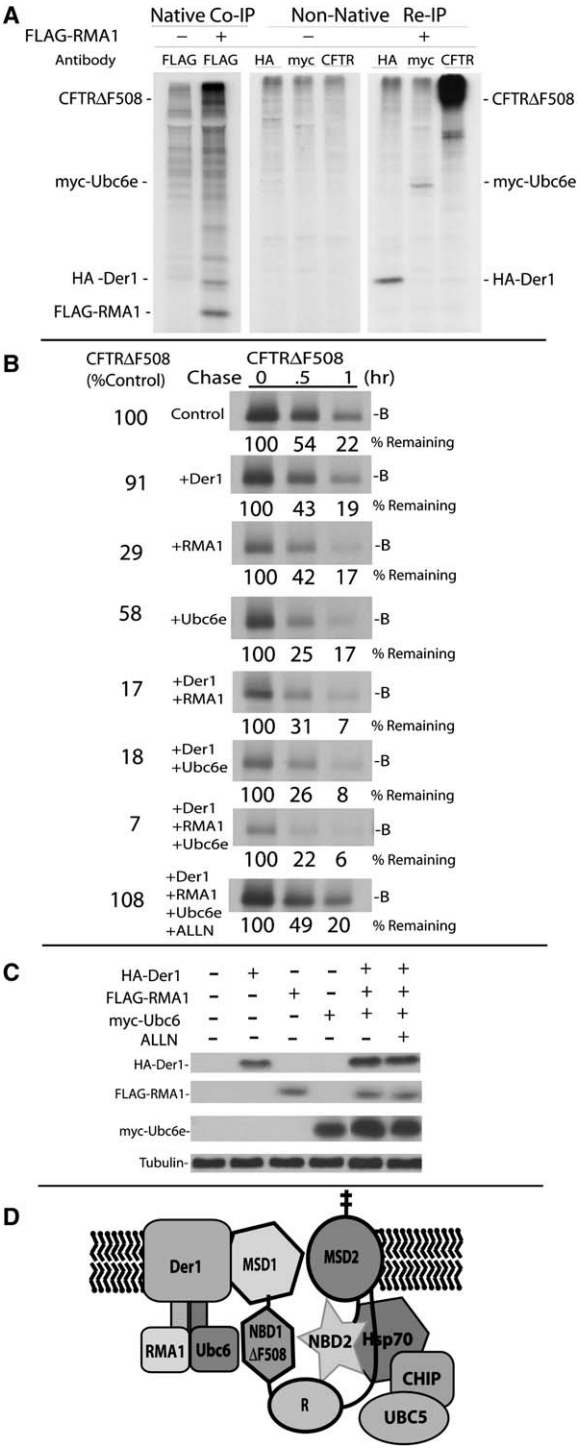

Figure 6. RMA1, Ubc6e, and Derlin-1 Functionally and Physically Interact to Promote Proteasome-Dependent Degradation of CFTRΔF508

(A) CFTRΔF508, HA-Derlin-1, and *myc*-Ubc6e are present in a coprecipitable complex with FLAG-RMA1. Cells were labeled with ^{35}S-translabel for 1 hr and solubilized in a buffer containing 0.1% Triton X-100. Coimmunoprecipitations and reimmunoprecipitations were then carried out as described in the Experimental Procedures section. Since a large number of proteins in the 36 kDa molecular weight range precipitate with FLAG-RMA1, it is difficult to detect a signal for Ubc6e above the background in the native immunoprecipitation. Yet, Ubc6e was clearly present in RMA1 precipitates, because it could be reimmunoprecipitated from native precipitates under denaturing conditions.

(B) Coexpression of RMA1 and Ubc6e with Derlin-1 potentiates the degradation of ^{35}S-CFTRΔF508. Total CFTRΔF508 present at the end of the 20 min labeling period was quantitated and is expressed as a percentage of the total signal in the control. Changes in the levels of CFTRΔF508 during the course of the indicated chase reaction were quantitated and expressed in percentage of the total signal at t = 0.

(C) Western blot analysis of transiently expressed HA-Derlin-1, FLAG-RMA1, and *myc*-Ubc6e when these proteins are expressed individually and in concert with each other.

(D) Schematic diagram representing the E3 ubiquitin ligase complexes that function to select CFTR and CFTRΔF508 for proteasomal degradation.

reduced the accumulation of ^{35}S-CFTRΔF508 to levels that were ~90% lower than the control and increased CFTRΔF508 turnover 2-fold (Figure 6B). Derlin-1, RMA1, and Ubc6e appear to physically and functionally interact to promote CFTR degradation.

It is possible that the reduction in CFTR accumulation observed when components of the RMA1 E3 complex were all jointly overexpressed is simply due to reduced CFTRΔF508 synthesis that is resultant from its coexpression with multiple proteins. However, the following observations indicate that elevation of the activity of the RMA1 E3 complex is the predominant cause for the reduced accumulation of CFTRΔF508 when RMA1, Ubc6e, and Derlin-1 are jointly expressed. First, when RMA1, Derlin-1, and Ubc6e were coexpressed, the cell is fully capable of synthesizing CFTRΔF508 because it accumulated above control levels when the proteasome inhibitor ALLN was present during cell labeling (Figure 6B). Second, the expression levels of individual forms of these proteins are not reduced by their joint coexpression (Figures 4C, 4D, and 6C). Thus, the data presented support a model for ERQC in which RMA1, Ubc6e, and Derlin-1 form an ER membrane-associated E3 ubiquitin ligase complex that acts alongside the cytosolic Hsc70/CHIP/UbcH5 E3 complex to target the B form of CFTR and CFTRΔ508 for proteasomal degradation (Figure 6D).

RMA1 and CHIP Promote the Degradation of Different Regions of CFTR

Since components of the RMA1 E3 are membrane inserted and expose their functional domains on the cytosolic face of the ER and components of the CHIP E3 complex are cytosolic, we investigated whether they could function to sense the folding status of different subdomains of CFTR. This question was investigated by determining whether there were differences in the stage of biogenesis at which different-length CFTR and CFTRΔF508 fragments that resemble translation intermediates became sensitive to RMA1 or CHIP (Figure 7A). Cells were transfected with quantities of CHIP and RMA1 expression plasmids that led to a partial reduction in the steady-state

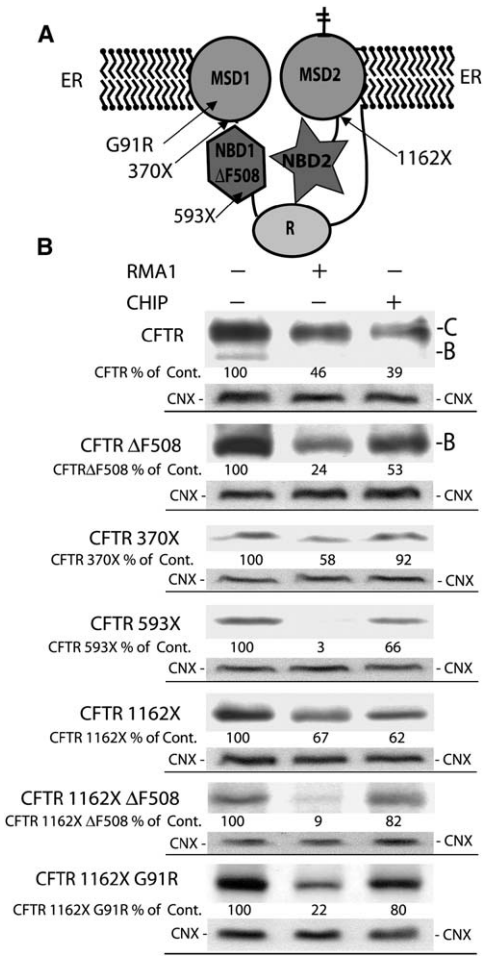

Figure 7. RMA1 and CHIP E3 Ubiquitin Ligases Exhibit Differences in Their Ability to Sense Folding Defects in CFTR Mutants

(A) A model depicting the position in CFTR where stop codons or point mutations were introduced to generate the tools utilized in panel (B).

(B) Steady-state levels of different-length CFTR fragments when RMA1 and CHIP activity are elevated. Cells were transfected with pcDNA3.1 that expresses the indicated form of CFTR and pcDNA3.1-RMA1 or pcDNA3.1-CHIP. The steady-state levels of CFTR and its different fragments were determined by Western blot with an antibody that specifically recognizes the N terminus of CFTR. Calnexin levels were determined to serve as load controls. Quantitation of CFTR fragment levels are expressed as percentage of control.

level of CFTR and CFTRΔF508 (Figure 7B). CFTR370X levels were partially reduced by RMA1, but were insensitive to CHIP. CFTR593X, which is truncated near the end of NBD1, was not detectable when RMA1 was overexpressed, but CHIP only reduced its expression level by ~30%. CFTR1162X is truncated after MSDII and appears to fold to a native-like conformation that is a poor substrate for chaperones (Meacham et al., 1999). Consistent with this interpretation, CFTR1162X was largely resistant to the action of both RMA1 and CHIP. To our surprise, deletion of F508 made CFTR1162XΔF508 sensitive to RMA1, but not CHIP.

Since the expression of MSDII reduced the sensitivity of CFTR fragments to RMA1, it is possible that the RMA1 E3 complex acts to sense the assembly status of CFTR's transmembrane domains. If this is the case, then RMA1 should be able to detect defects in CFTR folding that are caused by a disease-causing mutation in MSDI. Hence, we compared the ability of RMA1 and CHIP to sense the folding defect caused by the G91R mutation in transmembrane helix I, which prevents proper assembly of CFTR (Xiong et al., 1997). Indeed, CFTR1162X G91R was found to be sensitive to elevation of RMA1 activity, but not to CHIP. These data support the conclusion that the RMA1 and CHIP E3 complexes selectively interact with different subdomains of CFTR.

DISCUSSION

The RING domain protein RMA1 has been identified as a component of an ER membrane-associated E3 ubiquitin ligase complex that cooperates with the cytosolic Hsc70/CHIP E3 complex to monitor the conformation of different regions of nascent CFTR. The RMA1 QC checkpoint monitors the assembly status of amino-terminal regions of CFTR and appears to perform this function during or soon after translation. The CHIP QC checkpoint inspects the folding status of CFTR's cytosolic domains, and CFTR biogenic intermediates are most sensitive to CHIP action after NBDII synthesis. Hence, the CHIP E3 appears to play a posttranslational role in QC of CFTR.

Folding of CFTR and CFTRΔF508 appears to progress down similar pathways (Zhang et al., 1998), and both are substrates of the RMA1 and CHIP E3 complexes. However, the deletion of F508 from NBD1 appears to cause the folding of larger portion of nascent CFTRΔF508 than nascent CFTR to arrest in a conformation that is recognized by the RMA1 checkpoint. Yet, the folding defect in CFTRΔF508 that is sensed by components of the RMA1 E3 complex is not insurmountable. However, forms CFTRΔF508 that escape the RMA1 checkpoint ultimately fail QC at the CHIP checkpoint. Therefore, compounds developed to correct disease-causing folding defects in CFTR (Brown et al., 1996; Pedemonte et al., 2005) must enable CFTRΔF508 to pass through sequential QC checkpoints that are staffed by ER membrane-inserted and cytosolic E3 ubiquitin ligases.

The exact nature of the nonnative form(s) of CFTR and CFTRΔF508 that are recognized by components of the RMA1 E3 complex is not clear. Nevertheless, several observations we made suggest that the RMA1 E3 complex functions to detect folding defects related to the misassembly of CFTR's membrane-spanning domains. First, the elevation of RMA1 activity drives the degradation of CFTR biogenic intermediates that have the G91R mutation in MSDI, which is degraded by ERAD due to defects in MSD assembly (Xiong et al., 1997). Second, Derlin-1 retains CFTR in the ER and does so via a mechanism that may involve complex formation with MSDI. Finally, the sensitivity of early-stage CFTR biogenic intermediates,

which contain just MSDI and adjacent cytoplasmic domains, to RMA1 is reduced upon expression of MSDII. Since CFTRΔF5081162X fails to attain a conformation that is resistant to elevation of RMA1 activity, deletion of F508 hinders the ability of MSDII to assemble into a complex with amino-terminal regions of CFTR, which appear to include MSDI (Chen et al., 2004).

The region in CFTR that is ubiquitinated by the Hsc70/CHIP E3 are also unknown. Hsc70 is the substrate selector for the CHIP E3 complex (Younger et al., 2004) and can cooperate with Hdj-2 to suppress the aggregation of purified NBD1 (Meacham et al., 1999). Thus, it was surprising to observe that fragments of CFTR and CFTRΔF508 that contained just MSDI and NBD1 were relatively insensitive to elevation of CHIP levels and that sensitivity to CHIP E3 activity was greatly increased after NBDII synthesis. Fragments of CFTR that resemble early-stage biogenic intermediates may be less sensitive to cytosolic CHIP than RMA1 because CHIP cannot efficiently gain access to misfolded regions that may be readily accessed by the membrane-inserted components of the RMA1 E3 complex. In contrast, NBDII is the last domain in CFTR to be synthesized and might be more accessible to the CHIP E3. In addition, the posttranslational folding of NBDII appears to be the rate-limiting step in CFTR folding, and this process is defective in CFTRΔF508 (Du et al., 2005). Thus, it is logical that the Hsc70/CHIP E3 complex would recognize forms of CFTR and CFTRΔF508 in which NBDII, and possibly other cytosolic domains, have failed to fold or assemble efficiently.

The ubiquitin ligase of activity of the Hsc70/CHIP E3 complex has been reconstituted (Younger et al., 2004), but the mechanism by which the RMA1 E3 complex binds and ubiquitinates CFTR is unknown. Based on the data presented, we propose the following two-step mechanism for RMA1 E3 function in the selection of CFTR for proteasomal degradation. First, Derlin-1 may act alone or in combination with other factors to scan the assembly status of CFTR's membrane domains. If MSDI and/or MSDII fail to fold/assemble rapidly or correctly, then Derlin-1 enters into a complex with CFTR. The CFTR that is associated with Derlin-1 is brought into association with RMA1 and Ubc6e. Subsequently, RMA1 and Ubc6e cooperate to ubiquitinate a cytosolic region that is exposed in nonnative CFTR, but is masked in on-pathway biogenic intermediates. Since ΔF508NBD1 is prone to misfolding (Thibodeau et al., 2005), it is possible that the binding of Derlin-1 to MSDI is coupled to ubiquitination of regions in ΔF508NBDI by RMA1 and Ubc6e.

The proposal that Derlin-1 binds misassembled CFTR and brings it into contact with RMA1 and Ubc6e is based on the following observations. First, Derlin-1 forms complexes with the B form of CFTR and CFTRΔF508, and it can also be coimmunoprecipitated with MSDI of CFTR. Complex formation between Derlin-1 and the B form of CFTR correlates with the retention of CFTR in the ER. Second, the modulation of the cellular activity of Derlin-1 influences the partitioning of CFTR between its folding and degradation pathways. Yet, in contrast to studies with RMA1 and Ubc6e, the elevation of Derlin-1 activity alone does not lead to a dramatic reduction in the accumulation of nascent CFTR. Instead, Derlin-1 appears to bind and hold CFTR in the ER and requires interaction with additional factors, such as RMA1 and Ubc6e, to facilitate CFTR degradation.

Whether Derlin-1 can directly bind to CFTR independent of other cofactors is an open question. Nonetheless, the ability of Derlin-1 to enter into complexes with nonnative CFTR suggests that is has a chaperone-like activity that allows it to function as a substrate selector for the RMA1 E3 complex. This interpretation fits well with several recent studies that identify chaperone functions for membrane proteins (Lord and High, 2005). The *E. coli* protein YidC and its eukaryotic homologs function to suppress the posttranslational aggregation of membrane proteins and can facilitate protein folding and assembly reactions (Dalbey and Kuhn, 2004). In addition, the ER membrane protein INSIG-1 functions to recognize the conformational state of HMG-CoA reductase and targets cholesterol bound forms for ubiquitination by the transmembrane E3 gp78 (Song et al., 2005). The overexpression of INSIG-1 does not cause retention of CFTR in the ER (data not shown), but its identification provides precedence for a membrane protein serving as the substrate selector for an ER-associated ubiquitin ligase.

RMA1 and Ubc6e are members of an expanding list of ERQC factors that are isolated in complexes with Derlin-1. The known interaction partners of Derlin proteins include VIMP (Ye et al., 2004), N-glycanase (Katiyar et al., 2005), E3 ubiquitin ligases (Lilley and Ploegh, 2005; Ye et al., 2005), EDEM (Oda et al., 2006), and the retrotranslocation factor p97 (Lilley and Ploegh, 2004; Ye et al., 2004). Since these cofactors are all implicated to function in either substrate ubiquitination or retrotranslocation, their interactions with Derlin appear to direct it to function at multiple steps in the ERAD pathway. Based on the association of Derlin-1 with p97, one function of Derlin-1 is suggested to be that of a retrotranslocation channel (Ye et al., 2004). CFTR degradation requires p97, but p97 acts after the selection of CFTR for ERAD, and overexpression of p97 does not cause CFTR to be retained in the ER (Dalal et al., 2004). Yet, we have observed p97 to coprecipitate with Derlin-1, CFTR370X, CFTR, and CFTRΔF508 (data not shown). Thus, Derlin-1 appears to be a jack-of-all-trades that interacts with different ERQC factors to couple its ability to retain CFTR in the ER with CFTR's ubiquitination and retrotranslocation.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Miscellaneous Reagents

The plasmids utilized in this study for transfection of cultured cells were described previously (Younger et al., 2004). RMA1 and Derlin-1 were cloned by RT-PCR from total mRNA isolated from HEK293 cells and inserted into pcDNA3.1(+). The antibodies used for Western blots and/or immunoprecipitations were described previously (Younger et al., 2004). α-RMA1 is a rabbit polyclonal antibody that was produced

against Gst-RMA1ΔTM purified from *E. coli*. The specificity of rabbit α-RMA1 was authenticated by comparing its cross-reactivity to a mouse monoclonal antibody against RMA1/RNF5 (Didier et al., 2003). siRNA duplexes utilized in this study were purchased from Dharmacon.

Analysis of Protein Biogenesis

Culture of HEK293 cells for analysis of protein biogenesis was performed as previously described (Younger et al., 2005). Cells were typically transfected with 1.0 μg of pcDNA3.1(+)-CFTR and 1.5 μg of pcDNA3.1(+)-CFTRΔF508. For coexpression experiments, RMA1, Ubc6e, and UbcH5a were utilized at 0.25–0.5 μg/well of a 6-well plate. pcDNA3.1(+)-HA-Derlin-1 was introduced into cells at 0.01–0.1 μg/well. The details of the protocols for ^{35}S-labeling of cells, immunoprecipitations, and Western blots are described elsewhere (Meacham and Cyr, 2002). When CFTR biogenesis was assessed by immunoprecipitation from ^{35}S-labeled cells, the protein concentration of lystates was determined, and immunoprecipitations were carried out with the same total quantity of cell lysate protein (Meacham et al., 1999). When the steady-state level of CFTR and CFTRΔF508 was measured by Western blot, levels of the marker protein Hsc70, tubulin, or calnexin in different samples were compared as load controls. The colocalization of GFP-CFTRΔF508 with RMA1 was determined by fluorescence microscopy (Younger et al., 2005).

Isolation of the RMA1/Ubc6e/Derlin-1 E3 Ubiquitin Ligase Complex by Coimmunoprecipitation

Expression plasmids for CFTRΔF508, FLAG-RMA1, *myc*-Ubc6e, and HA-Derlin-1 were cotransfected into HEK293 cells, which were labeled 18 hr later with ^{35}S-translabel for 1 hr. Cells from 6 individual wells of a 6-well plate were pooled and lysed by incubation on ice at 4°C in 500 μl of 150 mM NaCl, 50 mM phosphate, 1 mM phenylmethysulfonyl fluoride (PMSF), 1× protease inhibitor cocktail(-)EDTA (Roche), 0.2% BSA, and 0.1% Triton X-100. Immunoprecipitations were conducted with cleared lysates using protein-G agarose beads that were precoupled with α-FLAG antibody. Precipitates were washed with a buffer that was identical to the one previously described, except BSA was omitted. Products of the coimmunoprecipitation reactions were analyzed by SDS-PAGE and fluorography. Verification of the identity of ^{35}S-proteins in complexes isolated by coimmunoprecipitation was accomplished by reimmunoprecipitation of the indicated protein from coimmunoprecipitates (Meacham et al., 1999).

ACKNOWLEDGMENTS

The authors thank Drs. Bonificano, Sommer, Stanton, and Ginsberg for providing plasmids; and Drs. Delaunay-Moisan and Roani for providing RMA1/RNF5 sera. This work is supported by the NIH and CFF. J.M.Y. is supported by a predoctoral fellowship from the AHA. A NIGMS postdoctoral fellowship from SPIRE supports M.F.N.R.

Received: November 15, 2005
Revised: April 20, 2006
Accepted: June 5, 2006
Published: August 10, 2006

REFERENCES

Brown, C.R., Hong-Brown, L.Q., Biwersi, J., Verkman, A.S., and Welch, W.J. (1996). Chemical chaperones correct the mutant phenotype of the delta F508 cystic fibrosis transmembrane conductance regulator protein. Cell Stress Chaperones *1*, 117–125.

Chang, G., and Roth, C.B. (2001). Structure of MsbA from E. coli: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. Science *293*, 1793–1800.

Chen, E.Y., Bartlett, M.C., Loo, T.W., and Clarke, D.M. (2004). The DeltaF508 mutation disrupts packing of the transmembrane segments of the cystic fibrosis transmembrane conductance regulator. J. Biol. Chem. *279*, 39620–39627.

Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan, C.R., and Smith, A.E. (1990). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell *63*, 827–834.

Cyr, D.M. (2005). Arrest of CFTRDeltaF508 folding. Nat. Struct. Mol. Biol. *12*, 2–3.

Dalal, S., Rosser, M.F., Cyr, D.M., and Hanson, P.I. (2004). Distinct roles for the AAA ATPases NSF and p97 in the secretory pathway. Mol. Biol. Cell *15*, 637–648.

Dalbey, R.E., and Kuhn, A. (2004). YidC family members are involved in the membrane insertion, lateral integration, folding, and assembly of membrane proteins. J. Cell Biol. *166*, 769–774.

Denning, G.M., Anderson, M.P., Amara, J.F., Marshall, J., Smith, A.E., and Welsh, M.J. (1992). Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature *358*, 761–764.

Didier, C., Broday, L., Bhoumik, A., Israeli, S., Takahashi, S., Nakayama, K., Thomas, S.M., Turner, C.E., Henderson, S., Sabe, H., and Ronai, Z. (2003). RNF5, a RING finger protein that regulates cell motility by targeting paxillin ubiquitination and altered localization. Mol. Cell. Biol. *23*, 5331–5345.

Du, K., Sharma, M., and Lukacs, G.L. (2005). The DeltaF508 cystic fibrosis mutation impairs domain-domain interactions and arrests post-translational folding of CFTR. Nat. Struct. Mol. Biol. *12*, 17–25.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Jensen, T.J., Loo, M.A., Pind, S., Williams, D.B., Goldberg, A.L., and Riordan, J.R. (1995). Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. Cell *83*, 129–135.

Katiyar, S., Joshi, S., and Lennarz, W.J. (2005). The retrotranslocation protein Derlin-1 binds peptide:N-glycanase to the endoplasmic reticulum. Mol. Biol. Cell *16*, 4584–4594.

Kreda, S.M., Mall, M., Mengos, A., Rochelle, L., Yankaskas, J., Riordan, J.R., and Boucher, R.C. (2005). Characterization of wild-type and deltaF508 cystic fibrosis transmembrane regulator in human respiratory epithelia. Mol. Biol. Cell *16*, 2154–2167.

Lenk, U., Yu, H., Walter, J., Gelman, M.S., Hartmann, E., Kopito, R.R., and Sommer, T. (2002). A role for mammalian Ubc6 homologues in ER-associated protein degradation. J. Cell Sci. *115*, 3007–3014.

Lilley, B.N., and Ploegh, H.L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. Nature *429*, 834–840.

Lilley, B.N., and Ploegh, H.L. (2005). Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. Proc. Natl. Acad. Sci. USA *102*, 14296–14301.

Lord, J.M., and High, S. (2005). Polytopic proteins: preventing aggregation in the membrane. Curr. Biol. *15*, R169–R171.

Matsuda, N., Suzuki, T., Tanaka, K., and Nakano, A. (2001). Rma1, a novel type of RING finger protein conserved from Arabidopsis to human, is a membrane-bound ubiquitin ligase. J. Cell Sci. *114*, 1949–1957.

McCracken, A.A., and Brodsky, J.L. (1996). Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP. J. Cell Biol. *132*, 291–298.

Meacham, G.C., and Cyr, D.M. (2002). Isolation of CFTR. Chaperone complexes by co-immunoprecipitation. Methods Mol. Med. *70*, 245–256.

Meacham, G.C., Lu, Z., King, S., Sorscher, E., Tousson, A., and Cyr, D.M. (1999). The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. EMBO J. *18*, 1492–1505.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M., and Cyr, D.M. (2001). The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat. Cell Biol. *3*, 100–105.

Oda, Y., Okada, T., Yoshida, H., Kaufman, R.J., Nagata, K., and Mori, K. (2006). Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. J. Cell Biol. *172*, 383–393.

Ostedgaard, L.S., Rich, D.P., DeBerg, L.G., and Welsh, M.J. (1997). Association of domains within the cystic fibrosis transmembrane conductance regulator. Biochemistry *36*, 1287–1294.

Pedemonte, N., Lukacs, G.L., Du, K., Caci, E., Zegarra-Moran, O., Galietta, L.J., and Verkman, A.S. (2005). Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. J. Clin. Invest. *115*, 2564–2571.

Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L., et al. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science *245*, 1066–1073.

Rowe, S.M., Miller, S., and Sorscher, E.J. (2005). Cystic fibrosis. N. Engl. J. Med. *352*, 1992–2001.

Sato, S., Ward, C.L., and Kopito, R.R. (1998). Cotranslational ubiquitination of cystic fibrosis transmembrane conductance regulator in vitro. J. Biol. Chem. *273*, 7189–7192.

Schuberth, C., and Buchberger, A. (2005). Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. Nat. Cell Biol. *7*, 999–1006.

Sharma, M., Pampinella, F., Nemes, C., Benharouga, M., So, J., Du, K., Bache, K.G., Papsin, B., Zerangue, N., Stenmark, H., and Lukacs, G.L. (2004). Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. J. Cell Biol. *164*, 923–933.

Song, B.L., Sever, N., and DeBose-Boyd, R.A. (2005). Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. Mol. Cell *19*, 829–840.

Thibodeau, P.H., Brautigam, C.A., Machius, M., and Thomas, P.J. (2005). Side chain and backbone contributions of Phe508 to CFTR folding. Nat. Struct. Mol. Biol. *12*, 10–16.

Van Goor, F., Straley, K.S., Cao, D., Gonzalez, J., Hadida, S., Hazlewood, A., Joubran, J., Knapp, T., Makings, L.R., Miller, M., et al. (2006). Rescue of {Delta}F508 CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. Am. J. Physiol. Lung Cell. Mol. Physiol. *290*, 1117–1130.

Varga, K., Jurkuvenaite, A., Wakefield, J., Hong, J.S., Guimbellot, J.S., Venglarik, C.J., Niraj, A., Mazur, M., Sorscher, E.J., Collawn, J.F., and Bebok, Z. (2004). Efficient intracellular processing of the endogenous cystic fibrosis transmembrane conductance regulator in epithelial cell lines. J. Biol. Chem. *279*, 22578–22584.

Ward, C.L., and Kopito, R.R. (1994). Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. J. Biol. Chem. *269*, 25710–25718.

Ward, C.L., Omura, S., and Kopito, R.R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. Cell *83*, 121–127.

Welsh, M.J., and Ostedgaard, L.S. (1998). Cystic fibrosis problem probed by proteolysis. Nat. Struct. Biol. *5*, 167–169.

Xiong, X., Bragin, A., Widdicombe, J.H., Cohn, J., and Skach, W.R. (1997). Structural cues involved in endoplasmic reticulum degradation of G85E and G91R mutant cystic fibrosis transmembrane conductance regulator. J. Clin. Invest. *100*, 1079–1088.

Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T.A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature *429*, 841–847.

Ye, Y., Shibata, Y., Kikkert, M., van Voorden, S., Wiertz, E., and Rapoport, T.A. (2005). Inaugural article: Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. Proc. Natl. Acad. Sci. USA *102*, 14132–14138.

Younger, J.M., Ren, H.Y., Chen, L., Fan, C.Y., Fields, A., Patterson, C., and Cyr, D.M. (2004). A foldable CFTR{Delta}F508 biogenic intermediate accumulates upon inhibition of the Hsc70-CHIP E3 ubiquitin ligase. J. Cell Biol. *167*, 1075–1085.

Younger, J.M., Fan, C.Y., Chen, L., Rosser, M.F., Patterson, C., and Cyr, D.M. (2005). Cystic fibrosis transmembrane conductance regulator as a model substrate to study endoplasmic reticulum protein quality control in mammalian cells. Methods Mol. Biol. *301*, 293–303.

Zhang, F., Kartner, N., and Lukacs, G.L. (1998). Limited proteolysis as a probe for arrested conformational maturation of delta F508 CFTR. Nat. Struct. Biol. *5*, 180–183.