

**Selection of Misfolded CFTR for Proteasomal Degradation  
by Sequential Quality Control Checkpoints**

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## ABSTRACT

J. Michael Younger: Selection of Misfolded CFTR for Proteasomal Degradation  
by Sequential Quality Control Checkpoints  
(Under the direction of Douglas M. Cyr)

CFTR $\Delta$ F508 exhibits a correctable protein-folding defect. Protein Quality Control components identify and degrade proteins such as CFTR $\Delta$ F508 that fail to acquire a native conformation. Loss of CFTR activity results in improper lung mucosa hydration and causes cystic fibrosis. Components that function in the CFTR biogenic pathway have been identified. However, to more fully understand the folding defect and to identify the biogenic intermediate that is selected for degradation it is necessary to identify the Quality Control components responsible for CFTR $\Delta$ F508 degradation. Herein we identify two novel multi-subunit E3 Ubiquitin Ligase complexes: the cytosolic Hsc70/CHIP/UbcH5a complex, and the ER localized Derlin-1/RMA1/Ubc6e complex. Inactivation of the Hsc70/CHIP/UbcH5a complex using a mutant dominant negative form of UbcH5a has led to the identification of a CFTR $\Delta$ F508 biogenic intermediate that can acquire a detergent soluble stable interaction with molecular chaperones and upon exposure to 26°C, can fold and be expressed at the plasma membrane (PM). Studies utilizing both the Hsc70/CHIP/UbcH5a complex and the Derlin-1/RMA1/Ubc6e complex to comparatively study the sensitivity of CFTR biogenic intermediates to each quality control component has led to the finding that the kinetically trapped folding intermediate caused by the  $\Delta$ F508 mutation is identified by the Derlin-1/RMA1/Ubc6e complex early in the

biogenic process. Thus, these studies have been instrumental in the discovery of two sequential Quality Control checkpoints that function to target CFTR $\Delta$ F508 for proteasomal degradation.

For my children, Linnea and Aaron

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## LIST OF ABBREVIATIONS

AAA-ATPase	AAA family of ATPases
ABC	ATP binding cassette
ADP	adenine diphosphate
ALLN	N-Acetyl-Leu-Leu-NorLeu-Al
AMP	adenine monophosphate
ApoB	apolipoprotein B
APS	Ammonium persulfate
ASN	asparagine
ATP	adenine triphosphate
BFA	brefeldin A
BiP	immunoglobulin heavy chain binding protein (also known as GRP78)
BSA	bovine serum album
CAAX	cystine-alanine-alanine-any amino acid
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFTR $\Delta$ F508	CFTR deletion phenylalanine 508
CHIP	Carboxyl-terminus of Hsp70 Interacting Protein
CHN	a <i>C. elegans</i> homologue of CHIP (CHIP homologue new)
CoP	coat protein complex
COS	monkey-African green-kidney cells
CNX	calnexin

CTD	COOH-terminal domain
Cue	coupling of ubiquitin conjugation to ER degradation
CYC	cycle
Der	Degradation in the ER
DIF	detergent insoluble fraction
DMEM	Dulbecos' modified eagle medium
DNA	deoxyribonucleic acid
Doa	degradation of alpha 2
DSF	detergent soluble fraction
DTT	dithiothreitol
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase
ECL	enhanced chemiluminescent substrate
EDEM	ER-degradation enhancing $\alpha$ -mannosidase-like protein
EDTA	Ethylenedinitrilo-tetraacetic acid disodium salt
EEVD	glutamic acid-glutamic acid-valine-aspartic acid
EndoH	endoglycosidase H
ER	endoplasmic reticulum
ERAD	ER associated degradation
Erdj	ER DnaJ homologue
ERQC	ER luminal Glycoprotein Quality Control System
F508	phenylalanine 508

G/F	glycine and phenylalanine rich region
GFP	green fluorescence protein
gp	glycoprotein
gst	glutathione s-transferase
GT	glucosyltransferase
HA	hemagglutinin
Hdj	human DnaJ
HECT	homologous to E6-AP Carboxyl Terminus
HEK	human EPH/ELK-like kinase
HMG-CoA	hydroxyl 3-methylglutaryl coenzyme A reductase
Hop	Hsp organizer protein
HPD	histidine-proline-aspartic acid
Hrd	HMG-CoA reductase degradation
hrs	hours
Hsc	heat shock cognate
Hsp	heat shock protein
htm	homologous to mannosidase 1
INSIG	insulin induced gene
IP	immunoprecipitation
IPTG	isopropyl-beta-d-thiogalactopyranoside
kDa	kilodalton
Mata2	mating type locus alpha 2 repressor
MBP-RMA1	Maltose Binding Protein – RMA1

MEM-Met	Minimum Essential Medium without L-methionine
mg	milligram
MHC	major histocompatibility complex
ml	milliliter
mm	millimeter
mM	milli Molar
mRNA	messenger ribonucleic acid
MSD	membrane spanning domain
myc	c-myc derived epitope tag
$\mu$ Ci	micro curries
$\mu$ g	microgram
N	asparagine
NaCl	sodium chloride
NBD	nucleotide binding domain
NMR	nuclear magnetic resonance
NPL	nuclear protein localization
p	protein
PBS	phosphate buffered saline
pH	potential of Hydrogen
PI	protease inhibitor cocktail
PM	plasma membrane
PMSF	phenylmethsulfonyl fluoride
PPPY	proline-proline-proline-tyrosine

PPxY	proline-proline-any amino acid-tyrosine
PrP	prion protein
QC	quality control
QQ	glutamine-glutamine
R	regulatory domain
RING	Really Interesting New Gene
RIPA	Radio-immunoprecipitation analysis
RMA1	RING membrane associated
RMA1 $\Delta$ TM	RMA1 without transmembrane
RNF	RING finger protein
RT-PCR	reverse transcriptase-polymerase chain reaction
S	sulfur
SDS	sodium dodecylsulfate
SDS-PAGE	SDS – polyacrylamide gel electrophoresis
Sec	Secretory
SiRNA	small interfering RNA
TCR $\alpha$	T-cell receptor subunit alpha
TEMED	Tetramethylethylenediamine
TMAO	trimethylamine-N-oxide
TPR	tetrotricopeptide repeat
TRAM	tetramethyl rhodamine isothiocyanate-labeled anti-myosin
Ubc	ubiquitin conjugating
Ufd	ubiquitin dependent degradation pathway

US11	unique short region 11
VIMP	VCP(p97) interacting membrane protein
WW	tryptophan-tryptophan
Y	tyrosine



## **Chapter One**

### **General Introduction**

## 1.1 Cystic Fibrosis

Historically it was known that a child born with salty sweat was likely to die at an early age (Busch, 1990) (Gibson and Cooke, 1959). Often, the child would survive repeated respiratory infections and acute gastro-intestinal discomfort into its early teens. However, at an unusually late onset of adolescence the child would become thin and weak due to inadequate nutrition and die from symptoms similar to pneumonia.

In 1938, a pathologist, Dr. Dorothy Anderson, determined that the cause of death was related to the progressive destruction of lung tissue and usually included significant damage to the pancreas (<http://www.cff.org/home>) (Kalin et al., 1999). She termed the disease “cystic fibrosis of the pancreas”, a description of the observed condition of the pancreatic tissue under the microscope, despite the damage observed in the lung tissue. Several years later, in 1946, a report supported by familial data concerning the genetic occurrence of cystic fibrosis (CF), indicated that the disease was a recessive condition that was probably due to a mutation in a single gene (<http://www.cff.org/home>).

The most successful treatments of CF that resulted in improved quality and extension of life involved strategies to clear the mucus from the lungs, suggesting that CF mortality was closely linked to damage to the lung tissue. Analysis of mucus from CF patients indicated increased viscosity, having a consistency comparable to a gel-like material in contrast to a slightly thickened fluid. The increased mucus viscosity prevented adequate ciliary clearance which resulted in the accumulation of debris and bacteria entrained mucus (Puchelle et al., 1985). Thus, the primary problem in the CF lung is the recurrence of mucosal obstruction and infection resulting in progressive lung damage and early onset cardio-pulmonary failure. These problems coupled with pancreatic insufficiency and failure to thrive due to poor nutrition led to

premature death (Weber et al., 1976). Strategies to address dietary concerns generally involved high calorie, low fat content diets which were augmented in the 1970's with the introduction of pancreatic enzyme supplements (Weber et al., 1976). Enzyme delivery innovations and improved nutrition management has helped dramatically to improve quality of life, and positively influenced the life span of those afflicted with CF (Farrell et al., 2001).

To date, strategies to address nutrition, mucus clearance and the prevention of microbial infection have been most instrumental in the extension of life for CF patients. A strategy used to promote clearance of mucus involves the use of physical therapy techniques such as clapping the chest and back to mechanically encourage disruption of mucosal blockages in airway passages. Mechanical encouragement strategies are often coupled with patient orientations that promote gravity assisted clearance. Continued research related to the composition of the mucus in the CF lung has led to the development of DNase based enzymes that are delivered directly to the lung mucosa via inhaler systems, and hypertonic saline treatments (Shak et al., 1990) (Fuchs et al., 1994). The DNase functions to reduce mucus viscosity by digesting DNA material deposited by dead neutrophils that have accumulated in response to bacterial infection. Hypertonic saline helps to restore the electrolyte composition of CF mucus to normal parameters in an effort to encourage airway cells to secrete more water and improve ciliary clearance properties (Robinson et al., 1996) (Pavia et al., 1978). A major complement to mucus clearance strategies, which include but are not limited to those discussed above, is the periodic use of aggressive antibiotic and antimicrobial regiments to minimize bacterial growth in the lung (Wood and Smyth, 2006) (Mendelman et al., 1985). Bacterial growth prevention helps to minimize immune system responses that result in lung tissue inflammation and the accumulation of dead neutrophils that promote thickening of the mucus, both of which

exacerbate the CF condition. Thus, antibiotics play a critical role to minimize damage to the CF lung tissue. The recent development of antibiotics that are effective against *Pseudomonas aeruginosa* has been very significant in the treatment of CF considering the high propensity of infection and negative impact this particular organism has on the CF lung, and consequently quality and length of life of the patient (Kelly and Lovato, 1984) (Rowe et al., 2005).

Although there have been incredible advances in the treatment of CF which have resulted in a much improved quality of life and an increase in life expectancy from early adolescence to a median age of 36.8 years, much improvement is still needed. Currently, there are about 70,000 people worldwide with CF, and about 30,000 reside in the U.S. Every year about 1000 new cases will be identified. Historically, the treatment of CF has been directed at controlling the symptoms related to the thick mucus characteristic of the CF lung, and in the near future, additional similar treatments may become available. However, these treatments appear to become less effective as the patient becomes older primarily due to the accumulative lung tissue damage and the inaccessible nature of the bacterial infections. Thus, the development of improved treatments for CF requires an understanding of the cause of CF at the molecular level.

In 1989, the gene responsible for CF was identified. Cloning the gene permitted preliminary studies that indicated the encoded protein functions as a  $\text{Cl}^-$  ion channel (Riordan et al., 1989). This finding helped to explain the salty sweat associated with CF and the imbalance in mucus electrolyte composition in the CF lung. The gene was also shown to be active in epithelial cells that line the airway, salivary glands, intestines, and reproductive tissues (Buchwald et al., 1991). Considering its implication in causing CF, and its putative  $\text{Cl}^-$  channel activity, it was named the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989).

Follow-up studies using a reconstituted *in vitro* system provided direct evidence that CFTR functions as a chloride ion channel (Anderson et al., 1991).

### **1.2 Classes of Disease Causing CFTR Mutations**

There are more than 1,000 disease causing mutations in the CFTR gene of CF patients (Rowe et al., 2005). These CFTR mutations have been categorized into six classifications based on the type of defect they cause (Rowe et al., 2005). Class I mutations result in nonsense mutations or translational stop codons that lead to truncations of CFTR. Class II mutations cause full length transcripts to be processed improperly during biogenesis. Improper processing is caused by introduced perturbations that affect folding or assembly and result in trafficking problems and premature degradation (Cheng et al., 1990) (Yang et al., 1993). Class III mutations cause CFTR to exhibit abnormal ion channel activity usually due to improper regulation. Class IV mutations cause channel defects in CFTR that cannot support Cl ion passage across the membrane. Class V mutations result in reduced CFTR transcript levels, while Class VI mutations reduce CFTR's half life at the apical cell surface (Swiatecka-Urban et al., 2005). Although each class of mutation has the potential to play a significant role in the development of CF (Frizzell, 1999), mutations that cause defects in early biosynthesis and folding (Class I, II and V) provide the major contribution to the disease phenotype in CF patients. Approximately 90% of patients identified have an allele in which F508 is deleted (Welsh and Smith, 1993) (Amaral, 2005). The mutation resulting in the deletion of F508 is the major contributor to the disease phenotype specifically due to its common occurrence.

### **1.3 CFTR Domain Structure and Biogenesis**

Human CFTR is a large integral membrane glycoprotein composed of 1480 amino acids (Riordan et al., 1989). Amino acid sequence analysis, suggests that CFTR is a member of the

ATP Binding Cassette (ABC) transporter superfamily. ABC transporter proteins are found in both prokaryotes and eukaryotes and function to transport a variety of substrates such as ions, lipids, and proteins across membranes (Higgins, 1992). CFTR's primary amino acid sequence and its topology are similar to other ABC transporter proteins (Higgins, 1992), which supports the generally accepted prediction for the presence of four of its structural subdomains: two membrane-spanning domains (MSD), two nucleotide binding domains (NBD); and a unique regulatory (R) domain. The MSDs are each composed of six membrane spanning helices positioned in the lipid bilayer with alternating orientation, while the NBDs and the R domain are positioned in the cytosol (Winter and Welsh, 1997) (Riordan et al., 1989).

Our knowledge concerning the exact positioning of the subdomains relative to each other, and defined direct contact points between subdomains, is limited. However, information from studies related to other ABC transporters (Smith et al., 2002) (Locher et al., 2002) (Hopfner et al., 2000) to understand the function of subdomains (Ostedgaard et al., 1997) (Chen et al., 2000), examination of subdomain boundaries (Wang et al., 2002) (Bianchet et al., 1997) (Riordan et al., 1989) (Chen et al., 2000) and crystallization of murine NBD1 (Lewis et al., 2004) have led to an improved understanding of CFTR's tertiary structure. Based on these studies it is likely that the MSDs interact with each other primarily within the lipid bilayer, and the nucleotide binding domains pack tightly with each other and regions of the MSDs that are exposed in the cytosol (Ostedgaard et al., 1997). The interaction between the NBDs is important for the complete formation of the Walker A-Walker B motif (Frelet and Klein, 2006) which enhances the catalytic ATPase activity of the protein (Ostedgaard et al., 1997) (Chen et al., 2000) (Higgins, 1992). The R domain which is not found in other ABC Transporter Family members, thus exclusive to CFTR, is believed to be positioned distal to the MSDs in direct

contact with both NBDs (Ostedgaard et al., 1997). The orientation of the R domain is anticipated due to the regulatory influence that its phosphorylation status has on the ADP-ATP exchange rate and the kinetics of ATP hydrolysis by both NBDs (Winter and Welsh, 1997). The fourth extra-cellular loop on CFTR has two asparagine glycosylation sites (Riordan et al., 1989) which have been predicted to function as signals for components of ERQC for nascent CFTR protein folding and degradation (Pind et al., 1994) (Farinha and Amaral, 2005) (Amaral, 2004).

CFTR is co-translationally inserted into the ER membrane (Pasyk and Foskett, 1995) (Oberdorf et al., 2005) (Sadlish and Skach, 2004). In general, co-translational insertion of integral membrane secretory proteins involves endoplasmic reticulum (ER) membrane localized translocation machinery. This translocation machinery includes components of the heterotrimeric Sec61 complex, the ribosome, and accessory proteins such as TRAM that provide specialized functions (Hegde et al., 1998). The Sec61 complex is composed of Sec61, Sec62 and Sec63 (Meyer et al., 2000) (Deshaies et al., 1991) which function together to form a pore that traverses the ER membrane bi-layer and provides a site for ribosome tethering and is referred to as the translocon (Sadlish and Skach, 2004). The ribosome in association with the translocon functions to properly insert transmembrane domains and ER luminal domains via polypeptide chain elongation (Fedorov and Baldwin, 1997). Accessory proteins function in cooperation with the translocon to facilitate activities, such as TRAM, which provide lateral movement of transmembrane domains from the translocon to the lipid bi-layer (Hegde et al., 1998) (Wang and Dobberstein, 1999) (Pitonzo and Skach, 2006).

Due to the large size of CFTR, completion of translation and insertion into the ER membrane requires approximately nine minutes (Ward and Kopito, 1994). During synthesis, individual

subdomains begin to acquire a folded state (Strickland et al., 1997) (Ostedgaard et al., 1997). Ideally upon completion of synthesis, CFTR can collapse to a stable native conformation in which all of the subdomains become tightly packed into a functional ion channel (Ostedgaard et al., 1997). Folding of CFTR subdomains reportedly requires interactions with molecular chaperones in both the cytosol (Meacham et al., 1999) (Yang et al., 1993) and in the ER membrane (Pind et al., 1994). These interactions will be discussed in a later section.

Once CFTR synthesis is complete and the folded subdomains pack to acquire a native conformation, interactions with the molecular chaperone system become transient (Meacham et al., 1999) and CFTR can be trafficked away from the ER (Wang et al., 2004) (Yoo et al., 2002). CFTR export is accomplished by the formation of COPII vesicles that bud from the ER and specialize in sequestering export competent soluble and membrane associated proteins (Tang et al., 2005) (Wang et al., 2004) (Hauri et al., 2002). Selection of CFTR for export is similar to other membrane embedded secretory proteins, and likewise, the initial step requires sorting to ER exit sites (Ellgaard and Helenius, 2003). The sorting process is thought to involve a level of selectivity that is sensitive to the folded or assembled status of proteins and protein complexes respectively, providing a level of protein quality control (Ellgaard and Helenius, 2003) (Helenius and Aebi, 2004). The exact mechanism responsible for sorting secretory proteins is not completely defined. However, it is clear that the glycosylation status of a glycoprotein and the ability to interact with lectin binding proteins plays an important role in the efficiency of COPII vesicle packaging and trafficking (Ellgaard and Helenius, 2003). Assembled or properly folded proteins are concentrated in ER exit sites and packaged into nascent COPII vesicles (Sato, 2004) (Mancias and Goldberg, 2005) (Fromme and Schekman, 2005) (Tang et al., 2005). Export implies transport through the cis-, then the medial- and then



the trans-compartments of the Golgi apparatus and expression at the cell surface (Puthenveedu and Linstedt, 2005). Each level of the Golgi apparatus has its own set of resident components. Passage of CFTR through the medial-Golgi apparatus compartment results in exposure to mannose trimming enzymes, which further trim the already trimmed core glycosylation, while passage through the trans-Golgi apparatus compartment results in the addition of an array of extensive of sugar moieties (de Graffenried and Bertozzi, 2004).

Since extensive glycosylation of CFTR is performed in the trans-Golgi apparatus compartment, and CFTR is destined for export to the PM, complex glycosylation is indicative of CFTR's acquisition of a folded state. A crude yet simple biochemical method for determining the biogenic status of CFTR and a correlating sub-cellular localization is to observe the CFTR mobility pattern on SDS-PAGE gels (Welsh and Smith, 1993). Complexly glycosylated (mature) CFTR has a mobility that corresponds to 150-160kDa (C-form), while nascent core glycosylated (immature) CFTR that has not been trafficked from the ER has a mobility of 140kDa (B-form). Immature B-form CFTR potentially represents two distinct pools of core glycosylated CFTR; nascent CFTR that has just been synthesized, and CFTR that is folded to a stable conformation (stable B-form), but has not been trafficked from the ER to the Golgi apparatus for glycolytic processing (Cheng et al., 1990). A third form of CFTR exists in which proper ER insertion and core glycosylation did not occur. This form of CFTR is likely found as an aggregate in the cytosolic compartment, with mobility on SDS-PAGE gel slightly faster than B-form and is commonly referred to as A-form CFTR.

CFTR $\Delta$ F508 appears on an SDS-PAGE gel as a single band with mobility similar to CFTR B-form. Histological studies indicate that CF patients with the CFTR $\Delta$ F508 mutation do not express CFTR at the plasma membrane (PM) of sweat glands (Kartner et al., 1992).

Information obtained from studies to address this finding suggested that CFTR $\Delta$ F508 was unable to fold and was degraded. This supported the notion that the loss of CFTR function was the central cause of CF and that decreased CFTR activity resulted in abnormal Cl<sup>-</sup> and water transport across the apical membrane of epithelia cells. Thus, the loss of activity was proposed to cause improper hydration and thickening of mucus lining the airway, the major contributor to the CF pathology. Later, human CFTR $\Delta$ F508 was expressed in *Xenopus oocytes* which are normally grown at temperatures less than 37°C (Denning et al., 1992). This system routinely permitted CFTR $\Delta$ F508 expression, similar to that of CFTR at the plasma membrane and demonstrated the ability of CFTR $\Delta$ F508 to exhibit some Cl<sup>-</sup> channel activity (Denning et al., 1992). This study provided the first evidence that the CFTR $\Delta$ F508 responsible for most cases of CF is a temperature sensitive folding mutant that under favorable folding conditions can obtain a conformation that supports Cl<sup>-</sup> ion channel activity at the PM. As a result much research is directed at understanding the perturbation in CFTR folding and the downstream consequence of that aberrant folding when the  $\Delta$ F508 mutation is present (Denning et al., 1992) (Lukacs et al., 1994). The hope is to use this understanding of the CFTR biogenic pathway to develop improved treatments that permit the expression of CFTR $\Delta$ F508 at the PM and recovery of its activity to restore adequate mucus hydration and clearance to prevent lung damaging microbial infections, and ultimately the elimination of CF.

#### **1.4 Chaperone Systems function in CFTR biogenesis**

CFTR folding is complicated because it exposes mass to the cytosol and the ER lumen and must form a pore in the membrane through interactions between MSD1 and MSD2 (Chen et al., 2004) (Ostedgaard et al., 1997). In addition, acquisition of a native conformation requires the correct and efficient co-translational folding of individual cytosolic subdomains (Sheppard et

al., 1994) (Meacham et al., 1999), and the formation of proper interdomain interactions (Cyr, 2005). Both cytosolic chaperones and ER luminal chaperones (Pind et al., 1994) play an integral role in CFTR's ability to obtain a native structure. The molecular chaperone system is composed of proteins that functionally bind solvent exposed hydrophobic patches and prevent inappropriate interactions between neighboring proteins that could lead to the formation of toxic intracellular aggregates (Bukau et al., 2006). In general, the molecular chaperone system is composed of a variety of different families such as the Heat Shock Protein Families; Hsp40, Hsp70, Hsp90, Hsp100, the small Hsp proteins, and the chaperonins (Ellis, 2000) (Fan et al., 2003) (Picard, 2002) (Hohfeld et al., 2001) (Porankiewicz et al., 1999) (Welsh and Gaestel, 1998) (Ellis, 1999). All of these members are important for the maintenance of normal cellular protein homeostasis; however, a role for them in eukaryotic protein quality control in the process of nascent membrane protein folding is somewhat limited to the members of the Hsp90 (Picard, 2002), Hsp70, and Hsp40 families (Fan et al., 2003), as well as a group of specialized ER luminal proteins such as calnexin (Ellgaard and Helenius, 2003). In the following sections, I will discuss these different chaperone systems which have been shown to function in CFTR quality control.

#### **1.4.1 ER Luminal Chaperones**

The ER luminal Glycoprotein Quality Control System functions to monitor the folded state of nascent integral membrane glycoproteins in the ER lumen and helps them fold (Ellgaard and Helenius, 2003), or targets them for degradation (Amaral, 2004) (Parodi, 2000). A glycoprotein is formed by the addition of a core glycosylation moiety to an Asparagine of a nascent polypeptide that exposes a glycosylation site (Asn-Xaa-Ser/Thr) to the ER lumen. The core glycosylation of the glycoprotein is first trimmed from  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ - to

Glc<sub>1</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>- by Glucosidase I and II. Glc<sub>1</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>- is a substrate for the lectin binding ER localized molecular chaperone, calnexin. Calnexin binds the glycoprotein substrate and upon trimming to Man<sub>9</sub>GlcNAc<sub>2</sub>- by glucosidase II, releases the substrate allowing the substrate to attempt folding or assembly to a native conformation (Ellgaard et al., 1999). If acquisition of a native conformation is not obtained, glucosyltransferase (GT) modifies the glycosylation status of the glycoprotein to Glc<sub>1</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>- and the cycle continues (Ellgaard et al., 1999). Discontinuation of the cycle is dependent on acquisition of a native conformation, or trimming of the glycosylation status to a condition that is recognized by EDEM (Hosokawa, 2004) (Oda et al., 2003). Acquisition of a native conformation results in trimming to a glycosylation status that calnexin cannot bind but permits selection for delivery to an ER exit site, and trafficking to the Golgi apparatus in a CopII dependent vesicle trafficking pathway (Ellgaard et al., 1999) (Cabral et al., 2001). Passage through the cis- and medial-Golgi exposes the high mannose glycoprotein to further trimming of mannose molecules, a process that imparts resistance to endoglycosidase H trimming (Seibert et al., 1996). Continued trafficking of the glycoprotein through the trans-Golgi results in the addition of an extensive network of glucose molecules by Glucosyltransferases that can function for N- and O-glycan chain elongation (Berger, 2002). In the case that a substrate fails to fold, this results in a trimming status that signals for an interaction with EDEM (Oda et al., 2003), a lectin binding protein that functions to target glycoproteins for degradation (Helenius and Aebi, 2004).

The calnexin system model suggests that glycoproteins such as CFTR must interact with calnexin for folding and that this system may function to target CFTR for degradation. Thus, role for calnexin to interact with CFTR in the ER lumen to assist CFTR folding has been

proposed based on studies in yeast that demonstrate this requirement. In addition, a role for EDEM to function in the degradation of CFTR has been suggested based on the requirement of Htm1p in yeast (Gnann et al., 2004).

An interaction between calnexin and CFTR has been demonstrated in mammalian cells utilizing co-immunoprecipitation studies (Farinha and Amaral, 2005), however, treatment of cells with castanospermine, an inhibitor of glucosidase I and II, to block CFTR interactions with calnexin does not cause CFTR to aggregate and become insoluble (Cyr lab, unpublished observations). It does however cause a 50 percent reduction in C-form formation, but no accumulated B-form. Thus, the role of calnexin remains inconclusive.

#### **1.4.2 Cytosolic Chaperones**

The cytosolic domains of CFTR account for about 70% of the protein, and are thought to contribute substantially to the complexity of CFTR folding (Strickland et al., 1997). The molecular chaperones that appear to play a significant role in the folding of cytosolic subdomains are members of the Hsp Family of Molecular Chaperones, Hsp/Hsc70, Hsp90 and the co-chaperone Hsp40 proteins; Hdj1 and Hdj2 (Strickland et al., 1997) (Yang et al., 1993) (Pind et al., 1994) (Meacham et al., 1999).

##### **1.4.2a Hsp90 Chaperone**

Hsp90 is a soluble 90kDa ATPase that is most commonly known to chaperone steroid hormone receptors and signaling kinases (Picard, 2002) (Xu and Lindquist, 1993) that are not in association with their ligand or have not been activated (Jakob et al., 1995). Hsp90 can interact with Hsp70 via the Hsp organizer protein (Hop), which has two TPR domains (Chen et al., 1996) (Smith et al., 1993) (Chen and Smith, 1998), to form a multichaperone complex (Bose et al., 1996). The formation of this complex appears to permit the transfer of substrates

between the chaperones, most likely from Hsp70 to Hsp90 (Prodromou et al., 1999). The chaperone activity of Hsp90 is regulated by its co-chaperone p23. p23 interacts directly with unfolded proteins as a chaperone (Freeman et al., 1996) (Weikl et al., 2000) and delivers them to the ATP bound form of Hsp90 (Sullivan et al., 1997). The interaction between Hsp90 and p23 promotes substrate release after ATP hydrolysis by Hsp90 (Young and Hartl, 2000) (Prodromou et al., 1999).

The Hsp90 molecular chaperone is thought to play a limited role in protein folding in that it can stabilize pre-formed cytosolic subdomain structures and suppress degradation (Xu et al., 2002) (Youker et al., 2004), but it is not thought to act generally in nascent protein folding (Nathan et al., 1997) . Data on the involvement of Hsp90 in the folding of CFTR is limited however, there is data which shows a distinct role for Hsp40 and Hsp90 to degrade CFTR in yeast (Youker et al., 2004).

#### **1.4.2b The Hsp70/Hsp40 Chaperone/Co-chaperone pair**

Hsp70 proteins reversibly bind proteins that are nascent, unfolded, or partially unfolded, in an ATP dependent cycle to prevent inappropriate hydrophobic interactions and to facilitate their folding (Gething and Sambrook, 1992) (Hartl et al., 1992). The Hsp70 family of molecular chaperones are soluble ~70kDa proteins that contain an NH<sub>4</sub>-terminal ATPase domain, a substrate binding domain, and a COOH-terminal domain. The ATPase domain has intrinsic ATPase activity, and is structurally composed of two lobes with a central cleft for binding ATP. The substrate binding domain is composed of two structures, a  $\beta$ -sandwich that forms a substrate binding cavity and an  $\alpha$ -helical component that forms a structure similar to a lid. The COOH-terminus may also contain an EEVD motif, depending on the species, that is important for interactions with tetratricopeptide repeat motif containing proteins. When the

Hsp70 ATPase domain has a bound ATP, the lid structure is open making the substrate binding pocket accessible to extended non-native polypeptides, however, the affinity for substrate is low and the substrate may quickly be bound and released (Mayer and Bukau, 2005). Interactions between the substrate and the substrate binding pocket of Hsp70 are generally hydrophobic interactions between amino acid side chains, however hydrogen bond interactions can also be formed. Hydrolysis of ATP to ADP by the ATPase domain of Hsp70 results in an intramolecular conformational change of the substrate binding domain such that the affinity for a substrate is increased and the lid-like structure moves into a position that appears to mechanically inhibit substrate release (Erbse et al., 2004). Upon exchange of ADP for ATP, the conformation of the Hsp70 molecule will return to the low affinity open lid conformation, and the substrate is likely to be released (Gething and Sambrook, 1992) (Hartl et al., 1992). This difference in affinity for substrates suggests a role for Hsp70 to interact with non-native proteins differentially; with a low affinity providing rapid association/disassociation properties, or with a high affinity providing rapid association and slow disassociation properties. Thus the non-native protein binding activity of Hsp70 proteins is highly dependent upon the activity level of its ATPase domain. Interestingly, although Hsp70 has intrinsic ATPase activity, it is generally known that this activity is regulated by interacting proteins called co-chaperones (Liberek et al., 1991) (Cyr et al., 1992).

The Hsp40 Family of Molecular Chaperones, which are also known as the J-protein family (Walsh et al., 2004), interact with and regulate the activity of Hsp70 proteins. Two mammalian Hsp40 proteins that function with Hsp70 to help proteins fold are Hdj1 and Hdj2. The structure of both Hdj1 and Hdj2 contains the NH<sub>4</sub>-terminal prototypic 70 amino acid J-domain that contains a conserved HPD tripeptide sequence (Kelley, 1998), a domain rich in glycine and

phenylalanine residues (commonly referred to as the G/F-rich region), two COOH-terminal domains (CTDI and CTDII) and a dimerization domain. The J-domain interacts with and functionally stimulates the activity of Hsp70s ATPase domain. This interaction is characterized to functionally regulate the affinity of Hsp70s substrate binding activity (Mayer and Bukau, 2005) (Wall et al., 1994). The G/F region is thought to function as a ‘flexible extender’ or ‘hinge’ region based on the absence of secondary structure that links the J-domain to the CTDs. The crystal structure of residues 171-352 which correspond to Sis1s COOH-terminal domains indicates the presence of a homodimerization domain and the presence of a hydrophobic patch on the barrel-like structure formed from CTDI sequence (Sha et al., 2000). Mutagenic analyses of residues that form the hydrophobic patch indicate that they are required for Sis1 to interact with non-native proteins (Lee et al., 2002), and a potential for involvement in molecular chaperone activity. Hdj2 differs from Hdj1 in that it also contains both a zinc finger motif and a CAAX sequence. The zinc finger motif contains 4 repeated CXXCXGXG sequences that coordinate zinc ions to form a structure that bind substrates. The CAAX sequence functions as a site for farnesylation by the prenyltransferase enzyme. This post translational modification functions to anchor Hdj2, similar to other farnesylated Hsp40 proteins, to the ER membrane (Meacham et al., 1999) (Caplan et al., 1992), which positions Hdj2 to function in protein metabolism at the cytosolic face of the ER.

Hdj2 has been shown to function with Hsp70 *in vitro* to inhibit protein aggregation and promote folding of denatured luciferase, and has been shown to interact with Hsc70:substrate complexes that represent co-translational membrane protein folding intermediates of CFTR (Meacham et al., 1999). This study led to the understanding that Hsc70, Hdj1 and Hdj2 interact with CFTR immediately following insertion of MSD1. Hdj2s interaction with the



Hsc70:CFTR complex is elevated, consistent with expression of CFTR cytoplasmic subdomains, but is reduced upon translation of MSD2 (Meacham et al., 1999). The increased Hdj2 interaction suggests a requirement for increased Hdj2 activity in the folding of CFTR subdomains and the reduction in Hdj2 interaction with the complex upon translation of MSD2 suggests that Hdj2 performs a function that is resolved consistent with completion of MSD2 translation. An additional important observation from this study was that interactions between the Hsc70/Hdj2 pair and full length CFTR $\Delta$ F508 are greater than those with CFTR (Meacham et al., 1999).

This study indicates that Hdj2 is the major Hsp40 co-chaperone that functions with Hsc70 to prevent aggregation and to promote folding of CFTR. It also supports the conclusion that CFTR $\Delta$ F508 is aggregation prone, and exposes structure that is observed by the molecular chaperone system similar to a CFTR translation intermediate that precedes translation of MSD2. Thus, the propensity of CFTR to misfold appears to be exacerbated by the  $\Delta$ F508 mutation, suggesting a requirement for degradation.

### **1.5 CFTR Degradation**

Chemicals that have been shown to promote the stabilization of protein structure *in vitro*, such as glycerol and TMAO have been added to cell culture media and have been shown to permit expression of functional CFTR $\Delta$ F508 at the PM of culture cells (Brown et al., 1996). However, the molar concentrations of these chemicals that are required for observation of CFTR $\Delta$ F508 at the PM in cultured cells would be toxic to patients if administered at the same level (Gelman and Kopito, 2002). Studies with chemical chaperones (Gekko and Ito, 1990) in addition to studies that demonstrate that the  $\Delta$ F508 causes a temperature sensitive folding defect (Denning et al., 1992) suggested that modification of the cellular environment that favor

the folding of proteins may function to help CFTR $\Delta$ F508 fold, and could potentially rescue the CF phenotype if enough CFTR activity could be observed at the PM. To further understand the molecular basis of CFTR $\Delta$ F508 dysfunction, and to develop strategies to rescue some of the CFTR activity at the PM, one approach is to develop a combined understanding of how CFTR and CFTR $\Delta$ F508 display structural differences, and how these differences are observed by Quality Control components that determine their fate. Approximately 60-75 percent of wild-type CFTR and nearly 99 percent of CFTR $\Delta$ F508 is unable to fold (Ward and Kopito, 1994), cannot satisfy ERQC requirements, is retained in the ER and is subsequently degraded in a non-lysosomal pre-Golgi compartment by the cytosolic proteasome (Cheng et al., 1990) (Ward and Kopito, 1994) (Lukacs et al., 1994) (Welsh and Smith, 1993) (Yang et al., 1993) (Jensen et al., 1995). There are two lines of evidence to indicate that CFTR is degraded in a non-lysosomal, pre-Golgi compartment. First is the ability of CFTR to be degraded in the presence of a fungal metabolite, Brefeldin-A, that inhibits ER to Golgi vesicle trafficking (Ward and Kopito, 1994) (Lukacs et al., 1994). These data indicate that CFTR is degraded prior to a stage that involves trafficking to the Golgi apparatus. Secondly, CFTR degradation is unaffected by the treatment of cell media with chemicals which disrupt lysosomal degradation of substrates (Lukacs et al., 1994). Thus it was proposed that CFTR was degraded by some other mechanism. The addition of proteasome inhibitors to cell culture media caused the accumulation of CFTR in cultured cells transiently expressing the protein (Ward et al., 1995). Thus it was proposed that CFTR was a substrate of the proteasome and an ERAD substrate. It was known that CFTR required Hsp70 to be degraded (Zhang et al., 2001), but the mechanism was unknown.

### **1.5.1 CHIP/Hsp70 Complex**

The identification of CHIP, an Hsp70 interacting E3 that could possibly function in a

complex with Hsp70 to selectively degrade misfolded proteins was an interesting candidate to test for a role in CFTR degradation (Ballinger et al., 1999). Indeed, a role for CHIP in CFTR degradation was demonstrated to require both the U-box domain and the Hsp70 interacting TPR domain (Meacham et al., 2001). Increased expression of CHIP resulted in both the inability of CFTR to fold and acquire mature glycosylation status, and near complete degradation of all nascent CFTR (Meacham et al., 2001). These data indicated that CHIP functions to modify Hsp70s chaperone activity for the selection of misfolded substrates for degradation. Furthermore, it indicated that elevated levels of CHIP shifted CFTRs fate toward a degradation pathway. When CHIP deleted for its U-box domain was co-expressed with CFTR, CFTR did not accumulate in either the ER or at the PM as expected, instead, CFTR accumulated as an insoluble aggregate in the cytosol (Meacham et al., 2001). This suggested that elevated levels of CHIP that could interact with Hsp70, but could not function to target CFTR for degradation acted to inhibit Hsp70s normal folding function and caused CFTR to aggregate. All of these data supported the notion that identification of CFTR for degradation may be a simple matter of kinetics. If CHIP activity could be inhibited, this may shift the fate of CFTR toward the folding pathway and provide CFTR more time to fold prior to being recognized for degradation by the Ubiquitin Proteasome System.

## **1.5.2 The Ubiquitin Proteasome System**

### **1.5.2a The Ubiquitin System**

The identification of the first physiological substrate of the Ubiquitin System was in 1987 (Shanklin et al., 1987). Today, it is well established that the Ubiquitin System is a means of specifically targeting a protein for degradation (Bonifacino and Weissman, 1998). To target a protein for degradation, components of the Ubiquitin System utilize ubiquitin protein and

energy derived from the hydrolysis of ATP to build ubiquitin chains on a target protein. The targeting process and the components involved are generally understood and will be discussed in greater detail below, however; an understanding for how the Ubiquitin System selectively identifies a target that must be ubiquitinated and degraded is in its infancy and is available only in the most general sense. It is clear that the identification process is highly specific (Weissman, 1997) (Hershko and Ciechanover, 1998) and the client base is large. Thus, it is conceivable that the identification and characterization of the selection criterion used by the Ubiquitin System may continue for many years to come.

### **1.5.2b Ubiquitination**

Ubiquitination of a substrate involves the modification of a target protein with a covalently attached protein called ubiquitin. An ubiquitin chain is formed via isopeptide linkages between the ε-amino group of K48 of an ubiquitin moiety and the COOH- terminus of the ubiquitin that will be added (Kerscher et al., 2006). The ubiquitin protein is expressed in all eukaryotic organisms and is highly conserved from yeast to humans (Wilkinson et al., 1995). The ubiquitin protein is a 76 amino acid molecule with a molecular mass of approximately eight kDa. It has a unique fold that is very heat stable yet is significantly hydrophobic considering its solubility in the cytosol and the nucleus. Ubiquitin itself is a substrate of the Ubiquitin System which allows the formation of ubiquitin chains. The formation of ubiquitin chains functions as a signal to other cellular systems that recognize an ubiquitin chain composed of at least four ubiquitin proteins as a signal for degradation (Deveraux et al., 1994) .

### **1.5.2c Ubiquitin System Enzymes**

Specific ubiquitination of substrates requires the coordinated activity of three classes of enzymes commonly referred to as the E1 ubiquitin activating enzyme, the E2 ubiquitin

conjugating enzymes and the E3 ubiquitin ligases (Pickart, 2001) (Glickman and Ciechanover, 2002) (Hochstrasser, 1996) (Weissman, 1997) (Hershko and Ciechanover, 1998). The E1 catalyzes the activation of ubiquitin by hydrolyzing ATP to AMP and utilizes the energy to produce a high energy thioester bond between its active site cysteine residue and the NH<sub>3</sub>-terminal glycine of ubiquitin. The activated ubiquitin is transferred from the E1 to an active site cysteine residue in the conserved Ubc domain of the E2 Ubiquitin Conjugating enzyme. An E3 ubiquitin ligase functions to coordinate the transfer of the activated ubiquitin to a specific substrate by facilitating the transfer (Hershko and Ciechanover, 1998).

The E1 enzyme is highly conserved among eukaryotes and is represented by a single gene. In mammalian cells there are approximately thirty E2 enzymes that have been identified. The specificity of interactions between E2s and their cognate E3s is not fully understood. In many cases, the interactions are determined experimentally by conducting biochemical assays that lead to the identification of components in E2:E3 complexes. Several E2 have been generally categorized according to their association with specific systems, or according to their subcellular localization, usually relative to information obtained in yeast studies. For example, the Yeast Ubc4/Ubc5 Family of E2s is generally categorized as a family of E2s that function to degrade short-lived and abnormal proteins (Seufert and Jentsch, 1990) (Girod and Vierstra, 1993), and yeast Ubc6 and Ubc7 are categorized as membrane localized E2s based on their subcellular localization to the ER membrane (Biederer et al., 1997) (Lenk et al., 2002). There are hundreds of E3 enzymes in the mammalian cell suggesting the high level of specificity these enzymes confer to the ubiquitin system. There are two known classes of E3 Ubiquitin Ligase enzymes, HECT (Homologous to E6-AP Carboxyl Terminus) E3s (Huibregtse et al., 1995) (Cyr et al., 2002) (Pickart, 2001) and RING (Really Interesting New Gene) E3s (Cyr et

al., 2002) (Freemont, 2000). The HECT domain E3s have a HECT domain that functions similar to an E2 Ubc domain, having an active site cysteine that can be charged with an activated ubiquitin molecule that is delivered by an E2 (Cyr et al., 2002). The NH<sub>2</sub>-terminus of each HECT domain E3 is specific for a particular group of substrates while the COOH-terminus functions to recruit specific E2s and facilitate ubiquitin chain elongation. RING E3s are structurally and functionally distinct from HECT E3s (Cyr et al., 2002). They contain a RING domain that is formed and structurally stabilized by coordinated ionic interactions between zinc and histidine or cysteine residues that are properly spaced (Hatakeyama and Nakayama, 2003). The RING domain functions to transiently recruit E2s to the E3:substrate complex suggesting a scaffold-like function for the RING E3s. The mechanism of ubiquitin transfer to the substrate is not known. RING E3s that can directly interact with substrates using a substrate recognition domain are considered single-subunit RING E3s while those that require an adaptor protein or substrate selector that specifically recognizes the substrate are considered multi-subunit RING E3s (Cyr et al., 2002).

The RING domain family has recently been expanded to include a sub-class of U-box proteins that have a U-box domain in contrast to a RING domain (Cyr et al., 2002). The structural similarity between the RING domain and the U-box domain has been substantiated by computer based structural modeling techniques (Aravind and Koonin, 2000), and more recently by NMR structural data generated with the U-box domain of Prp19 interacting with a fragment of its cognate E2, UbcH7 (Ohi et al., 2003). The data indicate that not only is the structure of the RING and U-box domains conserved, residues important for interactions between RING domains and their cognate E2 correlate with residues in the U-box domain that are important for their interaction with cognate E2s (Ohi et al., 2003). Thus, the U-box domain

is predicted to function as a non-canonical RING domain, but the structure of the domain is stabilized by hydrogen-bonding and ionic-bridging interactions in contrast to coordinated zinc ionic interactions found in RING domains (Ohi et al., 2003). It has been hypothesized that U-box proteins function differently from RING E3s and perform as an E4 poly ubiquitin chain elongation factor. This hypothesis is supported by the inability of the U-box containing protein, yeast Ufd2, to independently facilitate ubiquitin chain assembly on a substrate. However, Ufd2 is required for efficient poly ubiquitination and degradation of artificial substrates when the target substrate is first ubiquitinated by the HECT E3 Ufd4 (Koegl et al., 1999). Other U-box E3s such as CHIP, CYC4, Prp19 and UIP5 in addition to CHN1, a *C. elegans* homologue of CHIP, have been shown to function with E2 ubiquitin conjugating enzymes, and independent of other E3s, to specifically target substrates for efficient poly ubiquitin chain assembly (Cyr et al., 2002) (Hatakeyama and Nakayama, 2003). Thus, U-box proteins represent a new sub-class of the RING type E3s (Hatakeyama and Nakayama, 2003).

#### **1.5.2d Ubiquitin System and ER Membrane Proteins**

Until 1993, the Ubiquitin System was thought to target soluble cytosolic and nuclear proteins for degradation. Around that same time, questions concerning the mechanism by which misfolded mutant ER proteins are selected for degradation were being addressed. These concerns arose from studies indicating that misfolded proteins and misassembled protein subunits that are retained in the ER are degraded in a manner that seemed instantaneous, having no apparent accumulation of degradation intermediates in the cytosol, and no association with the endosomal compartment. The first piece of evidence to address these concerns came from a study indicating a role for the Ubiquitin System in the degradation of a mutant ER protein. The study demonstrated that defects in protein translocation, caused by a temperature sensitive

mutation in Sec61 that promoted its degradation at increased temperature, could be suppressed by mutating Ubc6 (Sommer and Jentsch, 1993). Thus, a connection between degradation of abnormal proteins in the ER and the Ubiquitin System was identified. Since this discovery, much research has focused on the mechanisms by which a variety of ER membrane proteins are selected for degradation by the Ubiquitin System, and how that ubiquitinated substrate is degraded by the proteasome.

### **1.5.2e The Proteasome**

Degradation of proteins is a major function in the cell. Degradation of proteins is not exclusively accomplished by the proteasome however; the role of the proteasome is substantial as indicated by the approximately  $5 \times 10^5$  proteasomes present in a cell which accounts for about 0.6 percent of cellular protein (Hendil, 1988). Almost half of all proteins in the cell are degraded within the first twenty-four hours of being synthesized (Rock et al., 1994), and nearly one-third of all newly synthesized proteins are degraded by the proteasome within minutes of their synthesis (Schubert et al., 2000) (Yewdell, 2001). The 26S proteasome is a multi-protein complex that is found in the nucleus and the cytosol of eukaryotic cells (Coux et al., 1996). It is composed of one or two 19S multimeric protein complex(s), and one core 20S multimeric protein complex. The 20S component has a cylindrical or barrel like structural arrangement formed by a stack of four rings each of which is composed of seven protein subunits (Lowe et al., 1995) (Groll et al., 1997). The interior of the hollow barrel is lined with trypsin-, chymotrypsin-, and postglutamyl hydrolase-like activity which actively performs the proteolytic function to degrade polypeptides that are threaded into its core (Coux et al., 1996). The activity of this protease is regulated by 19S components that individually resemble a cap, and act to cover the open ends of the 20S barrel (Groll et al., 2000). Each 19S particle is made



up of a lid and a base. The base has 6 ATPase subunits that participate in substrate unfolding (Braun et al., 1999), and have chaperone like activity (Strickland et al., 2000). The lid is composed of proteins that function in recognition of polyubiquitinated substrates (Thrower et al., 2000), restricting the entrance of polypeptides with extensive secondary structure (Lam et al., 1997), cleaving polyubiquitin chains from the substrate (Papa and Hochstrasser, 1993) (Lam et al., 1997) (Eytan et al., 1993) and to act as a docking site for components that present the substrate to the proteasome for degradation (Madura, 2004). The role for the 19S cap to recognize ubiquitinated substrates indicates the exclusive nature of the protease activity and indicates its association with a system regarded as highly specific thus, the Ubiquitin System and the Proteasome are typically regarded as the Ubiquitin- Proteasome System. The Ubiquitin-Proteasome System therefore does not participate to degrade proteins indiscriminately; in contrast, it functions in a highly regulated fashion to degrade proteins that have been post-translationally modified by a specific combination of enzymes that can be empirically shown to recognize specific substrates.

#### **1.5.2f Substrate Recognition**

Substrate recognition properties of the Ubiquitin Proteasome System are an area of intense investigation. It has been shown that proteasomes are recruited to locations within the cell where there is an abundance of ubiquitinated substrates in association with molecular chaperones (Garcia-Mata et al., 1999) (Anton et al., 1999) (Johnston et al., 1998). Thus it is clear that the molecular chaperone system functions to assist with the delivery of ubiquitin modified substrates that have been selected for degradation by the proteasome. The first piece of evidence that the molecular chaperone system is potentially involved in selecting misfolded ER membrane proteins for degradation was related to the finding that defects caused by a

mutant, temperature sensitive form of Sec61 that cannot support protein translocation, can be suppressed by mutating the Ubiquitin System component Ubc6 (Sommer and Jentsch, 1993). This finding not only identified a role for the Ubiquitin Proteasome System in the degradation of misfolded or unassembled ER membrane proteins, but it also introduced the notion that the misfolded protein selected for degradation could likely be found in association with components of the molecular chaperone system while being ubiquitinated. Later it was found that Hsp70 is required for degradation of misfolded substrates such as CFTR in yeast (Zhang et al., 2001). Critical pieces of evidence that provided additional support for this supposition was the identification of the interaction between Hsp70 and the U-box E3 Ubiquitin Ligase, CHIP (Carboxyl-terminus of Hsp70 Interacting Protein) (Ballinger et al., 1999) that could function to ubiquitinate and promote the degradation of the non-native forms of CFTR and CFTR $\Delta$ F508 (Meacham et al., 2001). The ability of the Ubiquitin System to ‘hi-jack’ cellular machinery not normally thought to be associated with protein degradation, and use them to functionally select and target specific proteins for degradation has become a general approach to studying protein degradation pathways. Viruses provide an interesting strategy that exploits this mode of operation displayed by the Ubiquitin System. The viruses’ genome encodes proteins that when expressed by the host cell function as scaffold proteins (Lilley and Ploegh, 2004). These proteins specifically link or recruit Ubiquitin System components such as E3 Ubiquitin Ligases to a target protein (Loureiro et al., 2006) (Lilley and Ploegh, 2005). This targeting results in proteins such as the MHC Class I molecule, a protein involved in advertising the viral infection on the cell surface, for degradation. Degradation of the MHC Class I molecule from the ER prevents antigen presentation at the cell surface and the virus remains undetected by immune system activity.

### **1.5.3 Endoplasmic Reticulum Associated Degradation**

Misfolded ER luminal and integral membrane proteins are retained in the ER and dislocated back into the cytosol to be degraded by a process commonly referred to as Endoplasmic Reticulum Associated Degradation (ERAD) (Hampton, 2002). This system is strictly the process by which a protein is extracted either out of the ER lumen or retrotranslocated out of the ER membrane, and processed for presentation to the proteasome for degradation (Hampton, 2002). In the case of ER luminal proteins, this requires that the substrate be processed by components of the calnexin cycle and be identified by the lectin binding protein EDEM/hml1, that functionally binds substrates having a glycosylation status identifiable as a signal for ER retention and degradation (Ellgaard et al., 1999) (Hosokawa et al., 2001). Once the substrate is identified, it is retrotranslocated across the ER membrane through a channel. Recent evidence of this process in yeast studies to identify components required for degradation of CFTR indicates several ERAD components such as Der3/Hrd1 (Bordallo et al., 1998) and Doa10 (Swanson et al., 2001), two ERAD E3s, and the p97/Cdc48-Ufd1-Npl4 (Bruderer et al., 2004) complex, an AAA-ATPase (to be discussed below) to be required. Interestingly, removal from the ER does not require activity from import machinery such as BiP, an ER Hsp70 homologue that provides energetic translocation assistance (Brodsky et al., 1999), nor does it require selective ubiquitination (Meyer et al., 2002), which is in contrast to earlier discussion concerning proteins that are exposed to the cytosol. Thus, it seems that the identification of ER luminal ERAD proteins is somewhat generic in that the major bulk of these proteins are ubiquitinated as they emerge from the ER by a single ubiquitin ligase complex, Hrd1/Der3 and the interacting E2s Ubc1, and Ubc7 in association with Cue1, a transmembrane protein that provides ER localization for Ubc7. There is evidence that an additional ER localized E3, gp78,

that also functions with Ubc7, may play a role in this process. The channel responsible for retrotranslocation of these proteins is controversial, however; evidence seems to point toward the passive Sec61 complex used in translocation that could conceivably function also in retrograde translocation (McCracken and Brodsky, 2003) (McCracken and Brodsky, 2005).

Integral membrane ERAD substrates can be separated into two categories; those that have cytosolic sub-domains and those that do not. The later proteins appear to require that their ER luminal domains be retrotranslocated and ubiquitinated similar to soluble ER luminal ERAD substrates (McCracken and Brodsky, 2005). The integral membrane ERAD substrates with cytosolic subdomains are identified by cytosolic Quality Control components such as members of the molecular chaperone system, and are selected for degradation by components of the Ubiquitin System (Meacham et al., 2001).

Independent of the mechanism by which the ERAD substrate is originally identified for degradation, beyond this point in the discussion there is no known definitive difference in the process for how ER luminal or ER membrane proteins are delivered to the proteasome, given that all of the ERAD substrates exhibit mass to the cytosol and have ubiquitin conjugates. Each of the substrates is proposed to interact with an AAA-ATPase protein, p97 (Lord et al., 2002). p97 is composed of two homoheptomeric rings that are stacked and form a central pore. The rings have ATPase activity that is utilized to cause a conformational change that generates force (Ye et al., 2003). The generated force mechanically drives a ratcheting type activity that is proposed to extract proteins from the ER membrane either through a channel, or directly from the lipid bi-layer. Interaction between p97 and the substrate is coordinated via the localization of p97 to the cytosolic face of the ER by two interacting proteins Ufd1-Npl4 (Meyer et al., 2002). Exactly how the p97, Ufd1-Npl4 complex is recruited to the membrane is

not clear. Ufd1-Npl4 has been shown to bind polyubiquitin chains via a zinc finger domain in Npl4 (Meyer et al., 2002) (Wang et al., 2003) and mediate substrate binding by p97 (Meyer et al., 2002). Another model suggests that p97 can bind ubiquitin independently (Dai and Li, 2001) (Rape et al., 2001). In resolution, p97 appears to interact with a substrate first via direct interaction with the substrate and then through interactions with ubiquitin (Ye et al., 2003).

The extraction process pulls the protein through the central pore of the p97 complex and is proposed to unfold the substrate for presentation to the proteasome. Unfolding of substrates will inherently expose hydrophobic patches. Thus, chaperone activity is likely required, unless p97 directs the substrate into the 19S with minimal cytosolic exposure. Considering that the 19S regulator also functions as an ATPase similar to p97, the 19S can pull the substrate into the proteolytic core of the 20S subunit for degradation (Navon and Goldberg, 2001).

It has been proposed that some substrates may not require p97 action and may initially interact with the 19S regulator. Studies have demonstrated that the 19S particle can extract a protein substrate from ER membrane *in vitro* (Navon and Goldberg, 2001). In either case, the ubiquitin conjugate enters the 19S through coordinated activity between the p97 complex and proteins associated with the cap. Rpn10 in yeast (Kominami et al., 1997), or S5a in mammalian cells (Deveraux et al., 1994) functions as a component of the 19S regulator to interact with ubiquitin, however the activity is dispensable with only mild proteasome activity reduction (Wilkinson et al., 2000). Additionally, prior to degradation, the ubiquitin chain is removed from the substrate by de-ubiquitination enzymes (Wilkinson, 2000). Once the protein enters the core, it is degraded to small peptides that are digested in the cytosol by free proteases.

Ubc6, a short-lived E2 that is localized to the ER by a COOH-terminus hydrophobic tail, is

extracted from the ER membrane independent of Sec61 activity (Walter et al., 2001). There is also evidence that some ERAD substrates, such as Carboxyl peptidase Y\*, (Hiller et al., 1996) and MHC class I proteins (Blom et al., 2004), are found in the cytosol with attached glycosylations. Thus, these proteins must be exported from the ER with their glycosylations attached. Crystallization of bacterial SecYEG, which is highly homologous to Sec61, indicates that the pore size should accommodate only the passage of unfolded polypeptides (Van den Berg et al., 2004). Thus, these proteins with attached sugar moieties are bulky, and are not expected to be Sec61 export competent. Therefore, a channel that can accommodate not only glycosylated, but also ubiquitinated (Hiller et al., 1996) and partially folded proteins such as Green Fluorescence Protein (Fiebiger et al., 2002) (Tirosh et al., 2003) is predicted.

Derlin-1, a mammalian homologue of Der1 found in yeast is predicted to have four transmembrane domains and is localized to the ER. This protein was found in association with US11, an ER membrane viral protein that promotes the degradation of MHC Class I molecules, and p97 (Lilley and Ploegh, 2004) (Ye et al., 2004) thus, Derlin-1 was proposed to function as a pore for protein export (Ye et al., 2004). Direct evidence for Derlin-1 to function as a specialized pore or to accommodate the export of bulky protein structures from the ER membrane is not available.

## **1.6 Identification of Novel CFTR Quality Control Components**

Based on our model for how CFTR was being selected for degradation, inhibiting CHIPs function would require the identification and production of a dominant negative form of CHIPs cognate E2. There was evidence that members of the UbcH5 family of E2 could function with CHIP *in vitro* to generate polyubiquitin chains. Thus, we proposed to study this mechanism of CHIP mediated degradation of CFTR to determine if the fate of CFTR could be shifted toward

a folding pathway, and to elucidate information as to the nature of the CFTR folding intermediate that the CHIP/Hsp70 complex selected for degradation.

At the onset of our studies we also hypothesized the possibility that multiple E2/E3 complexes could be responsible for CFTR quality control. This idea came from studies suggesting a role for Ubc6e to function in CFTR degradation. The data specifically indicated that CFTR $\Delta$ F508 co-expressed with an inactive E2, Ubc6e C91S, has reduced turnover kinetics (Lenk et al., 2002). Interestingly, Ubc6e also functions to degrade TCR $\alpha$  a membrane protein that exposes the majority of its mass in the ER lumen (Lenk et al., 2002). Considering that Ubc6e is an ER localized transmembrane protein with its E2 domain facing the cytosol (Sommer and Jentsch, 1993), its only interface with TCR $\alpha$  is either in the ER membrane lipid bi-layer, or very near the cytosolic face of the ER where TCR $\alpha$  exposes 5 amino acids. Therefore, we thought it possible that Ubc6e functions with an ER localized integral membrane E3, to ubiquitinate CFTR when the membrane spanning domains fail to satisfy ERQC requirements for transmembrane domain assembly important for membrane channel function. Thus, as we were concluding experiments in regard to modulating the activity of the CHIP/Hsp70 complex, we were beginning to focus our attention on E3 candidates that could function with Ubc6e in parallel with the CHIP/Hsp70 E3 ubiquitin ligase complex.

Two E3s were of intense interest due to their implied role in ERAD, Doa10/TEB4 and gp78. The human homolog of Doa10 (TEB4), had not been tested as an ERAD E3, however, based on sequence it was expected to function similar to Doa10. (Swanson et al., 2001). Doa10 functions in yeast to degrade two endogenous ERAD substrates, Ubc6 which is turned over rapidly, and Mat $\alpha$ 2 (Swanson et al., 2001), and is important for yeast viability (Swanson et al., 2001). Deletion strains for Doa10 shows reduced growth and increased sensitivity to stress.

Both E2s Ubc6 and Ubc7 can function with Doa10, if both are deleted in addition to Ubc1, yeast die (Swanson et al., 2001). Based on sequence, TEB4 contains a RING domain and a WW domain (Swanson et al., 2001). The WW domain is an interaction site for the consensus sequence PPxY for protein:protein interactions (Swanson et al., 2001). Ubc6 homologues are the only E2s known to contain PPxY (PPPY) sequences, therefore, TEB4, similar to yeast Doa10, might function with Ubc6e (Swanson et al., 2001).

gp78 is a transmembrane glycoprotein RING E3 that has a domain homologous to Cue-1 (Fang et al., 2001). The Cue-1 domain is thought to function for the recruitment of Ubc7 to the ER in yeast (Fang et al., 2001). At the time gp78 had been shown to function with mouse Ubc7 for the degradation of two ERAD substrates; unassembled CD3- $\delta$  and ApolipoproteinB100 in over-expression studies in cultured cells, and ubiquitinate ApolipoproteinB48 *in vitro* (Fang et al., 2001) (Liang et al., 2003). However, whether or not it functions with Ubc6 homologues is not known.

A third E3 was considered due to the fact that it was the only other E3 known to be ER localized. RMA1 is a small, single COOH-terminal transmembrane domain, ER localized ring domain E3, originally identified in plants and has since been found to be conserved in humans (Matsuda et al., 2001). It has been shown to function *in vitro* with both Ubc4 and UbcH5a for self-ubiquitination of a Maltose Binding Protein-RMA1 (MBP-RMA1) fusion, suggesting a role in ERAD for ER localized cytosolic facing substrates (Matsuda et al., 2001). This function was also tested in cultured cell over-expression studies, but endogenous substrates for RMA1 had not been identified (Matsuda et al., 2001). Thus we proposed utilizing these components to test them for a role in ER localized CFTR Quality Control.



## 1.7 References

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## **Chapter Two**

### **A Foldable CFTR $\Delta$ F508 Biogenic Intermediate Accumulates Upon Inhibition of the Hsc70/CHIP E3 Ubiquitin Ligase**

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## 2.1 Abstract

CFTR $\Delta$ F508 exhibits a correctable protein-folding defect that leads to its misfolding and premature degradation, which is the cause of cystic fibrosis (CF). Herein we report on the characterization of the CFTR $\Delta$ F508 biogenic intermediate that is selected for proteasomal degradation and identification of cellular components that polyubiquitinate CFTR $\Delta$ F508. Non-ubiquitinated CFTR $\Delta$ F508 accumulates in a kinetically trapped, but folding competent conformation, that is maintained in a soluble state by cytosolic Hsc70. Ubiquitination of Hsc70 bound CFTR $\Delta$ F508 requires CHIP, a U-box containing cytosolic co-chaperone. CHIP is demonstrated to function as a scaffold that nucleates the formation of a multisubunit E3 ubiquitin ligase whose reconstituted activity toward CFTR is dependent upon Hdj2, Hsc70, and the E2 UbcH5a. Inactivation of the Hsc70/CHIP E3 leads CFTR $\Delta$ F508 to accumulate in a soluble conformation, which upon lowering of cell growth temperature can fold and reach the cell surface. Inhibition of CFTR $\Delta$ F508 ubiquitination can increase its cell surface expression and may provide an approach to treat CF.

## 2.2 Introduction

CFTR is a plasma membrane  $\text{Cl}^-$  ion channel that is localized to the apical surface of epithelial cells that line lung airways (Riordan et al., 1989). More than 90% of patients afflicted with cystic fibrosis (CF) inherit the  $\text{CFTR}\Delta\text{F508}$  mutant allele.  $\text{CFTR}\Delta\text{F508}$  is missing F508 in nucleotide binding domain 1 (NBD1) and is synthesized, but exhibits a temperature-sensitive folding defect that causes its premature degradation by the ubiquitin proteasome system (Cheng et al., 1990; Denning et al., 1992; Jensen et al., 1995b; Ward et al., 1995). Loss of CFTR function at the cell surface causes mortality in CF patients because the hydration of airway epithelia is altered and this gives rise to persistent microbial infections with resultant lung fibrosis (Welsh and Smith, 1993).

An interesting feature of  $\text{CFTR}\Delta\text{F508}$  biology is that it can function as a regulated  $\text{Cl}^-$  ion channel at the plasma membrane if cellular growth conditions are altered to allow it to fold and escape the endoplasmic reticulum quality control system (ERQC) that monitors its conformation (Brown et al., 1996; Denning et al., 1992). Thus, the development of agents that promote folding or block the degradation of nascent  $\text{CFTR}\Delta\text{F508}$  has the potential to provide a therapeutic avenue for the treatment of cystic fibrosis (Gelman and Kopito, 2002). Rational design of such therapeutics requires a basic understanding of the mechanism for  $\text{CFTR}\Delta\text{F508}$  misfolding and the identification of the ERQC machinery that selects  $\text{CFTR}\Delta\text{F508}$  for degradation.

Assembly of CFTR is complicated because it is a 1480 residue glycomembrane protein whose function requires the formation of intramolecular contacts between its two transmembrane domains, two cytoplasmic NBDs and a regulatory domain (R-domain) (Meacham et al., 1999; Ostedgaard et al., 1997; Xiong et al., 1997). CFTR and  $\text{CFTR}\Delta\text{F508}$

biogenesis is inefficient with 60-75% of CFTR and nearly 99% of CFTR $\Delta$ F508 being degraded prior to reaching the cell surface (Ward and Kopito, 1994). These data indicate that the kinetics of CFTR and CFTR $\Delta$ F508 folding are slow and that non-native intermediates of each are selected for degradation by ERQC systems (Lukacs et al., 1994). The nature of the CFTR $\Delta$ F508 biogenic intermediate that is selected for degradation is unknown, but CFTR and CFTR $\Delta$ F508 appear to assume similar conformations at early stages of assembly (Zhang et al., 1998). Since F508 is located on the surface of nucleotide binding domain 1 (NBD1), CFTR $\Delta$ F508 assembly is proposed to go off pathway at a late stage where NBD1 makes intramolecular contacts with the transmembrane domains (Lewis et al., 2004). The inability of CFTR $\Delta$ F508 to assemble properly is proposed to make it prone to aggregation (Qu and Thomas, 1996), and cause its degradation intermediates to accumulate in detergent insoluble aggregates (Gelman et al., 2002).

The mechanism by which the cell monitors the conformational state of CFTR $\Delta$ F508 biogenic intermediates and makes protein triage decisions that determine their fate is unclear. Current models suggest that the folded state of CFTR and CFTR $\Delta$ F508 is surveyed by the cytosolic chaperones Hsc70 and Hsp90 (Loo et al., 1998; Strickland et al., 1997; Yang et al., 1993; Zhang et al., 2001) and the ER luminal lectin binding chaperone calnexin (Okuyoneda et al., 2004; Okuyoneda et al., 2002; Pind et al., 1994). Calnexin forms complexes with the immaturely glycosylated B-form of CFTR $\Delta$ F508 and its overexpression causes CFTR $\Delta$ F508 to be retained in the ER (Okuyoneda et al., 2004; Pind et al., 1994). However, a functional requirement for calnexin in CFTR folding and/or degradation is yet to be demonstrated.

Cytosolic Hsc70, on the other hand, has been demonstrated to function in complexes with either folding or degradatory co-chaperones to mediate steps in CFTR folding and degradation

(Meacham et al., 1999; Meacham et al., 2001). The Type I Hsp40 co-chaperone Hdj-2 is farnesylated and localized to the cytoplasmic face of the ER where it recruits Hsc70 to bind ribosome associated CFTR to promote early stages of its assembly (Meacham et al., 1999). Hsc70 can also interact with the degradatory co-chaperone CHIP to facilitate the proteasomal degradation of ER localized forms of CFTR and CFTR $\Delta$ F508 (Meacham et al., 2001). Thus, the cytosolic Hsc70 chaperone system appears to play a major role in CFTR folding and the selection of CFTR $\Delta$ F508 for degradation.

Mechanistic insight into how CHIP functions as a degradatory co-chaperone is required to understand how the cell makes protein triage decisions for Hsc70 substrates such as nascent CFTR $\Delta$ F508. One outstanding question pertains to the nature of the CFTR $\Delta$ F508 biogenic intermediate that is selected by CHIP for degradation. In addition, the mechanism by which CHIP functions to target CFTR and CFTR $\Delta$ F508 for proteasomal degradation is not clear. CHIP is a multiple domain protein that contains three N-terminal tetratricopeptide repeat motifs (TPR) that enable it to interact with the C-terminus of Hsc70 and a non-canonical RING domain, termed the U-box, which promotes interactions with E2 enzymes (Ballinger et al., 1999; Jiang et al., 2001). Deletion analysis demonstrated that the TPR repeat motifs and U-box of CHIP are essential for it to mediate CFTR and CFTR $\Delta$ F508 degradation (Meacham et al., 2001). Thus, CHIP was proposed to interact with Hsc70 to form an E3 ubiquitin ligase that selectively ubiquitinates non-native CFTR $\Delta$ F508 and thereby targets it for proteasomal degradation. However, CHIP has been observed to function in a U-box independent manner to alter the Hsc70 polypeptide binding and release cycle and negatively influence the folding of some client proteins (Ballinger et al., 1999; Cardozo et al., 2003; Connell et al., 2001; Dai et al., 2003; Xu et al., 2002). Thus, it is plausible that CHIP targets CFTR and CFTR $\Delta$ F508 for

degradation by acting as an anti-folding factor.

Herein, we reconstituted CFTR ubiquitination with purified components and demonstrated that Hsc70 and CHIP functionally interact with the E2 UbcH5a to form a multisubunit E3 ubiquitin ligase that polyubiquitinates the cytosolic sub-domains of CFTR. We also observed that inactivation of Hsc70/CHIP E3 activity in cultured cells caused the accumulation of a foldable CFTR $\Delta$ F508 degradation intermediate. These collective data support a model for quality control in which the solubility of CFTR and CFTR $\Delta$ F508 biogenic intermediates is maintained by Hsc70 and Hdj-2. However, when soluble CFTR $\Delta$ F508 accumulates in a kinetically trapped non-native state, CHIP acts to promote its polyubiquitination and degradation by attracting UbcH5a to Hsc70: CFTR $\Delta$ F508 complexes.

### 2.3 Results

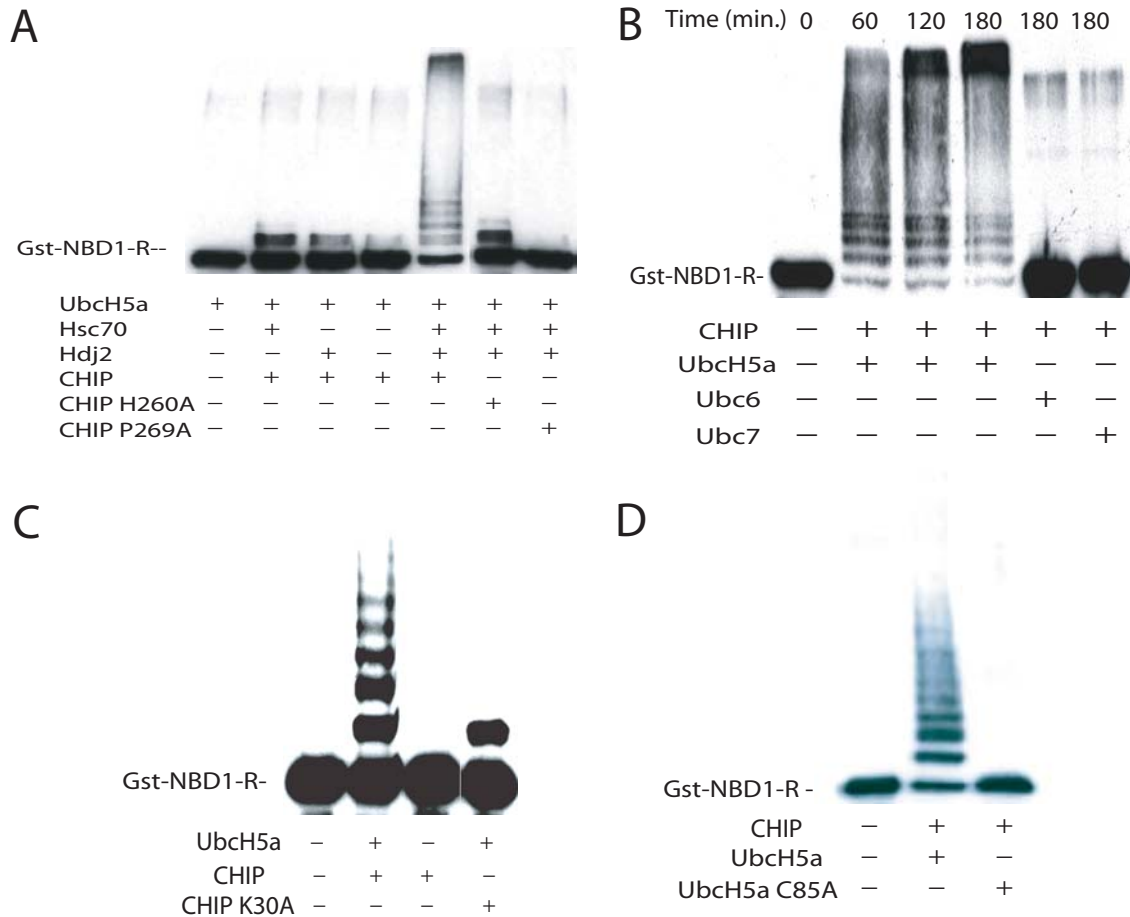
**Purified CHIP functions as an Hdj-2, Hsc70 and UbcH5a dependent E3 ubiquitin ligase to polyubiquitinate CFTR** -- To investigate whether CHIP targets CFTR for degradation by acting as an U-box dependent E3 ubiquitin ligase we sought to reconstitute CFTR ubiquitination with purified components. To accomplish this goal CHIP, Hsc70, Hdj-2 and the E2 UbcH5a were overexpressed and purified from the soluble fraction of *E. coli* cell extracts. UbcH5a was chosen as the E2 for these experiments because it was previously demonstrated to cooperate with CHIP to promote polyubiquitin chain assembly (Jiang et al., 2001). The CFTR substrate utilized for this ubiquitination reaction was gst-NBD1-R, which contains glutathione S-transferase and CFTR residues 371-855 (Naren et al., 1999). This region of CFTR contains the cytosolic NBD1 and the R-domain and which are recognized by Hdj-2 and Hsc70 (Meacham et al., 1999). The ubiquitination of gst-NBD1-R was assayed after incubating it with different combinations of chaperones and ubiquitination enzymes by

monitoring the retardation of its apparent mobility on SDS-PAGE gels (Figure 2.1A).

E1 and UbcH5a were unable to facilitate ubiquitination of *gst-NBD1-R* and the addition of CHIP lead to the formation of a small quantity of ubiquitinated *gst-NBD1-R*. The addition of Hsc70 or Hdj-2 in combination with CHIP further stimulated the ubiquitination action of UbcH5a, but the efficiency of this reaction remained low with less than 3% of *gst-NBD1-R* being detected as mono-, di- or tri-ubiquitinated species. However, greater than 85% of *gst-NBD1-R* was polyubiquitinated when E1, UbcH5a, Hsc70, Hdj-2 and CHIP were jointly present. Mutation of highly conserved residues in the U-box of CHIP, H260A and P269A, greatly reduced the polyubiquitination of *gst-NBD1-R*. In addition, mutation of K30A in the TPR repeat domain of CHIP reduced its ubiquitination activity to levels observed when CHIP and Hdj-2 were present, but Hsc70 was omitted from reaction cocktails (Figure 2.1A and C). Thus, CHIP cooperates with UbcH5a, Hsc70 and Hdj-2 in a TPR repeat and U-box dependent manner to facilitate CFTR polyubiquitination.

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

To evaluate the specificity of CHIP's ubiquitination activity, its ability to cooperate with the mammalian E2s Ubc6 and Ubc7 (Lenk et al., 2002; Tiwari and Weissman, 2001) to facilitate *gst-NBD1-R* ubiquitination was examined (Figure 2.1D). Ubc6 and Ubc7 were chosen for this



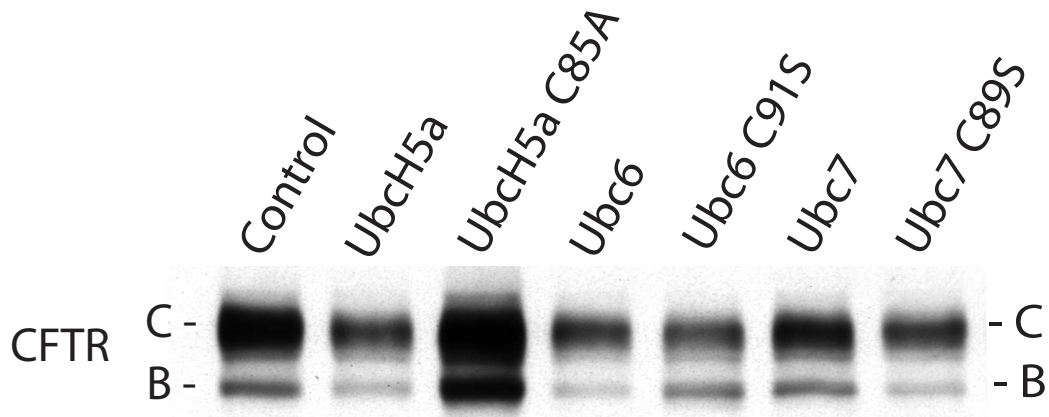
**Figure 2.1 Reconstitution of CFTR ubiquitination.** (A) Polyubiquitination of purified *gst-NBD1-R*. (B) *Ubc6* and *Ubc7* cannot cooperate with the *Hsc70*/*CHIP* complex to polyubiquitinate *gst-NBD1-R*. (C) *CHIP K30A* exhibits a defect in polyubiquitination activity. (D) *Ubch5a C85A* is unable to polyubiquitinate *gst-NBD1-R*. Ubiquitination of *gst-NBD1-R* (1 $\mu$ M) was carried out at 37 $^{\circ}$ C in a reaction cocktail composed of 20mM HEPES, pH 7.4, 50mM NaCl, 5mM MgCl<sub>2</sub>, 2mM DTT, 2.5mM ATP, 10 $\mu$ M ubiquitin and 0.15 $\mu$ M E1. Additional components were present, where indicated, at the following concentration: *Ubch5a* (4 $\mu$ M), *Ubch5a C85A* (4 $\mu$ M), *Ubc6* (4 $\mu$ M), *Ubc7* (4 $\mu$ M), *CHIP* (3 $\mu$ M), *CHIP K30A* (3 $\mu$ M), *Hsc70* (2 $\mu$ M), and *Hdj-2* (4 $\mu$ M). In panels B-D *Hsc70* and *Hdj-2* were present in all reactions except for lane 1. Incubation times for were for 2 hrs. unless otherwise noted. Conjugation of ubiquitin to *gst-NBD1-R* was measured by monitoring changes in the mobility of *gst-NBD1-R* on SDS-PAGE gels by Western blot with  $\alpha$ -R-domain antibody. In the X-ray films shown in panels A-D the band that corresponds to *gst-NBD1-R* is overexposed to allow for the visualization of its ubiquitinated forms. Quantification of shorter exposures of this X-ray film by laser densitometry indicates that greater than 85% of *gst-NBD1-R* is polyubiquitinated by the joint action of *Ubch5a*, *Hsc70*, *Hdj-2* and *CHIP* (panel A lane 5). In the absence of *Hsc70* or *Hdj-2* (panel A lane 2 and 3), *Ubch5a* and *CHIP* converted less than 3% of *gst-NBD1-R* to an ubiquitinated species.

experiment because these E2s mediate the ubiquitination of ERAD substrates in cultured cells and their purified forms function *in vitro* to facilitate polyubiquitin chain assembly (Tiwari and Weissman, 2001). In reaction cocktails that contained E1, UbcH5a, Hsc70, Hdj2 and CHIP, gst-NBD1-R was converted to a polyubiquitinated species in time dependent fashion (Figure 2.1 B). However, after 180 min. of incubation a polyubiquitinated form of gst-NBD1-R was not detected when the purified E2 domain of Ubc6 or Ubc7 was substituted for UbcH5a in otherwise identical reaction cocktails. Thus, CHIP appears to recognize UbcH5a in a specific manner.

Next, the ability of UbcH5a C85A to facilitate the polyubiquitination of gst-NBD1-R was determined (Figure 2.1D). UbcH5a C85A is a form of UbcH5a in which its active site cysteine that accepts a charged ubiquitin from E1 has been mutated. UbcH5a C85A is predicted to interact with the U-box on CHIP, but should not facilitate polyubiquitin chain assembly because ubiquitin cannot be conjugated to it. This supposition was found to be true as UbcH5a C85A was unable to cooperate with CHIP to promote the polyubiquitination of gst-NBD1-R. Hence, the Hsc70 and Hdj-2 dependent reconstitution of CFTR polyubiquitination requires the action of the CHIP TPR and U-box domains and the active site cysteine of UbcH5a.

**Overexpression of UbcH5a C85A in cultured cells inhibits CFTR and CFTR $\Delta$ F508 degradation** -- To access whether or not UbcH5a is an *in vivo* component of Hsc70/CHIP E3, the effect that UbcH5a and UbcH5a C85A overexpression in HEK293 cells had on CFTR biogenesis was determined (Figure 2.2). In addition, we compared the effect of UbcH5a or UbcH5a C85A overexpression on CFTR biogenesis to that of wild type and dominant negative mutant forms of the human E2s Ubc6 and Ubc7 (Figure 2.2). Elevation of UbcH5a levels caused a decrease in the accumulation of the immaturely glycosylated ER localized B-form and



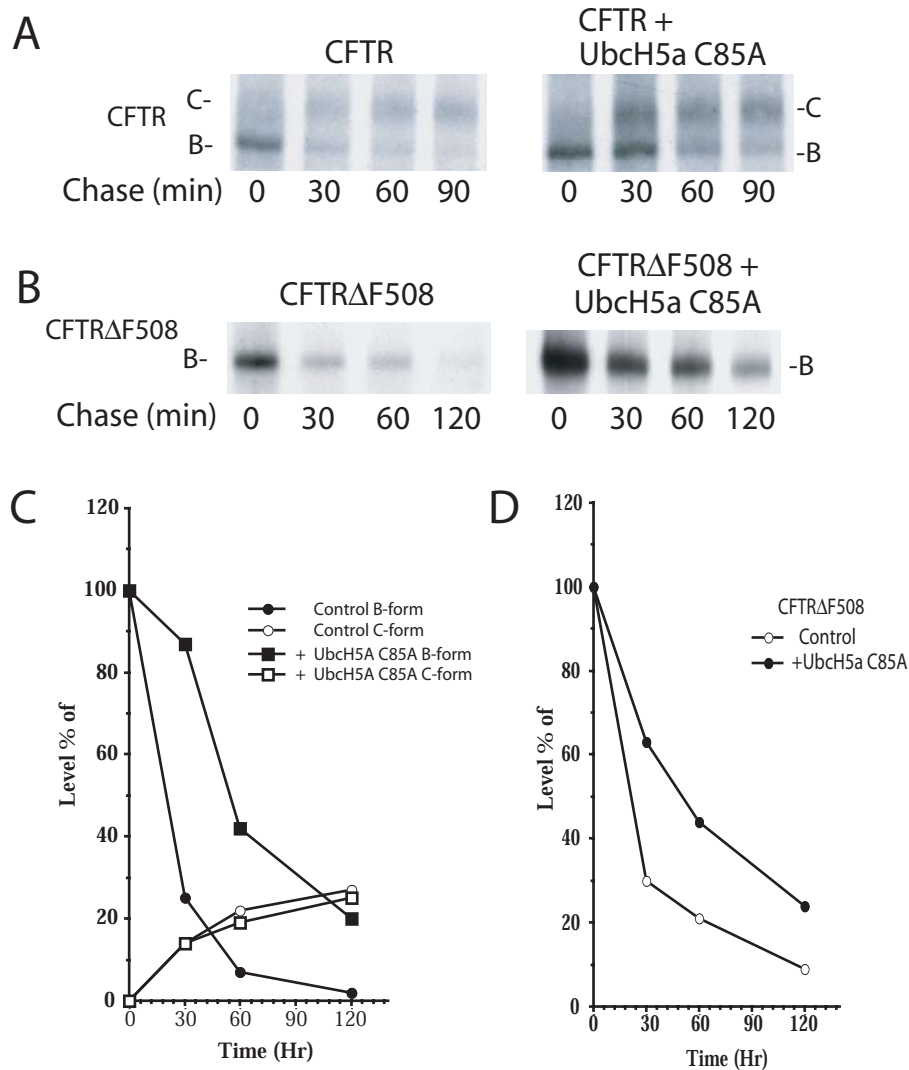


**Figure 2.2 Degradation of CFTR and CFTR $\Delta$ F508 is inhibited by overexpression of Ubch5a C85A.** Western blot analysis of CFTR levels when the E2s Ubch5a, Ubc6 and Ubc7 are overexpressed. HEK293 cells were transiently transfected with pCDNACFTR either alone or in combination with pCAGGS His6Ubch5a, pCAGGS His6Ubch5a C85A, pCMVPLD myc-Ubc6, pCMVPLD myc-Ubc6 C91S, pCDNA myc-Ubc7, or pCDNA myc-Ubc7 C89S. Total cell extracts were prepared 24 hrs after transfection in SDS-PAGE sample buffer. Proteins in cleared cell lysates were resolved on SDS-PAGE gels and then transferred to nitrocellulose. Membranes were probed with  $\alpha$ -CFTR and developed with the ECL reagent. The immaturely glycosylated ER localized B-form and maturely glycosylated plasma membrane associated C-form of CFTR are denoted as B and C, respectively.

the maturely glycosylated, plasma membrane localized C-form of CFTR. In contrast, overexpression of UbcH5a C85A led to a several fold increase in the steady-state level of the B- and C-form of CFTR. Ubc6 and Ubc6 C91S overexpression were also observed to influence CFTR expression levels, but the effect that Ubc6 C91S had on the accumulation of the B-form of CFTR was modest when compared to results obtained with UbcH5a C85A. On the other hand, Ubc7 and Ubc7 C89S overexpression did not cause a detectable change in the steady-state level of CFTR and CFTR $\Delta$ F508. Since Ubc6 does not appear to interact with CHIP (2.1), the effect that its overexpression has on CFTR biogenesis appears to result from its ability to function with additional quality control factors that monitor the folded state of CFTR (Gnann et al., 2004).

To explore the reason why overexpression of UbcH5a C85A drove the B-form of CFTR to accumulate, its effect on the kinetics of CFTR and CFTR $\Delta$ F508 degradation was determined (Figure 2.3A and B). In pulse-chase experiments, UbcH5a C85A overexpression increased the quantity of the B-form of CFTR and CFTR $\Delta$ F508 present at the beginning of the chase period approximately 1.5 to 3-fold (Legend to Figure 2.3). The UbcH5a C85A induced increase in CFTR and CFTR $\Delta$ F508 levels appeared to result from impaired degradation on nascent CFTR and CFTR $\Delta$ F508 because the half-life of the B-form of CFTR and CFTR $\Delta$ F508 was increased 2-3 fold (Figure 2.3C and D). In addition, the maturation efficiency of CFTR in the presence or absence of UbcH5a C85A was around 25% (Figure 2.3C). Thus, inhibition of the Hsc70/CHIP E3 complex via UbcH5a C85A overexpression inhibits CFTR degradation, but does not interfere with CFTR folding efficiency.

To gain additional support for the interpretation that CHIP and UbcH5a interact with each other to select nascent CFTR and CFTR $\Delta$ F508 for degradation we demonstrated that the co-

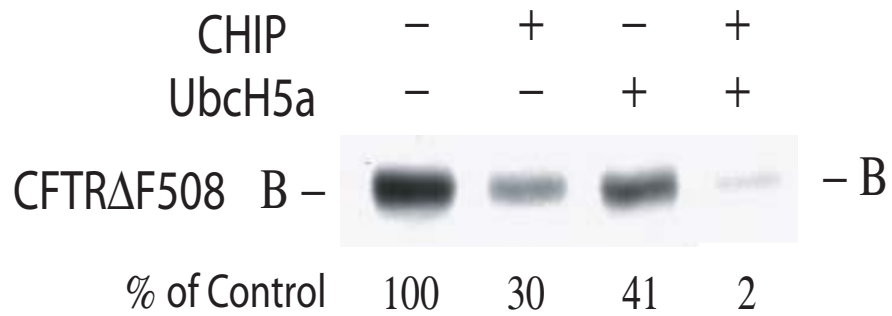


**Figure 2.3 UbchH5a C85A overexpression slows the rates of CFTR and CFTR $\Delta$ F508 degradation.** (A and B) Cells were transfected as described above and then labeled for 20 min with <sup>35</sup>S-translabel. A chase period was initiated by the addition of cycloheximide and, at the indicated times, <sup>35</sup>S-CFTR and <sup>35</sup>S-CFTR $\Delta$ F508 were immunoprecipitated from cell extracts. CFTR and CFTR $\Delta$ F508 isolated in this manner were then detected by SDS-PAGE and fluorography. (C and D) are graphs that illustrate the processing efficiency and half-life of CFTR and CFTR $\Delta$ F508. Relative CFTR and CFTR $\Delta$ F508 levels were quantified by laser densitometry of the X-ray films shown in panels B and C, respectively. Values were normalized to the quantity of the B-form of CFTR and CFTR $\Delta$ F508 present at T=0 under the indicated experimental condition levels. T=0 values for the B-form of CFTR were 1.1 and 1.8 O.D./m.m, in the absence and presence of UbchH5a C85A, respectively. T=0 values for the B-form of CFTR $\Delta$ F508 were 2.3 and 7.0 O.D./m.m, in the absence and presence of UbchH5a C85A, respectively.

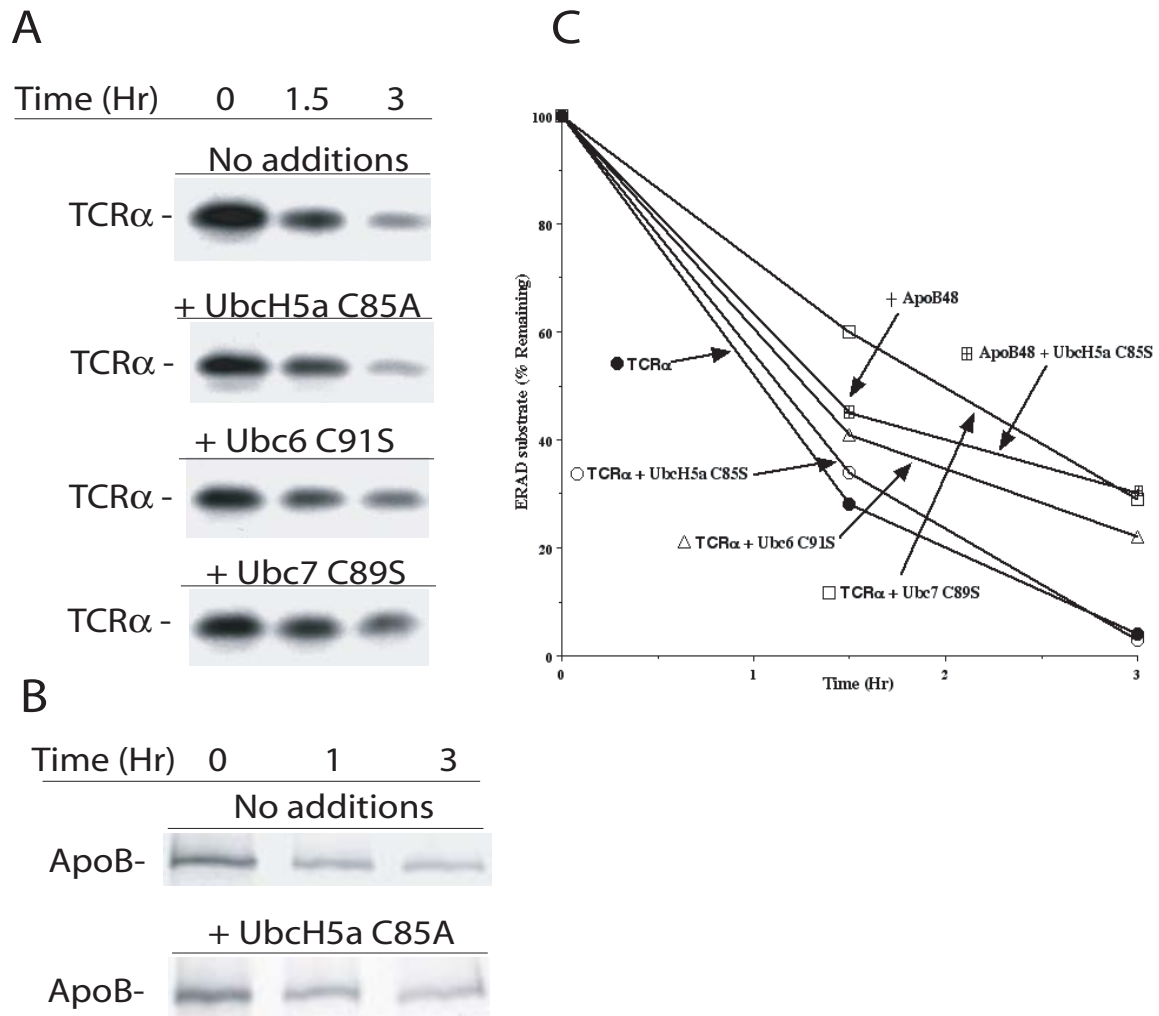
expression of CHIP and UbcH5a enhanced the effect that individual forms of each had on CFTR $\Delta$ F508 degradation (Figure 2.3C). When CHIP or UbcH5a was overexpressed alone, steady state levels of CFTR $\Delta$ F508 were reduced to 30 and 41% of control levels, respectively. Yet, when CHIP and UbcH5a were co-expressed, CFTR $\Delta$ F508 accumulation was reduced by greater than 98% (Figure 2.4).

**Overexpression of UbcH5a C85A does not generally inhibit ERAD** -- To ascertain whether or not UbcH5a C85A overexpression generally inhibited ERAD or was specifically required for CFTR degradation, its effect on the degradation of the T-cell receptor  $\alpha$ -subunit (TCR $\alpha$ ) was examined (Figure 2.5A). TCR $\alpha$  is a transmembrane protein that exposes a large extracellular domain in the ER lumen whose unassembled form is degraded via an ERAD pathway that utilizes the E2s Ubc6 and Ubc7 (Bonifacino et al., 1989; Lenk et al., 2002; Tiwari and Weissman, 2001). Pulse chase analysis revealed that TCR $\alpha$  had a half-life of around 1 hr in absence or presence of UbcH5a C85A (Figure 2.5A and C). In addition, the overexpression of CHIP was not observed to influence the rate of TCR $\alpha$  turnover (data not shown). In contrast, overexpression of either Ubc6 C91S or Ubc7 C89S led to an increase in the half-life of TCR $\alpha$  from 1 hr to 1.5 to 2 Hrs, respectively. Thus, UbcH5a C85A overexpression does not detectably hinder the turnover of a transmembrane ERAD substrate whose degradation can be blocked by interference with the action of the cytosolic E2s Ubc6 and Ubc7.

Since the membrane topology of TCR $\alpha$  differs from that of CFTR we also examined the sensitivity of Apolipoprotein B48 degradation to overexpression of UbcH5a C85A. ApoB48 is a 2152 amino acid residue secretory protein whose nascent form has features that are similar to CFTR because it exposes surfaces in the ER lumen and cytosol, and is a substrate of cytosolic Hsp70 and Hsp90 (Gusarova et al., 2001). ApoB48 folding and exit from the ER involves its



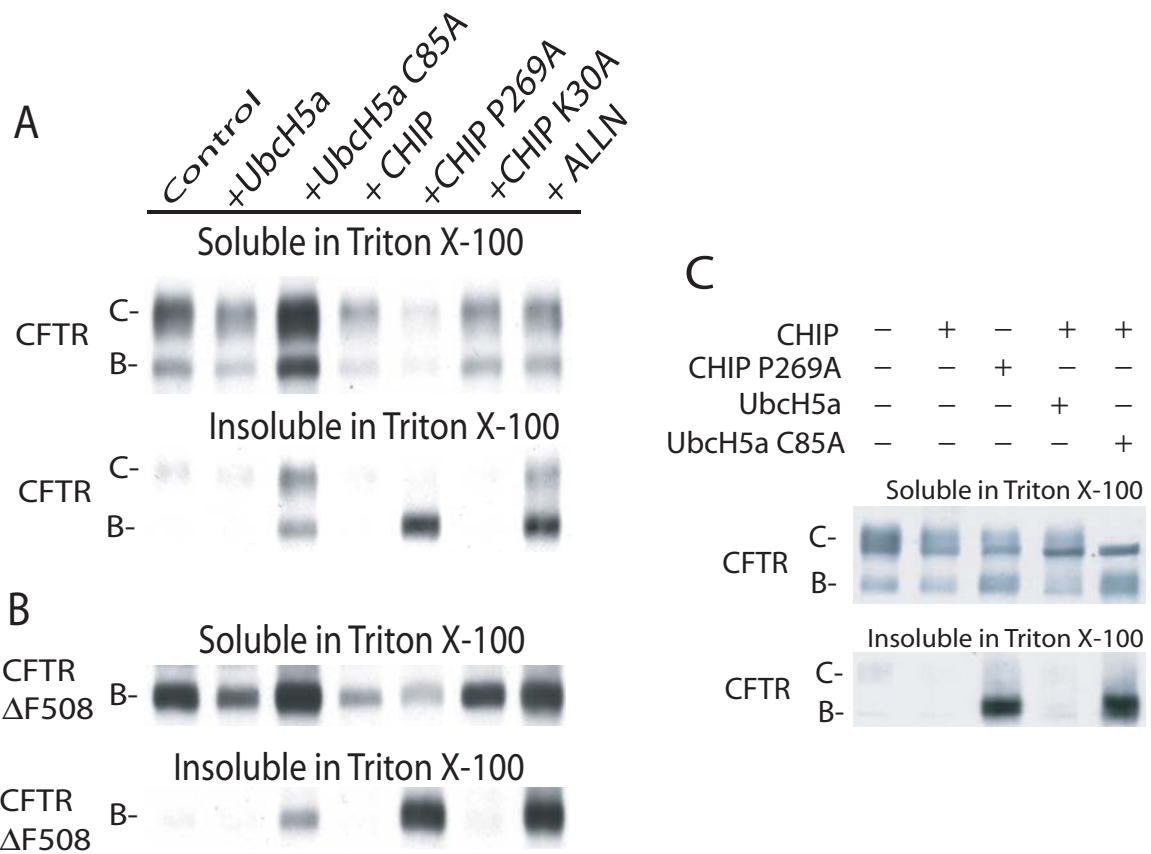
**Figure 2.4 CHIP and UbcH5a jointly reduce steady state levels of CFTR $\Delta$ F508.** CHIP and UbcH5a were overexpressed alone or in combination, and the effect this had on the accumulation of CFTR $\Delta$ F508 was determined by Western blot. Quantification of CFTR $\Delta$ F508 is expressed as a percentage of the total CFTR $\Delta$ F508 present in lane 1.



**Figure 2.5 Sensitivity of ERAD substrates other than CFTR to inhibition of the Hsc70/CHIP E3.** (A) TCR degradation is insensitive to UbcH5a C85A overexpression. HEK293 cells were transiently transfected with pCDM82B4 TCR either alone or in combination with pCAGGS His6UbcH5a C85A, pCMVPLD myc-Ubc6 C91S, or pCDNA myc-Ubc7 C89S. After a 24 hr post-transfection period cells were labeled for 20 min with <sup>35</sup>S-translabel. A chase period was initiated by the addition of cycloheximide and, at the indicated times, cells were harvested and lysed. TCR was immunoprecipitated from cell extracts and detected by SDS-PAGE and autoradiography. (B) ApoB48 turnover is insensitive to UbcH5a C85A overexpression. HEK293 cells were transiently transfected with pCDNA-ApoB48 either alone or in combination with pCAGGS His6UbcH5a C85A. Rates of ApoB48 degradation were then determined by pulse chase analysis. (C) Quantification of TCR and ApoB48 levels. The relative quantity of TCR and ApoB48 in matched samples was determined by scanning densitometry and the amount present at T=0 is expressed as 100% of control.

assembly into complexes with lipids, and unassembled forms are degraded via a pathway that involves the transmembrane E3, gp78 (Liang et al., 2003), which can interact with cytosolic Ubc7 (Cyr et al., 2002). The overexpression of CHIP does not accelerate ApoB48 degradation (Meacham et al., 2001) and, herein we report that the overexpression of UbcH5a C85A does not have a detectable effect on the rate ApoB48 degradation (Figure 2.5B and C). Thus, the collective data presented in Figure 2.5 indicate that the overexpression of UbcH5a C85A does not pleiotropically inhibit the function the cellular quality control machinery.

**A detergent soluble CFTR $\Delta$ F508 degradation intermediate accumulates upon inactivation of the Hsc70/CHIP E3 complex** -- CFTR and CFTR $\Delta$ F508 biogenic intermediates appear to be aggregation prone and therefore are selected for ubiquitination and proteasomal degradation (Qu and Thomas, 1996). When the proteasome is inhibited, polyubiquitinated CFTR $\Delta$ F508 accumulates in detergent insoluble aggregates (Ward et al., 1995), but non-ubiquitinated CFTR $\Delta$ F508 biogenic intermediates are not well characterized. To investigate the aggregation state of non-ubiquitinated CFTR $\Delta$ F508, we modulated the activity of the Hsc70/CHIP E3 and determined the detergent solubility of the CFTR and CFTR $\Delta$ F508 biogenic intermediates that accumulated (Figure 2.6). Overexpression of UbcH5a and CHIP caused an overall decrease in the soluble pool of CFTR and CFTR $\Delta$ F508 and none of the B-form was detected in the Triton X-100 insoluble fraction. When UbcH5a C85A was overexpressed, several-fold more of the B and C form of CFTR accumulated in the Triton X-100 soluble fraction. To our surprise, we also observed UbcH5a C85A overexpression caused a several fold increase the quantity of the B-form of CFTR $\Delta$ F508 that accumulated in a Triton X-100 soluble state. Under these experimental conditions, we also observed a small quantity of the C-form of CFTR in the detergent insoluble fraction. Since the C-form of CFTR is typically



**Figure 2.6 Triton X-100 soluble CFTR and CFTR $\Delta$ F508 degradation intermediates accumulate in response to overexpression of UbcH5a C85A.** (A and B) Analysis of the solubility of CFTR or CFTR $\Delta$ F508. CFTR or CFTR $\Delta$ F508 was transiently expressed in HEK293 cells either alone or in combination with pCAGGS His6UBCH5a, pCAGGS His6UBCH5a C85A, pCDNA3 CHIP, pCDNA3 CHIP P269A or pCDNA3 CHIP K30A. Where indicated, ALLN (200 $\mu$ M) was added to the growth medium four hours preceding cell lysis to inhibit the proteasome. (C) Co-expression of CHIP and UbcH5a C85A blocks CFTR folding and causes its biogenic intermediates to aggregate. CFTR and CFTR $\Delta$ F508 present in cell extracts were detected by Western blot. The fractionation of cell extracts into Triton X-100 soluble and insoluble material was performed as described in the materials and methods section. The upper part of each panel represents Triton X-100 soluble material; whereas the lower part represents Triton X-100 insoluble material. The immaturely glycosylated B-form of CFTR and CFTR $\Delta$ F508, and maturely glycosylated C-form of CFTR are denoted as B and C, respectively.



soluble, this material appears to represent a minor contamination of the detergent insoluble fraction with Triton X-100 soluble material.

Paradoxically, when the action of the Hsc70/CHIP E3 was blocked via overexpression of CHIP P269A we observed markedly different results. CHIP P269A inhibited degradation of the B-form of CFTR and CFTR $\Delta$ F508, but the degradation intermediate that accumulated was insoluble in Triton X-100 (Figure 2.6A and B). In addition, CHIP P269A blocked the glycolytic maturation of CFTR from the B to C form. The effect that CHIP and CHIP P269A had on CFTR and CFTR $\Delta$ F508 biogenesis were resultant from interactions with Hsc70 because mutation of CHIPS' TPR domain in CHIP K30A abolished the influence of CHIP on CFTR biogenesis (Figure 2.6).

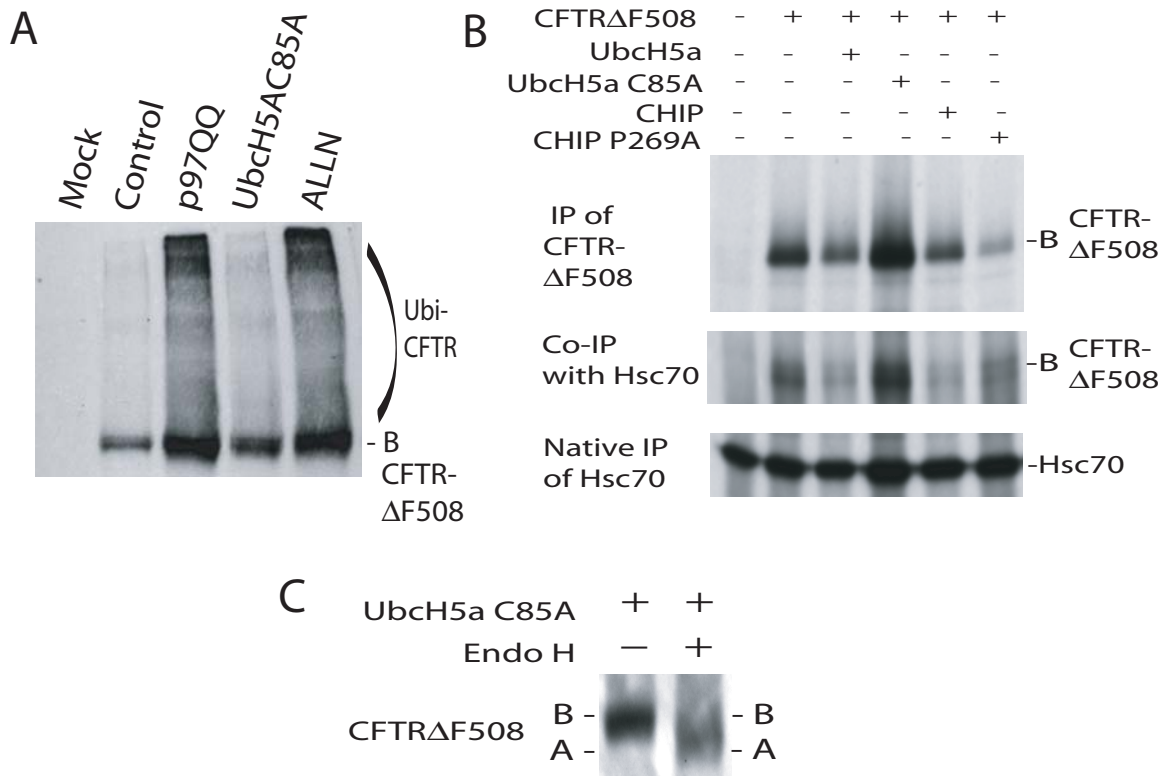
How do we explain the observation that the overexpression of Ubch5a C85A and CHIP P269A block CFTR degradation, but yet cause the degradation intermediates that accumulate to exhibit differential solubility? Since, CHIP P269A can block the glycolytic maturation of CFTR; it has retained the ability of CHIP to interact with Hsc70 to arrest the folding of CFTR (Meacham et al., 2001) and other Hsc70 clients (Ballinger et al., 1999). Hence, since CHIP P269A can alter Hsc70 chaperone function, but can't promote CFTR degradation, and thereby causes non-native CFTR to aggregate. In contrast, CFTR degradation intermediates remain soluble when Ubch5a C85A is overexpressed because Ubch5a C85A competes with endogenous Ubch5a for binding to CHIP and Hsc70 is present in HEK293 cells at levels that are around 10 fold higher than those for CHIP (Meacham et al., 2001). Thus, Hsc70 levels exceed those of the CHIP:Ubch5a C85A complexes and Hsp70s overall ability to suppress CFTR aggregation is not effected by overexpression of Ubch5a C85A.

If the aforementioned interpretation is correct and Ubch5a C85A specifically inactivates the

U-box of CHIP to block CFTR degradation, then the co-expression of UbcH5a C85A and CHIP should have the same effect on CFTR biogenesis as CHIP P269A. Indeed, we observed that the simultaneous overexpression of CHIP and UbcH5a C85A blocked the glycolytic maturation and degradation of CFTR and drove CFTR degradation intermediates to accumulate in detergent insoluble aggregates (Figure 2.6, lane 1 vs 5).

The data presented in Figure 2.6 are important for the following reasons. First, these data indicate that interference with Hsc70/CHIP E3 activity drives the accumulation of a novel detergent soluble CFTR $\Delta$ F508 biogenic intermediate. Second, these data demonstrate that proper chaperone function of Hsc70 is critically important for maintenance of non-native CFTR and CFTR $\Delta$ F508 in a soluble state. Finally, the observation that UbcH5a C85A and CHIP co-expression makes CHIP behave like CHIP P269A supports the interpretation that CHIP and UbcH5a functionally interact *in vivo* to select CFTR for ubiquitination.

**Characterization of the soluble CFTR $\Delta$ F508 degradation intermediate** -- To investigate the nature of the soluble CFTR $\Delta$ F508 degradation intermediate that accumulated when UbcH5a C85A was overexpressed, we compared its ubiquitination state to that of degradation intermediates that accumulate in response to proteasome inhibition by ALLN, or overexpression of a dominant negative form of p97 (p97 QQ). p97 is a cytosolic chaperone that functions to extract polyubiquitinated proteins from the ER (Ye et al., 2001) and participates in CFTR $\Delta$ F508 degradation (Dalal et al., 2004) (Figure 2.7A). In the control lane, non-ubiquitinated CFTR $\Delta$ F508 was observed to migrate on gels with the apparent molecular weight of the 140KDa immature B-form. Polyubiquitinated CFTR $\Delta$ F508, which accumulated when its degradation was blocked by ALLN or p97 QQ, migrates on SDS-PAGE gels as a high molecular weight smear. The CFTR $\Delta$ F508 that accumulated in response to UbcH5a C85A



**Figure 2.7 Characterization of the CFTR $\Delta$ F508 biogenic intermediate that accumulates when Ubch5a C85A is overexpressed.** (A) Western blot analysis of CFTR $\Delta$ F508 levels when its proteasomal degradation is inhibited by different methods. CFTR $\Delta$ F508 was transiently expressed in HEK293 cells alone or in combination with a dominant negative p97 QQ, or Ubch5a C85A. Cells were harvested in SDS sample buffer 24 hrs after transfection. Where indicated, the proteasome inhibitor ALLN (200 $\mu$  M) was added to culture media 4 hrs prior to cell harvest. The mobility of CFTR $\Delta$ F508 on SDS-PAGE gels was determined by Western blot. Ubiquitinated CFTR $\Delta$ F508 runs as a high molecular smear on SDS-PAGE gels and is denoted. (B) Analysis of complex formation between Hsc70 and CFTR $\Delta$ F508 when different forms of CHIP and Ubch5a are overexpressed. Cells were transfected with the indicated expression plasmids as described in the legend to Figure 2.6. After a 24 hr post-incubation period, cells were radiolabeled for 30 min with <sup>35</sup>S-translabel, harvested and then split. Half of each lysate was prepared under denaturing or native buffer conditions and immunoprecipitations were carried out and analyzed by fluorography. (C) CFTR $\Delta$ F508 that accumulates in the presence of Ubch5a C85A is glycosylated. HEK293 cells that transiently express CFTR $\Delta$ F508 alone or in combination with His6Ubch5a C85A were cultured for 24 hrs post transfection. Cells were harvested and half of the lysate was treated with endoglycosidase H while the other half served as the control (see the materials and methods section for details). The A and B denote the mobility of non-glycosylated, and immaturely glycosylated forms of CFTR $\Delta$ F508, respectively.

overexpression did not migrate as a high molecular smear on SDS-PAGE gels and, therefore, represents a non-ubiquitinated species. These results are consistent with the notion that UbcH5a C85A blocks the ubiquitination of CFTR $\Delta$ F508 and thereby inhibits its degradation.

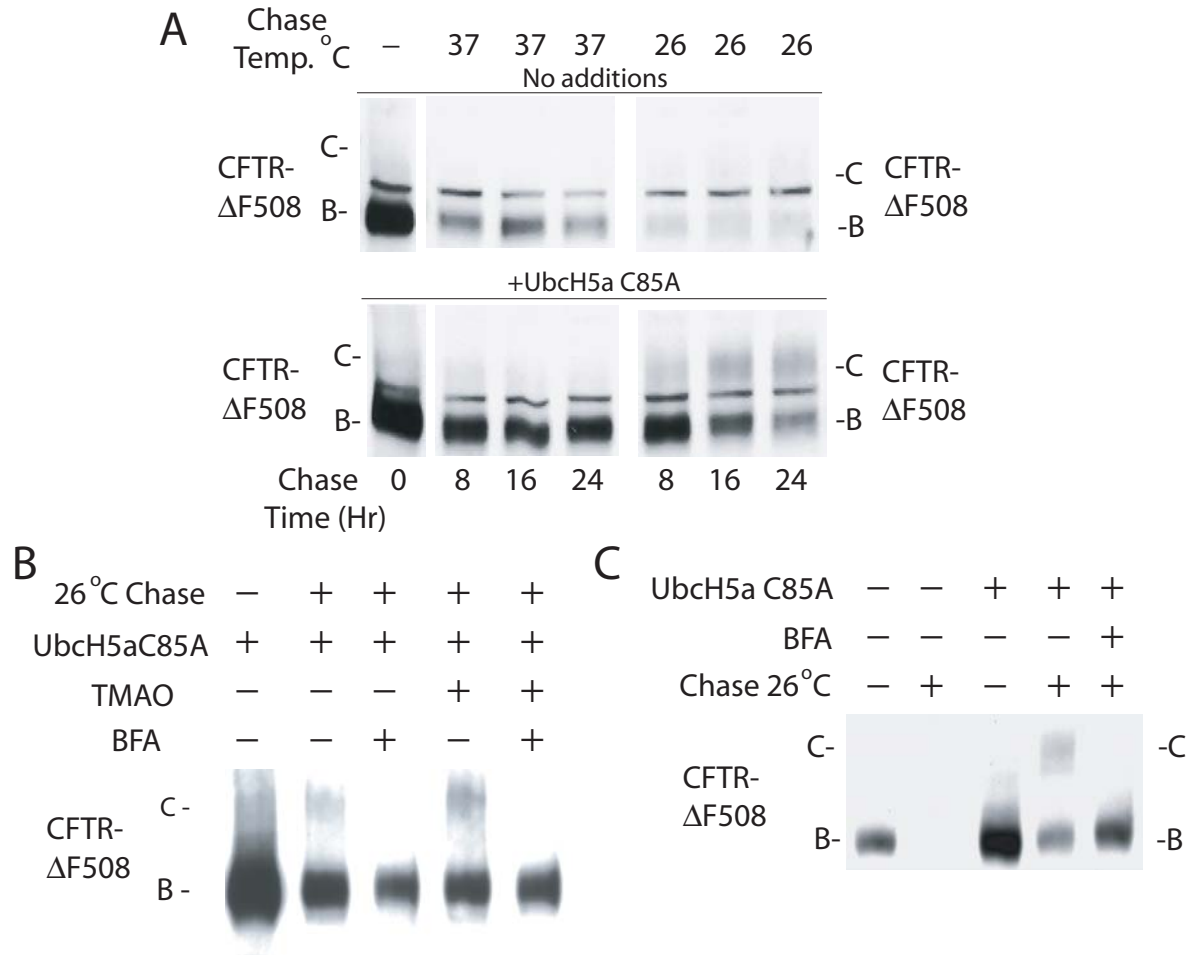
Next, we examined the effect that modulating Hsc70/CHIP E3 action had on complex formation between Hsc70 and CFTR $\Delta$ F508 degradation intermediates (Figure 2.7B). This was accomplished by co-immunoprecipitating Hsc70:CFTR $\Delta$ F508 complexes with  $\alpha$ -Hsc70 antibody from radiolabeled cells that were transfected with the indicated form of UbcH5a or CHIP. Overexpression of UbcH5a or CHIP reduced the levels of immunoprecipitable CFTR $\Delta$ F508, whereas UbcH5a C85A overexpression caused immunoprecipitable CFTR $\Delta$ F508 to accumulate several fold. On the other hand, CHIP P269A, which inhibits CFTR degradation and caused CFTR $\Delta$ F508 to aggregate (Figure 2.6), reduced the total amount of CFTR $\Delta$ F508 that could be immunoprecipitated from cell extracts. The newly synthesized pool of Hsc70 detected by immunoprecipitation was not significantly altered when the levels of the components of the Hsc70/CHIP/UbcH5a E3 complex were altered. Yet, we did observe that the levels of Hsc70:CFTR $\Delta$ F508 complexes were lower when UbcH5a and CHIP were overexpressed, and were elevated when UbcH5a C85A was overexpressed. Nonetheless, under all of the aforementioned experimental conditions tested, the changes in the levels of Hsc70:CFTR $\Delta$ F508 complexes appeared proportional to changes in the total amount of immunoprecipitable CFTR $\Delta$ F508 present in the cell extracts. The major conclusion that we would like to draw from these results is that the soluble CFTR $\Delta$ F508 degradation intermediate that accumulates when UbcH5a C85A is overexpressed is found in a complex with Hsc70.

The data presented thus far suggest that inhibition of Hsc70/CHIP ubiquitin ligase activity drives the accumulation of an ER membrane inserted CFTR $\Delta$ F508 biogenic intermediate that is

arrested at a stage where it has the potential to either fold or be degraded. If this is the case then the CFTR $\Delta$ F508 that accumulates in response to UbcH5a C85A overexpression should be glycosylated. Indeed, we observed that the gel mobility of the CFTR $\Delta$ F508 that accumulates in response to UbcH5a C85A overexpression was increased when total cell extracts were treated with endoglycosidase H, which removes ASN-linked glycans from glycoproteins (Figure 2.7C). Thus, inhibition of the Hsc70/CHIP E3 activity promotes the accumulation of an immaturely glycosylated and detergent soluble form of CFTR $\Delta$ F508 that can be isolated in complexes with Hsc70.

**CFTR $\Delta$ F508 that accumulates in the ER when Hsc70/CHIP action is blocked can fold to the native state** -- To determine whether the CFTR $\Delta$ F508 biogenic intermediate that accumulates in response to inhibition of Hsc70/CHIP E3 function is capable of folding we explored whether it could be chased to its maturely glycosylated C-form (Figure 2.8A). Transiently transfected HEK293 cells were grown for 24 hrs post-transfection and then treated with cycloheximide to inhibit new protein synthesis. Then the fate of the accumulated CFTR $\Delta$ F508 was monitored by Western blot after the indicated chase incubation at 37 °C or 26 °C (Figure 2.8A). The low temperature chase incubation was incorporated into the design of this experiment because nascent CFTR $\Delta$ F508 exhibits a temperature-sensitive folding defect and can fold to the native state and accumulate in its maturely glycosylated C-form when cells are cultured at 26 °C (Denning et al., 1992).

When control cells were allowed to synthesize CFTR $\Delta$ F508 at 37 °C and were then incubated at either 37 °C or 26 °C, greater than 90% of the total protein present at T=0 was degraded during the 8 hr chase period. When UbcH5a C85A was co-expressed with CFTR $\Delta$ F508, and a chase incubation was carried out at 37 °C, 40 to 50% of the total



**Figure 2.8 Reduction of cell growth temperatures enables CFTR $\Delta$ F508 degradation intermediates that accumulate in response to UbcH5a C85A overexpression to escape the ER.** (A) The immaturely glycosylated B-form CFTR $\Delta$ F508 that accumulates when UbcH5a C85A is overexpressed becomes maturely glycosylated when cell growth temperatures are reduced to 26°C. HEK293 cells were transiently transfected with pCDNA3.1 CFTR $\Delta$ F508 either alone or in combination with pCAGGS His6Ubc5a C85A. After a 24 hr post transfection period cycloheximide (25 $\mu$ g/ml) was added to growth media, and cells were either shifted to 26°C or maintained at 37°C for the indicated time. (B) Glycolytic processing of CFTR $\Delta$ F508 at 26°C is blocked by BFA and enhanced by the chemical chaperone TMAO. Cells were transfected as described above and either BFA (10 $\mu$ M) or TMAO (75 $\mu$ M) was added to cell growth media with cycloheximide. Chase incubations were then carried out at 37°C or 26°C for 16 hrs. (C) COS-7 cells are capable of maintaining CFTR $\Delta$ F508 degradation intermediates in a folding competent state. This experiment was carried out as described for panel A above except that the chase time was for 24 hrs at 26°C. Cell extracts were prepared in SDS-sample buffer and analyzed for CFTR $\Delta$ F508 expression by Western blot with  $\alpha$ -CFTR. The immaturely glycosylated and maturely glycosylated forms of CFTR $\Delta$ F508 are denoted as B and C, respectively.

CFTR $\Delta$ F508 present at T=0 remained in the cell for up to 24 hrs. However, even though UbcH5a C85A stabilized the B-form of CFTR $\Delta$ F508, its conversion to the C-form was not detected during the chase reaction at 37 °C. In contrast, when chase incubations were carried out at 26 °C, a significant portion of the CFTR $\Delta$ F508 that was stabilized in the B-form by UbcH5a C85A, was converted to the maturely glycosylated C-form. The formation of the maturely glycosylated C-form of CFTR $\Delta$ F508 was proportional to the loss of B-form and could be detected in cells after 8 hrs of chase time and appeared to be complete after 16 hrs. The glycolytic maturation of CFTR $\Delta$ F508 observed in the presence of UbcH5a C85A during the chase incubation at 26 °C was inhibited by Brefeldin A (BFA) and appears to result from trafficking of CFTR $\Delta$ F508 out of the ER (Figure 2.8B). To determine if we could further increase the folding efficiency of the CFTR $\Delta$ F508 that accumulated in the presence of UbcH5a C85A, cells were treated with the chemical chaperone TMAO just prior to the initiation of the chase reaction (Brown et al., 1996; Brown et al., 1997). TMAO treatment of cells increased the quantity of UbcH5a C85A stabilized CFTR $\Delta$ F508 that could be processed to a maturely glycosylated C-form around 2-fold. Thus, a portion of CFTR $\Delta$ F508 that accumulated in response to inhibition of its ubiquitination remains in a foldable state that can be brought back on pathway by alteration of cell growth temperatures or chemical chaperones.

To probe whether inhibition of the Hsc70/CHIP complex stabilizes CFTR $\Delta$ F508 in a folding competent conformation in more than one cell type, we examined the effect that UbcH5a C85A overexpression had on CFTR $\Delta$ F508 expression and folding in COS7 cells (Figure 2.8C). UbcH5a C85A overexpression was again observed to drive the accumulation of the B-form of CFTR $\Delta$ F508. In addition, a significant portion of the B-form that accumulated in the presence

of Ubc5Ha C85A could be chased at 26 °C, in a BFA sensitive manner, to the maturely glycosylated C-form. Hence, the Hsc70/CHIP complex can regulate biogenesis of CFTR $\Delta$ F508 in more than one cell type.

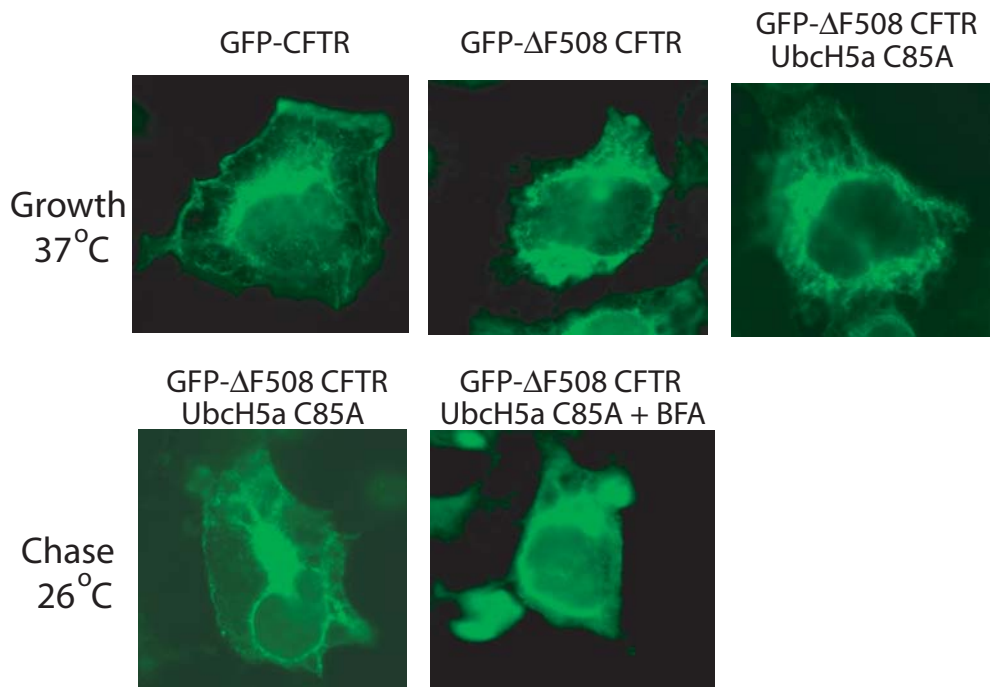
To demonstrate that the CFTR $\Delta$ F508 that was converted to the C-form during the 26 °C chase incubation was trafficked to the cell surface the localization of GFP-CFTR $\Delta$ F508 was examined under these experimental conditions and compared to that of GFP-CFTR (Figure 2.9). At 37 °C, GFP-CFTR was detected both at the cell surface and in a perinuclear location that corresponds to the ER (Moyer et al., 1998). At 37 °C, in the presence or absence of UbcH5a C85A, GFP-CFTR $\Delta$ F508 was only detected in its soluble ER form. However, when UbcH5a C85A transfected cells were cultured for 24 hrs at 37 °C, treated with cycloheximide and then incubated at 26 °C for 4 hrs, a pool of GFP-CFTR $\Delta$ F508 accumulated, in a BFA sensitive fashion, at the cell surface.

The collective data presented in Figures 2.7, 2.8 and 2.9 demonstrate that when the activity of the Hsc70/CHIP ubiquitin ligase is reduced, CFTR $\Delta$ F508 accumulates as an immaturely glycosylated species that is not a dead-end folding intermediate. Instead, the cell can maintain a pool of kinetically trapped CFTR $\Delta$ F508 folding intermediates in a soluble foldable state.

## **2.4 Discussion**

Herein we provide new insights into how CFTR and CFTR $\Delta$ F508 biogenic intermediates are partitioned between folding and degradation pathways. The data presented suggest a model for quality control in which newly synthesized CFTR and CFTR $\Delta$ F508 initiate folding, but intermediates of each accumulate in a kinetically trapped conformation that is maintained in a soluble state by Hsc70. CHIP then interacts with Hsc70 and acts via a two-step mechanism to





**Figure 2.9 Localization of GFP-CFTR $\Delta$ F508 in HEK293 cells.** Cells grown on glass cover slips were transiently transfected with GFP-CFTR or GFP-CFTR $\Delta$ F508 with or without Ubch5a C85A. Cells were then cultured for 24 hrs at 37°C and fixed. Where indicated 25 $\mu$ g/ml cyclohexamide and/or Brefeldin A (10 $\mu$ M) was added to culture media. Then after 4 hrs of culture at 26°C, cells were fixed and the coverslips were mounted on glass slides. Images were collected and processed as described in the materials and methods section.

attract UbcH5a into Hsc70:CFTR complexes and the ubiquitin ligase thus formed polyubiquitinates CFTR.

Interestingly, inhibition of Hsc70/CHIP E3 activity caused the accumulation of a soluble and ER localized CFTR $\Delta$ F508 biogenic intermediate that remained bound by Hsc70. Addition of chemical chaperones to growth media and reduction of cell growth temperatures permitted this non-ubiquitinated CFTR $\Delta$ F508 degradation intermediate to fold, exit the ER, and accumulate on the cell surface. These are the first data that describe a non-ubiquitinated CFTR $\Delta$ F508 biogenic intermediate and they demonstrate that it can be maintained in a soluble and foldable state. This new information suggests that the development of drug cocktails that contain ubiquitination blockers and chemical chaperones could increase the cell surface expression of CFTR $\Delta$ F508 and provide a treatment for cystic fibrosis.

The nature of the folding defect that arrests the progression of CFTR $\Delta$ F508 through its folding cascade and what causes it to be selected for proteasomal degradation is not entirely clear. One school of thought is that CFTR $\Delta$ F508 is highly prone to misfolding and aggregation and is therefore selected for ERAD. Such a notion is supported by the observation that inhibition of the proteasome blocks CFTR $\Delta$ F508 degradation and drives the accumulation of ubiquitinated forms of CFTR $\Delta$ F508 in Triton X-100 insoluble aggregates (Ward and Kopito, 1998; Ward et al., 1995). However, since the inactivation of the Hsc70/CHIP E3 ligase leads to the accumulation of a soluble ER localized CFTR $\Delta$ F508 biogenic intermediate; the data we present support a different view. It appears that inhibition of the proteasome leads polyubiquitinated CFTR $\Delta$ F508 to aggregate because it can be extracted from the ER membrane by the p97/UFD1/NPL4 complex (Ye et al., 2003), but since it can not be degraded,

polyubiquitinated CFTR $\Delta$ F508 accumulates in aggresomes (Ward and Kopito, 1998). On the other hand, the non-ubiquitinated CFTR $\Delta$ F508 that accumulates in response to inhibition of the Hsc70/CHIP E3 remains soluble because it is inserted into the ER membrane and is bound by cytosolic Hsc70. Thus, while CFTR $\Delta$ F508 has a folding defect that prevents it from passing quality control and escaping the ER, it does not appear to be overly aggregation prone and cellular chaperones can maintain it in a soluble foldable state.

Since the cellular activity of CHIP and UbcH5a influence the partitioning of CFTR biogenic intermediates between folding and degradation pathways, we were interested in investigating whether inhibition the Hsc70/CHIP E3 would influence the processing efficiency of CFTR and CFTR $\Delta$ F508. In pulse-chase experiments UbcH5a C85A overexpression increased the half-life of the B-form of CFTR and CFTR $\Delta$ F508 from 2-3 fold. Therefore, UbcH5a C85A overexpression increased the steady state levels of CFTR and CFTR $\Delta$ F508 several fold. However, the processing efficiency of CFTR from its B-form to its C-form remained at around 25% whether or not the Hsc70/CHIP E3 complex was active. Thus, while the B-form of CFTR can be diverted away from its folding pathway through the elevation of the Hsc70/CHIP E3 activity, the ability of full length CFTR to stay on pathway and collapse to the native state appears to be limited by its intrinsic folding pathway and/or additional quality control factors.

We conclude that the E2 UbcH5a is a cytosolic factor that functions with Hsc70 and CHIP to mediate CFTR ubiquitination. This conclusion is supported by 3 lines of experimental evidence. First, purified CHIP and UbcH5a cooperated to facilitate the polyubiquitination of CFTR. Second, when CHIP and UbcH5a were co-expressed together they appeared to act synergistically to reduce the steady state levels of CFTR $\Delta$ F508. Third, the co-expression of UbcH5a C85A with CHIP blocked CHIPs ability to degrade CFTR and converted it into a

protein that behaved like the CHIP U-box mutant P269A.

UbcH5a is a member of a family of conserved E2 proteins that include UbcH5b and UbcH5c that are nearly 90% identical to each other (Jensen et al., 1995a; Scheffner et al., 1994). In addition to UbcH5a, purified CHIP can interact with UbcH5b and UbcH5c and mRNAs for each of these E2 proteins is present in all tissues tested (Jiang et al., 2001; Jensen et al., 1995a). Thus, we propose that CHIP functions with an UbcH5 E2 family member to ubiquitinate CFTR and other Hsc70 substrates, but we are not able to state whether it prefers one family member to the other. At this point, it is interesting to note that UbcH5 proteins are related to the yeast Ubc4/5 proteins that function to target misfolded proteins for degradation and protect cells from protein denaturing physiological stress (Seufert and Jentsch, 1990). In fact, one member of the Ubc4/5 family, Ubc1, has been shown to function on the ER surface to ubiquitinate ERAD substrates (Bays et al., 2001). Thus, it is logical that UbcH5 E2s would function as a component of an E3 complex that contains molecular chaperones and serve to prevent the accumulation of toxic protein aggregates.

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

The E2s Ubc6 and Ubc7 function with E3s such as gp78 and Doa10 on the cytoplasmic face

of the ER to ubiquitinate a variety of substrates (Cyr et al., 2002). Hence, it is plausible that E2/E3 complexes that contain Ubc6 and/or Ubc7 function to select CFTR and CFTR $\Delta$ F508 for degradation. Sommer and colleagues have explored this model and found that overexpression of Ubc6, but not Ubc7, modulates the rate of CFTR $\Delta$ F508 degradation (Lenk et al., 2002). When we compared the effect that dominant negative forms of Ubc6, Ubc7 and UbcH5a had on CFTR and CFTR $\Delta$ F508 expression, UbcH5a C85A and Ubc6 C91S drove the accumulation of the B-form of CFTR, whereas HsUbc7 had no apparent effect. The effect that UbcH5a C85A had on the accumulation of the B-form of CFTR and CFTR $\Delta$ F508, was markedly more dramatic than that of Ubc6 C91S, yet Ubc6 does appear to play a role in quality control of CFTR. Studies in yeast demonstrate that Ubc6 cooperates with the transmembrane E3 Doa10 to degrade membrane and cytosolic proteins (Swanson et al., 2001). In addition, DoA10 is required for efficient CFTR turnover in yeast (Gnann et al., 2004). Thus, the Doa10/Ubc6 E3 and may function along side the Hsc70/CHIP/UbcH5 E3 to mediate quality control of CFTR. The Hsc70/CHIP/UbcH5 E3 recognizes cytosolic regions of CFTR, whereas the Doa10/Ubc6 E3 may recognize unassembled transmembrane regions. This scenario would explain why turnover of the B-form of CFTR and CFTR $\Delta$ F508 is delayed, but not completely blocked by the inactivation of the Hsc70/CHIP E3 complex.

A critical question about the function of CHIP as quality control factor pertains to the mechanism by which it regulates Hsc70 polypeptide binding and protein folding activity. The data we present suggest that CHIP functions via a two-step mechanism to determine the fate of Hsc70 clients such as CFTR. The first step involves the binding of CHIP to the C-terminal EEVD motif in the lid domain of Hsc70 (Ballinger et al., 1999). This event alters the Hsc70 polypeptide binding and release cycle to arrest CFTR folding and may involve the transient

stabilization of Hsc70:CFTR complexes, which would allow the U-box on CHIP to attract UbcH5 to Hsc70:CFTR complexes.

Interestingly, the ability of CHIP to ubiquitinate Hsc70 clients can be modified by other co-chaperones. Data presented herein demonstrates that Hdj-2 can assist Hsc70 and CHIP in mediating substrate ubiquitination. However, the co-chaperone HspBP1, which is a member of a family of nucleotide exchange factors that promote substrate release from Hsc70, appears to block the ability of CHIP to ubiquitinate CFTR (Alberti et al., 2004). Thus, the fate of proteins that are bound to Hsc70 is regulated by its interactions with multiple cellular factors. To understand this process, the temporal relationship and driving force for interactions between Hsc70 it's folding or degradatory co-chaperones needs to be determined.

## 2.5 Materials and Methods

**Plasmids and Antibodies** -- The plasmids utilized in this study for transfection of cultured cells were from the indicated sources: pCDNA3.1CFTR and pCDNA3.1 CFTR $\Delta$ F508 (Meacham et al., 2001); pEGFPC2-CFTR and pEGFPC2-CFTR $\Delta$ F508 (Moyer et al., 1998); pCDM8 2B4 TCR- $\alpha$  (Bonifacino et al., 1989); pCDNA-ApoB48 (H. Ginsberg); pCAGGS His<sub>6</sub>UBCH5A C85A and pCAGGS His<sub>6</sub>UBCH5A (Jiang et al., 2001); pCMVPLDmyc-UBC6 and pCMVPLDmyc-UBC6 C91S (Lenk et al., 2002); pCDNA myc-UBC7 and pCDNA myc-UBC7 C89S (Tiwari and Weissman, 2001); pCDNA3.1CHIP, pCDNA3.1CHIP K30A, and pCDNA3.1CHIP P269A (Jiang et al., 2001; Meacham et al., 2001). pGEXCFTR 371-855 was termed pNBD1-R (Naren et al., 1999).

The following plasmids were utilized for overexpression of the indicated proteins in *E. coli*; pET9d Hdj2 (Meacham et al., 1999) and pET11a Hsc70 (Freeman et al., 1995). Plasmids prepared for this study were pET11a His<sub>6</sub>UbcH5a, pET11a-His<sub>6</sub>UbcH5a C85A, pET11d

His<sub>6</sub>Ubc7, pET11a His<sub>6</sub>Ubc6 1-243, pET30 His<sub>6</sub>CHIP, pET30 His<sub>6</sub>CHIP K30A, pET30 His<sub>6</sub>CHIP H260A and pET30 His<sub>6</sub>CHIP P269A. E1 was purchased from Calbiochem.

The antibodies utilized for Western blots and/or immunoprecipitations were  $\alpha$ CFTR clone MM13-4 from Upstate Biotechnology and  $\alpha$ CFTR R-domain antibody from R&D systems.  $\alpha$ TCR was from PharMingen and  $\alpha$ Hsc70 was from Stressgen Biotechnologies.

**Protein purification** -- The proteins utilized in *in vitro* ubiquitination assays were purified after overexpression in *E. coli*. Hsc70 was purified by a combination of ATP-agarose and anion exchange chromatography (Cyr et al., 1992). Hdj-2 was purified by anion exchange and hydroxyapatite chromatography (Meacham et al., 1999). UbcH5a, UbcH5a C85A, Ubc7, Ubc6 1-243, CHIP, CHIP K30A, CHIP H260A, and CHIP P269A were His<sub>6</sub> tagged and purified by metal chelate chromatography (Lu and Cyr, 1998). Proteins in peak fractions were greater than 90% pure, concentrated to 1-2 mg/ml, dialyzed in a buffer that contained 20mM Hepes, pH 7.4, 150mM NaCl, 1mM  $\beta$ -mercaptoethanol and 0.1mM phenyl methyl sulfonyl fluoride, and then snap frozen and stored at  $-80^{\circ}\text{C}$ . Gst-NBD1-R was expressed from pGEX-5X-NBD1-R in *E. coli* strain BL21 (DE3) and cells from a 600ml culture were harvested after a 16-hour induction at  $30^{\circ}\text{C}$  with 0.2 mM IPTG. Cell pellets were re-suspended and incubated for 30 min. on ice in a buffer composed of 10mM Tris-HCl pH 8.0, 1mM EDTA, 150mM NaCl, 1mM phenylmethyl sulfonyl fluoride, 1mM DTT, and lysozyme 0.1 $\mu\text{g}/\text{ml}$ . The cell suspension was then supplemented with 1% sarkosyl and the slurry was sonicated. Cleared extracts were supplemented with glutathione-agarose beads and incubated at  $4^{\circ}\text{C}$  for 1 hr. Gst-NBD1-R was eluted from washed beads with 50mM Tris-HCl that contained 10mM reduced glutathione, had a final concentration near 2 mg/ml, and was flash frozen and stored at  $-80^{\circ}\text{C}$ .

**Reconstitution of CFTR ubiquitination** -- The experimental conditions for the

reconstitution of gst-NBD1-R ubiquitination were described previously (Koegl et al., 1999). Ubiquitination assays were performed in a reaction buffer composed of 20mM Hepes, pH 7.4, 50mM NaCl, 5mM MgCl<sub>2</sub>, 2.5mM ATP, 2mM DTT, 10 μM bovine ubiquitin, 0.1 μM rabbit E1 (CalBiochem) and 1 μM Gst-NBD1-R. The indicated E2 protein was added at 4 μM and the other factors were included at the indicated concentration; CHIP (3 μM), Hsc70 (2 μM), Hdj2 (4 μM). Incubations were carried out at 37 °C for the indicated time and terminated by the addition of 20 μl of SDS sample buffer to 25 μl reaction cocktails. Proteins in samples were resolved on 7% SDS-PAGE gels and then transferred to nitrocellulose membranes at 100V for 60 min in a Bio-Rad mini gel wet transfer apparatus. Changes in the mobility of gst-NBD1-R were determined by decorating nitrocellulose membranes with α-R -domain antibody and developing them with ECL reagent (Amersham).

**Assays for CFTR Biogenesis** -- HEK293 and Cos-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum and a mixture of 1% penicillin and streptomycin at 37°C and transfected with CFTR and CFTRΔF508 expression plasmids as previously described (Meacham and Cyr, 2002). Steady state levels of CFTR and CFTRΔF508 levels were determined by western blot (Meacham et al., 1999). CFTR processing efficiency was measured by pulse chase analysis (Meacham and Cyr, 2002). Details of the protocols for direct immunoprecipitations or co-immunoprecipitations were as previously described (Meacham and Cyr, 2002; Meacham et al., 1999; Meacham et al., 2001). To verify the identity of CFTR isolated from chaperone complexes CFTR was re-immunoprecipitated from co-immunoprecipitates with α-CFTR (Meacham et al., 1999). To determine the glycosylation state of CFTR cell extracts were treated with Endo H (Meacham et al., 1999).

**Fluorescence microscopy** -- HEK293 cells were cultured on glass cover slips and



transiently transfected with 1 $\mu$ g pEGFPC2-CFTR or pEGFPC2-CFTR $\Delta$ F508 either alone or in combination with 3 $\mu$ g pCAGGS His<sub>6</sub>UBCH5A C85A. After a 24 hr post-transfection period, cells were washed twice for 5 min with 2 ml of PBS and fixed with 4% paraformaldehyde at room temperature. Where indicated, cells were grown for 24 hrs post-transfection and then treated with 25 $\mu$ g/ml cyclohexamide and incubated for an additional 4 hrs at 26°C. Coverslips were mounted on glass slides with the preservative Fluoromount-G. Images were collected using a Nikon E600 microscope and a Princeton Instruments CCD camera. Images were processed with Metamorph (Universal Imaging Corporation) and Adobe Photoshop software.

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## **Chapter Three**

**The ER Membrane Associated E3 Ubiquitin Ligase RMA1 and the Cytosolic  
E3 CHIP Detect Folding Defects in Different Regions of CFTR $\Delta$ F508**

### 3.1 Abstract

Cystic fibrosis arises from the misfolding and premature degradation of CFTR $\Delta$ F508. Yet, the quality control machinery that selects CFTR $\Delta$ F508 for degradation and the mechanism for its misfolding are not well defined. We identified an ER membrane associated E3 ubiquitin ligase complex that contains RMA1, Ubc6e, and Derlin-1 that cooperates with the cytosolic Hsc70/CHIP E3 complex to triage CFTR and CFTR $\Delta$ F508. Derlin-1 serves to retain CFTR in the ER membrane and interacts with the E3 RMA1 and E2 Ubc6e to promote CFTR's proteasomal degradation. The RMA1 E3 is capable of recognizing folding defects in CFTR $\Delta$ F508 coincident with translation, whereas the CHIP E3 appears to act post-translationally. A folding defect in CFTR $\Delta$ F508 that is detected by RMA1 involves the inability of MSDII to productively interact with CFTR's amino-terminal domains. The RMA1 and CHIP E3 ubiquitin ligases act sequentially in ER membrane and cytosol to monitor the folding status CFTR and CFTR $\Delta$ F508.



### 3.2 Introduction

CFTR is a Cl<sup>-</sup> 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 CFTR $\Delta$ F508 allele, which is missing F508 from nucleotide binding domain (NBD) I. CFTR $\Delta$ F508 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 CFTR $\Delta$ F508 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 CFTR $\Delta$ F508 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 CFTR $\Delta$ F508 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 CFTR $\Delta$ F508 folding or block the degradation of folded CFTR $\Delta$ F508 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 CFTR $\Delta$ F508 misfolding and the identification of the ERQC machinery that selects CFTR $\Delta$ F508 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<sup>-</sup> ion channel (Ostedgaard et al., 1997; Xiong et al., 1997). In most cell types tested, the biosynthesis of CFTR takes approximately 10 minutes and the assembly of CFTR and CFTR $\Delta$ F508 into a channel is inefficient with around 60-75% of CFTR and nearly 99% of newly synthesized CFTR $\Delta$ F508 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 CFTR $\Delta$ F508 does not reach the native state and is degraded prematurely in human airway cells (Kreda et al., 2005). CFTR and CFTR $\Delta$ F508 appear to assume similar conformations at early stages of assembly (Zhang et al., 1998), but CFTR $\Delta$ F508 folding becomes arrested at a poorly defined step.

Current models suggest two complementary mechanisms by which deletion of F508 arrests folding of CFTR. First, F508 is located in a surface exposed loop on NBD1 that makes contacts with MSD1 in bacterial ABC transporters (Chang and Roth, 2001). Therefore, 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  $\Delta$ F508 NBD1 appear to cause CFTR $\Delta$ F508 to be targeted for degradation, but how ERQC detects such folding defects is not clear.

Defects in the folding pathway of CFTR $\Delta$ F508 described above suggest that it accumulates in a conformation that exposes non-native 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 CFTR $\Delta$ F508 (Meacham et al., 1999; Meacham et al., 2001; Younger et al., 2004). Yet, upon inactivation of the Hsc70/CHIP E3 complex, CFTR $\Delta$ F508 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 CFTR $\Delta$ F508.

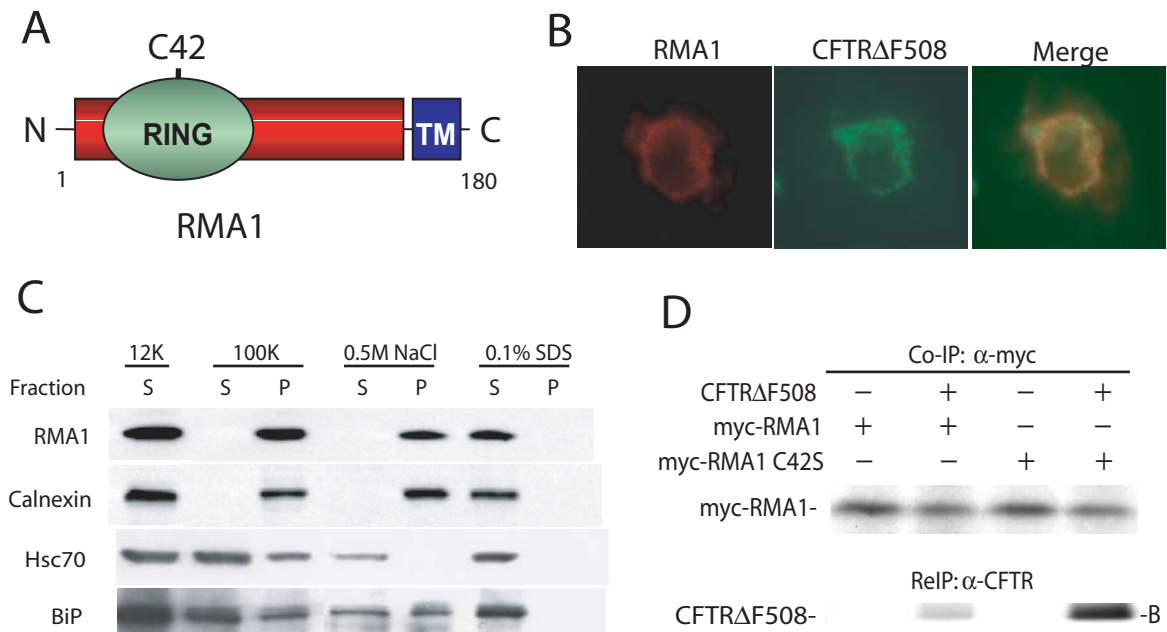
Studies in mammalian cells are yet to identify an ER localized E3 ubiquitin ligase that functions in ERAD of CFTR. Nonetheless, there is evidence to suggest that a tail anchored and ER localized E2 ubiquitin conjugating (Ubc) enzyme, Ubc6e, facilitates CFTR degradation (Lenk et al., 2002). Ubc6e does 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 NBDII synthesis and is defective in CFTR $\Delta$ F508. The CHIP E3 appears to act after NBDII synthesis and detects folding defects that involve terminal steps in CFTR assembly.

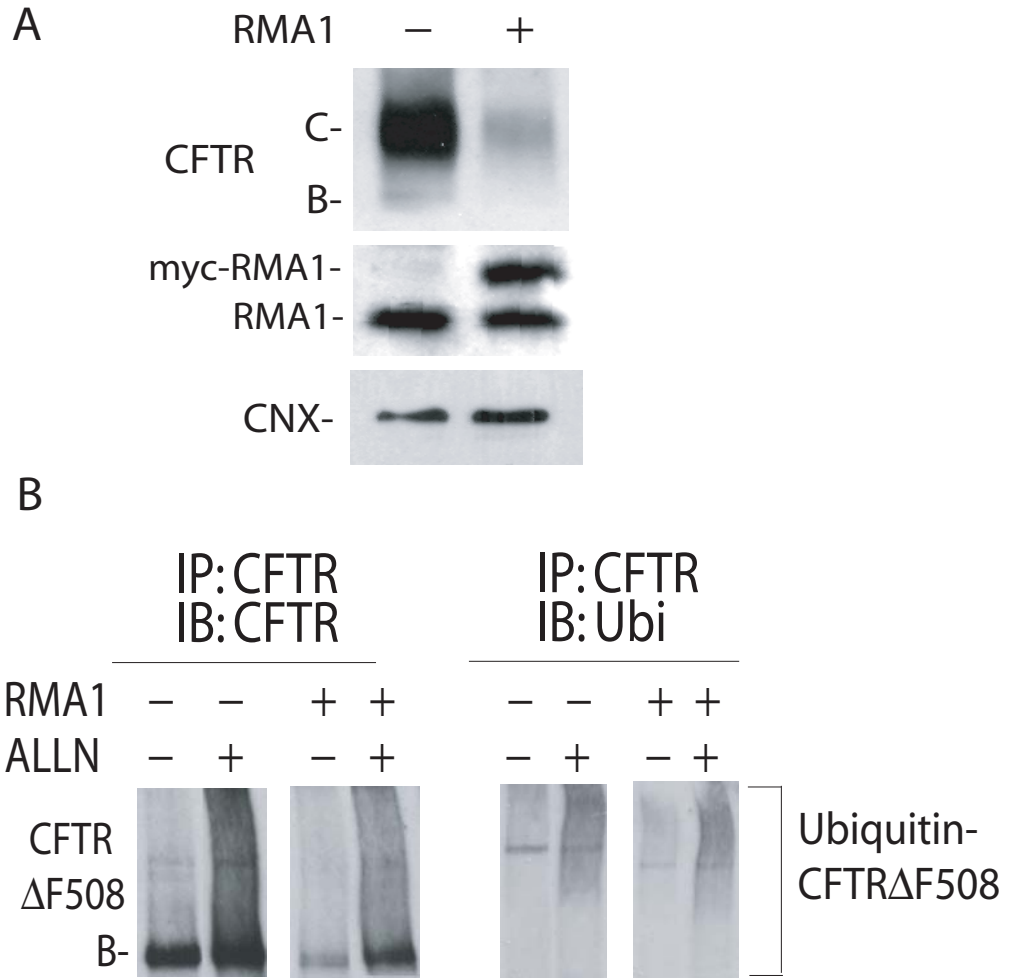
### 3.3 Results

**RMA1 cooperates with Ubc6e to target CFTR $\Delta$ F508 for degradation** -- To identify new QC checkpoints that function to triage CFTR and CFTR $\Delta$ F508 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 CFTR $\Delta$ F508 for proteasomal degradation. RMA1 is a 180 amino acid residue tail anchored ER membrane protein that contains a cytoplasmic RING domain (Figure 3.1A). RMA1 co-localizes with GFP-CFTR $\Delta$ F508 in the ER membrane (Figure 3.1B) and co-fractionates with the ER marker calnexin (Figure 3.1C). In addition, the B-form of CFTR $\Delta$ F508 (Figure 3.1D) and CFTR (data not shown) co-immunoprecipitate 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 CFTR $\Delta$ F508 co-precipitated with the RMA1 mutant. Thus, RMA1 is an ER localized E3 protein that enters into complexes with CFTR $\Delta$ F508 (Figure 3.1D).

To determine if 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, non-native B- and maturely glycosylated, folded C- form of CFTR was determined (Figure 3.2A). A 2-fold increase in RMA1 levels dramatically reduced the steady-state level the C-form of CFTR and this action was not accompanied by the accumulation of the B-form (Figure 3.2A). Treatment of cells with the proteasome inhibitor ALLN prevented RMA1 from reducing CFTR $\Delta$ F508 levels and caused a large quantity of the CFTR $\Delta$ F508, which would normally have been degraded, to accumulate as an ubiquitin conjugate (Figure 3.2B).



**Figure 3.1 RMA1 is an ER membrane associated E3 ubiquitin ligase that co-precipitates with CFTR F508** (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 carboxyl-terminal ends of RMA1 respectively. (B) Co-localization of myc-RMA1 and GFP-CFTR $\Delta$ F508 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-RMA1, calnexin, Hsc70 and BiP by Western blot. Where indicated, membranes that pelleted when extracts were spun at 100,000 x g were washed with 0.5M NaCl or resuspended in 0.1% SDS and then re-isolated. S and P denote the supernatant and pellets of centrifuged samples. (D) RMA1 and CFTR $\Delta$ F508 can be co-precipitated with each other. CFTR $\Delta$ F508 was co-expressed with myc-RMA1 or myc-RMA1C42S and extracts from 35S-labeled HEK293 cells were prepared in PBS supplemented with 0.1% Triton X-100. Co-immunoprecipitations were carried out with myc and then CFTR $\Delta$ F508 was re-immunoprecipitated with  $\alpha$ -CFTR. Autoradiographs of bands corresponding to RMA1 and CFTR $\Delta$ F508 are shown.

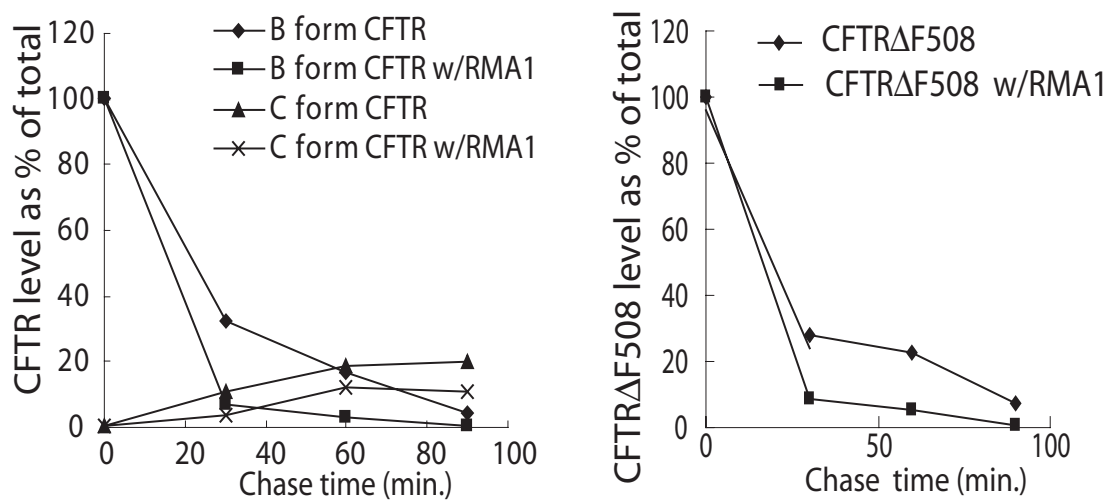
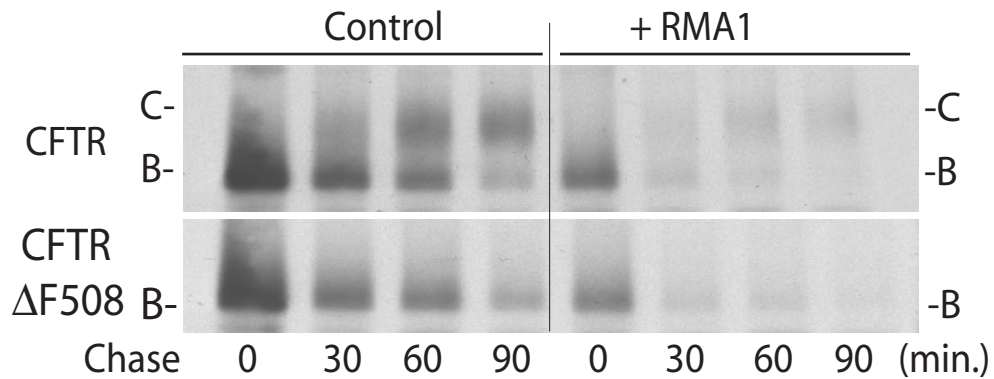


**Figure 3.2 RMA1 promotes the ubiquitination and degradation of both CFTR and CFTR $\Delta$ F508** (A) Overexpression of RMA1 reduces the steady-state level of CFTR. Western blots of extracts prepared from cells in which myc-RMA1 (pCDNA3.1-mycRMA1, 1.0 $\mu$ g) was co-expressed with CFTR. (B) Reduction of CFTR $\Delta$ F508 levels by RMA1 is blocked by ALLN. CFTR $\Delta$ F508 was co-expressed with RMA1, and ALLN (200 $\mu$ M) was added to cells 4 hrs prior to lysis. Extracts were prepared in lysis buffer supplemented with 5mM N-ethyl maleimide and the protein concentration of extracts was determined with a detergent compatible Bradford assay. CFTR $\Delta$ F508 was then immunoprecipitated from 30 $\mu$ g of protein extract. Immunoprecipitates were then probed for the presence of CFTR $\Delta$ F508 or ubiquitin by Western blot.

Pulse-chase analysis showed that elevation of RMA1 caused a 60% reduction in the accumulation of  $^{35}\text{S}$ -CFTR and  $^{35}\text{S}$ -CFTR $\Delta$ F508 during the 20 min. pulse-labeling period (Figure 3.3). Then in the chase period, RMA1 blocked the glycolytic maturation of CFTR and accelerated degradation of the CFTR $\Delta$ F508 B-form around 2-fold. The ability of RMA1 to divert the B-form of CFTR and CFTR $\Delta$ F508 from its folding pathway was lost when RMA1 C42S or RMA1 $\Delta$ TM was substituted for RMA1 in co-transfections (Figure 3.4A).

When RMA1 and Ubc6e levels were reduced by treatment of cells with siRNA oligonucleotides, CFTR $\Delta$ F508 levels increased nearly 4-fold and this effect correlated with reduced expression of RMA1 and Ubc6e (Figure 3.4B). Thus, RMA1 is an ER localized RING E3 that can be isolated in co-precipitates with the B-form of CFTR and the modulation of its activity influences the fate of nascent CFTR and CFTR $\Delta$ F508.

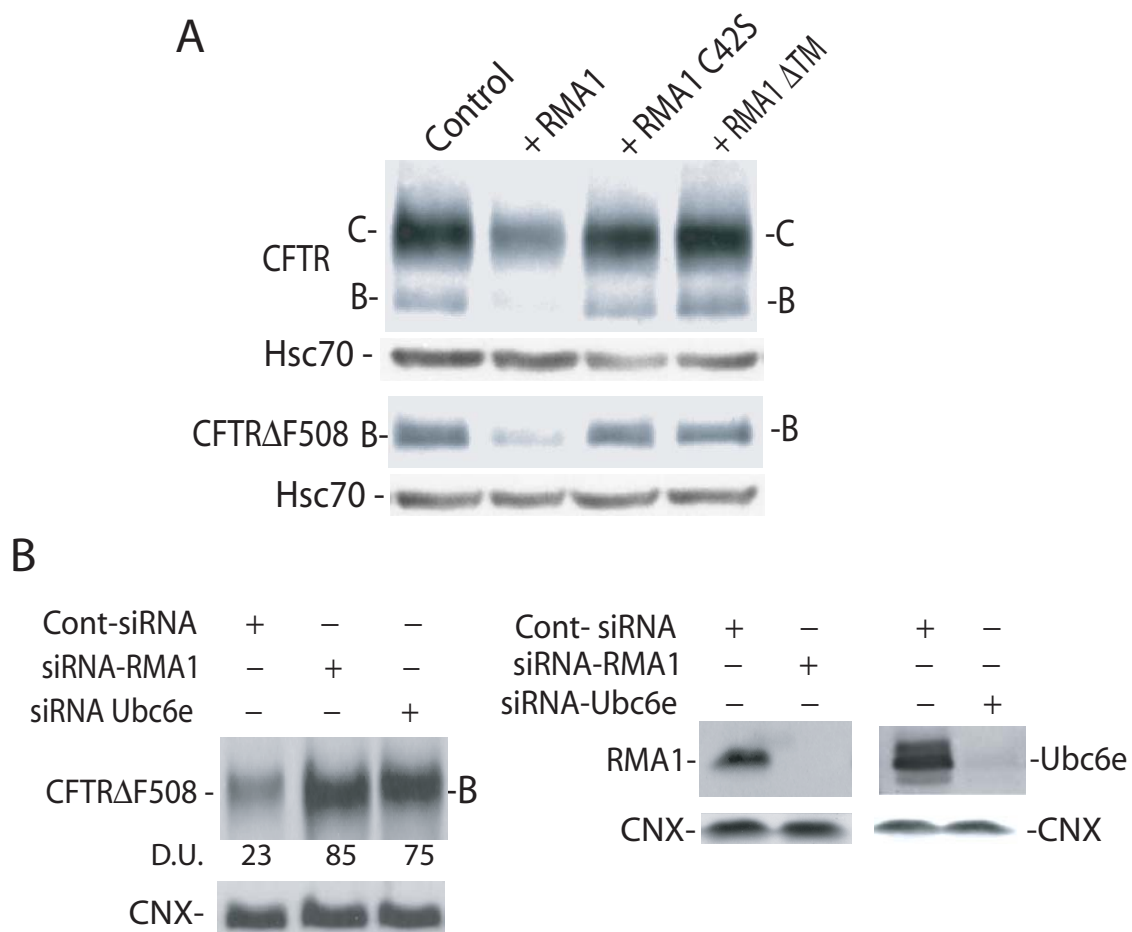
Data generated thus far indicates that RMA1 is a component of an ERQC complex that degrades CFTR and CFTR $\Delta$ F508 equally well. However, it remained possible that the deletion of  $\Delta$ F508 causes more nascent CFTR $\Delta$ F508 than CFTR to accumulate in a conformation that is recognized by RMA1 and/or its interacting partners. To explore this possibility, we compared the sensitivity of CFTR and CFTR $\Delta$ F508 to elevation of RMA1 again, but reduced the level of RMA1 by utilizing 0.5 $\mu\text{g}$  of RMA1 expression plasmid instead of 1.0 $\mu\text{g}$  (Figure 3.5). Under these conditions RMA1 overexpression only reduced CFTR levels, by around 30%, but CFTR $\Delta$ F508 accumulation was reduced by around 90%. Thus, triage of CFTR $\Delta$ F508 is more sensitive to elevation of RMA1 activity than CFTR. Yet, since RMA1 promotes the degradation of both CFTR and CFTR $\Delta$ F508, 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



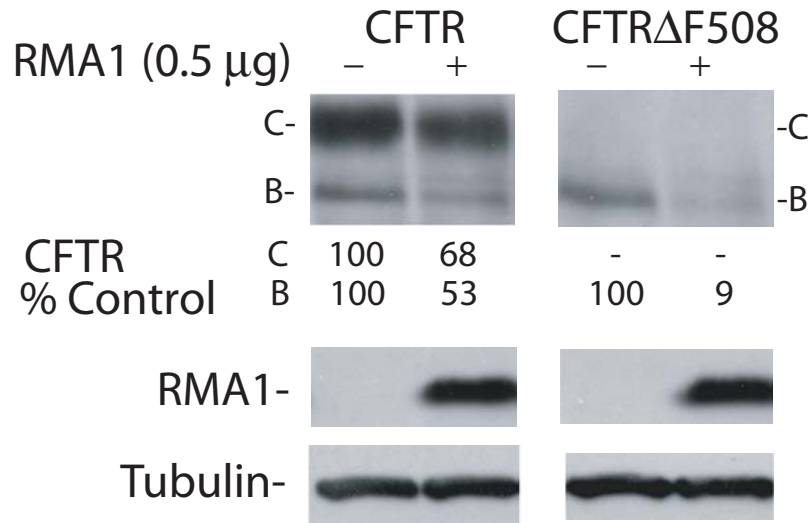
**Figure 3.3 The effect of RMA1 on the kinetics of CFTR and CFTR $\Delta$ F508 biogenesis.**

Cells were labeled with  $^{35}\text{S}$ -translabel for 20 min and after a given chase period CFTR or CFTR $\Delta$ F508 was immunoprecipitated and processed for autoradiography. Values from quantitation of CFTR and CFTR $\Delta$ F508 represent the % of the B-form of CFTR and CFTR $\Delta$ F508 that was present at T=0. Values are representative of 2 experiments and the quantitation displayed is from the data shown in the above panels.





**Figure 3.4 Elevation and reduction in RMA1 activity affects CFTR and CFTR $\Delta$ F508 levels.** (A) Steady-state levels of CFTR and CFTR $\Delta$ F508 when co-expressed with wild type or mutant forms of RMA1. (B) Steady-state levels of CFTR $\Delta$ F508 were determined 24 hrs after co-transfection with siRNA duplexes (50nM) against a non-specific 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 $\mu$ g of pcDNA3.1 RMA1 or Ubc6e was determined. Western blots of calnexin (CNX) or Hsc70 serve as gel loading controls.



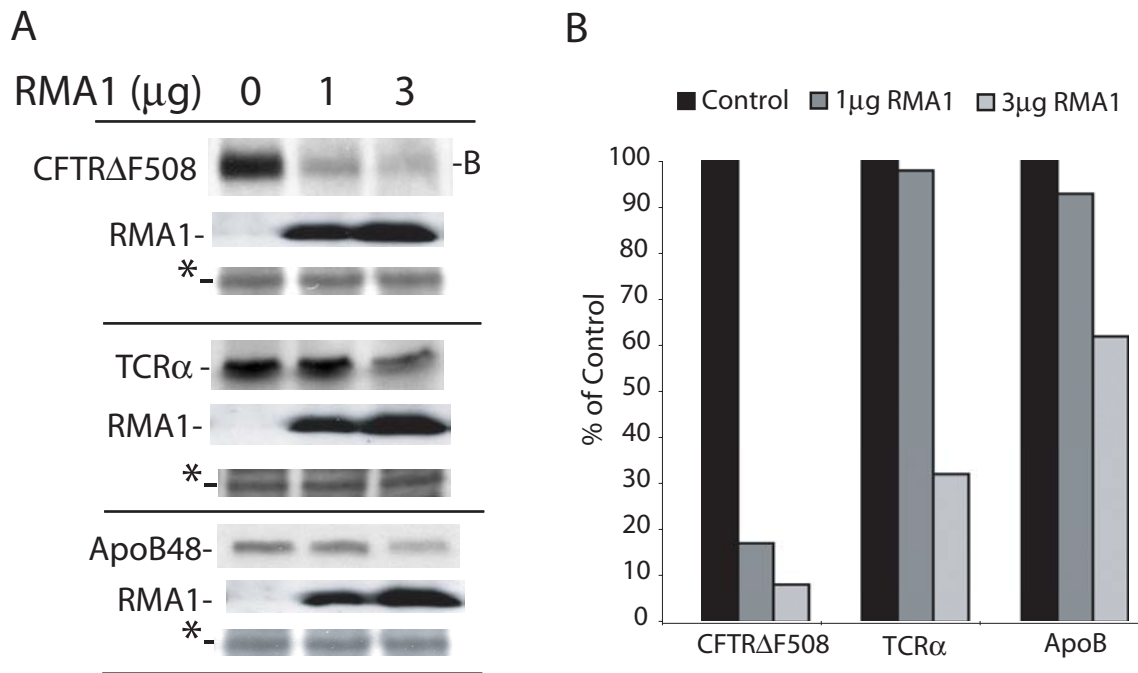
**Figure 3.5 CFTR $\Delta$ F508 is more sensitive to RMA1 overexpression than CFTR.**

A side by side comparison of the sensitivity CFTR and CFTR $\Delta$ F508 display to a lower level of RMA1 overexpression. Instead of co-transfecting cells with CFTR or CFTR $\Delta$ F508 expression plasmids and 1.0 $\mu$ g of pCDNA3.1-myc-RMA1 as in previous figures, cells were transfected with CFTR expression plasmids and 0.5 $\mu$ g of pCDNA3.1-myc-RMA1.

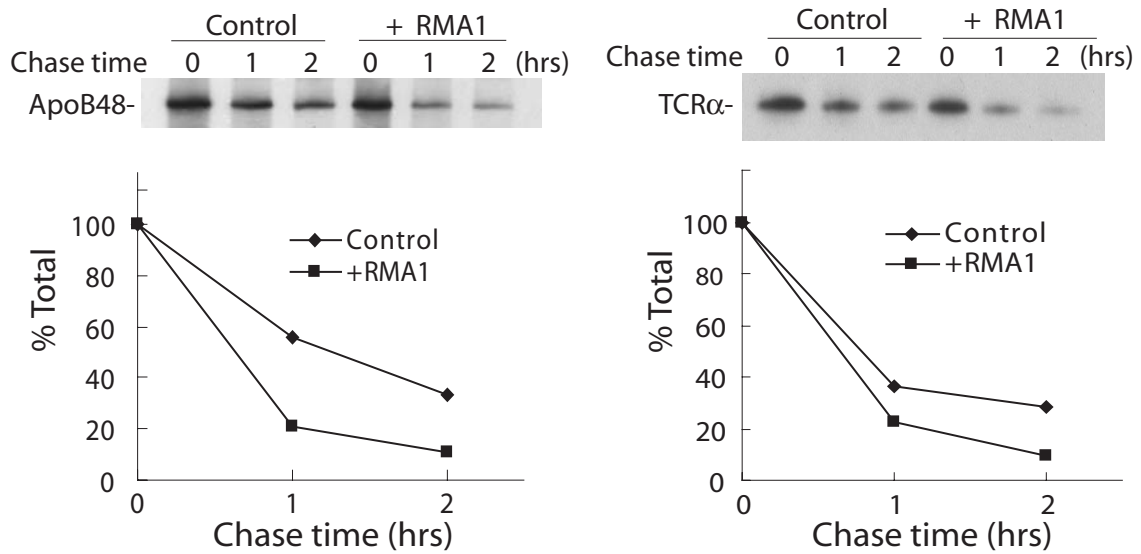
portion of nascent CFTR $\Delta$ F508 than nascent CFTR arrests at a stage where its non-native conformation can be recognized by components of the RMA1 E3 complex.

To evaluate the range of proteins that are selected for ERAD by RMA1 the sensitivity of CFTR $\Delta$ F508, TCR $\alpha$  and ApolipoB48 to RMA1 overexpression were compared (Figure 3.6A). The steady-state level of the B-form of CFTR $\Delta$ F508 was reduced by around 85% when cells were transfected with 1  $\mu$ g of pcDNA-RMA1. Under these same conditions, the accumulation of TCR $\alpha$  and ApoB48 was largely unaffected. Yet, when cells were transfected with 3  $\mu$ g of pcDNA-RMA1 a decrease in the steady state-levels of TCR $\alpha$  and ApoB48 was observed (Figure 3.6A). Decreased steady-state levels of TCR $\alpha$  and ApoB48 resultant from RMA1 overexpression correlated with a 2-fold increase in the degradation rates of these proteins (Figure 3.7A and B). The observed differences in the sensitivity of CFTR $\Delta$ F508, TCR $\alpha$  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.

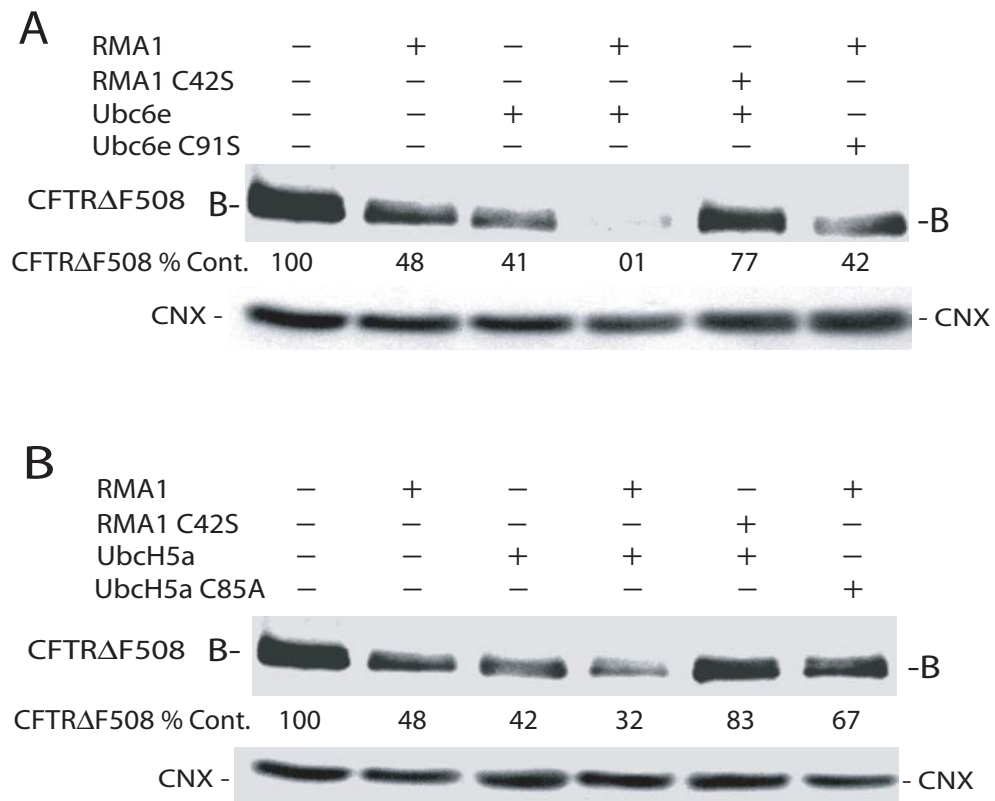
To determine if RMA1 and Ubc6e functionally interact, we analyzed whether they could act jointly to enhance CFTR $\Delta$ F508 degradation (Figures 3.8, 3.9 and 3.10). Individual levels of Ubc6e and RMA1 were adjusted to reduce CFTR $\Delta$ F508 accumulation by 50-60%. When RMA1 and Ubc6e were co-expressed at this same level, there was a dramatic reduction in CFTR $\Delta$ F508 accumulation (Figures 3.8A and 3.9A). 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 CFTR $\Delta$ F508. Thus, RMA1 and Ubc6e appear



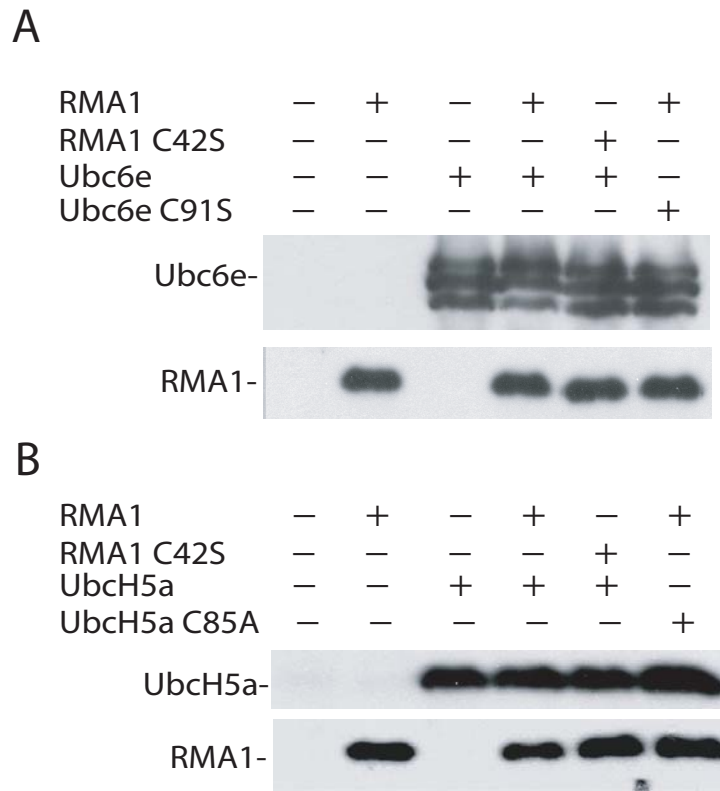
**Figure 3.6 The influence of RMA1 overexpression on CFTR $\Delta$ F508, TCR, and Apolipoprotein B48 levels.** (A) The indicated ERAD substrates were expressed alone or with pcDNA-RMA1 (1.0 or 3.0 $\mu\text{g}$ ). The steady-state level of the indicated protein was determined 18 hrs post-transfection by Western blot. (B) Quantification of the steady-state levels of CFTR $\Delta$ F508, TCR, and Apolipoprotein B48 from autoradiographs shown in panel A.



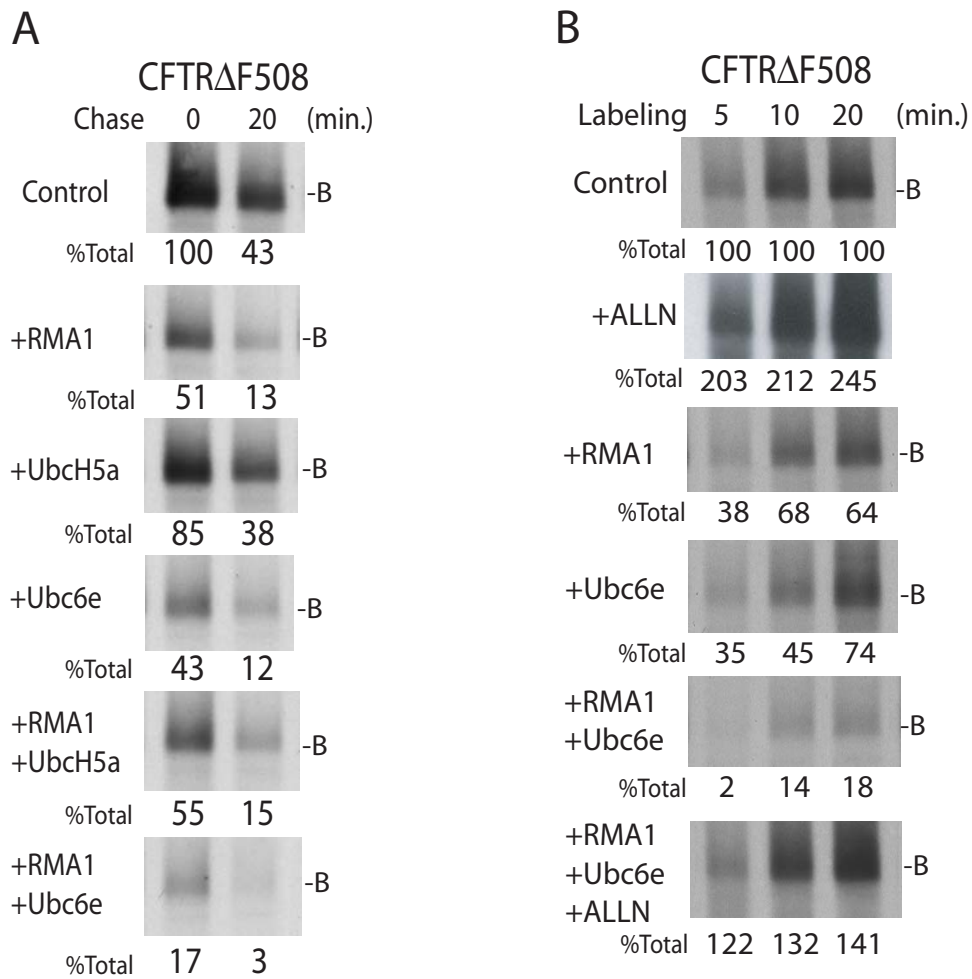
**Figure 3.7 Kinetics of Apolipoprotein B48 and TCR degradation.** Where indicated, cells were co-transfected with 3.0 $\mu$ g of pcDNA3.1-RMA1. Cells were labeled with  $^{35}$ S-translabel for 20 min and chased for the indicated times. Levels of immunoprecipitated Apolipoprotein B48 or TCR were expressed as a % of the total amount present at T=0.



**Figure 3.8 RMA1 and Ubc6e co-expression dramatically reduces CFTR $\Delta$ F508 levels.** (A and B) Western blots for CFTR $\Delta$ F508 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.



**Figure 3.9 RMA1, Ubc6e, and UbcH5a overexpression levels are compared.** (A and B) Western blots of cells extracts that show the expression level of different forms of FLAG-RMA1 and myc-Ubc6e or myc-UbcH5a when they were co-expressed with CFTR $\Delta$ F508. 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.



**Figure 3.10 RMA1 and Ubc6e cooperate to promote CFTR $\Delta$ F508 degradation.** (A) CFTR $\Delta$ F508 was immunoprecipitated from 35S-labeled cells at the beginning and end of a 20 min chase period and detected by autoradiography. (B) Synthesis of 35S-CFTR $\Delta$ F508 under the indicated conditions. When present, ALLN (200 $\mu$ M) was added to cell culture media 1 hr before the addition of 35S-translabel. Bands were quantified by laser densitometry and are expressed as a % of total CFTR $\Delta$ F508 present at T=0 in the control.



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 CFTR $\Delta$ F508 levels in cultured cells (Figure 3.8B). The combined overexpression of RMA1 and UbcH5a did not result in a synergistic reduction in the steady state levels of CFTR $\Delta$ F508 (Figures 3.8B and 3.9B). 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 3.10A). In contrast, the co-expression of RMA1 and Ubc6e synergistically reduced the quantity of <sup>35</sup>S-labeled CFTR $\Delta$ F508 that accumulated during a 20 min. labeling period (Figure 3.10B). 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 CFTR $\Delta$ F508 for degradation.

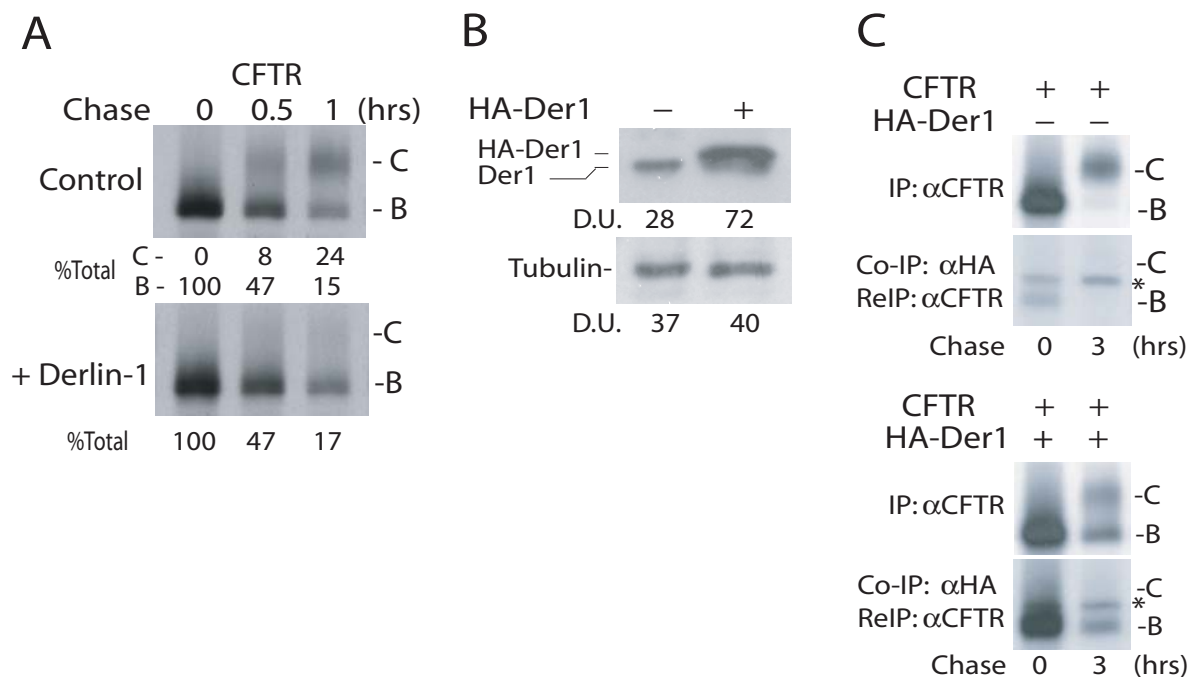
The pulse-chase analysis of CFTR and CFTR $\Delta$ F508 biogenesis (Figures 3.3 and 3.10A) shows that significantly less of the B-form of these proteins accumulates when RMA1 activity is increased. Therefore, it is possible that RMA1 and Ubc6e recognize folding defects in CFTR $\Delta$ F508 during or soon after translation. Alternatively, the co-expression of RMA1 and Ubc6e with CFTR $\Delta$ F508 might simply reduce its expression level. To address these questions, we examined the effect of RMA1 and Ubc6e on CFTR $\Delta$ F508 biosynthesis in the absence and presence of the proteasome inhibitor ALLN (Figure 3.10B). <sup>35</sup>S-CFTR $\Delta$ F508 accumulated with time over the course of a 20 min. labeling period and ALLN was observed to increase the accumulation of CFTR $\Delta$ F508 around 2-fold at every time point. Yet, in cells where RMA1 or

RMA1 and Ubc6e were overexpressed, the accumulation of CFTR $\Delta$ F508 was reduced at all time points. However, the co-expression of RMA1 and Ubc6e did not hinder synthesis of CFTR $\Delta$ F508, because treatment of cells with ALLN, restored the labeling of CFTR $\Delta$ F508 to a level that was greater than that observed in the control. Thus, a large portion of CFTR $\Delta$ F508 appears to be degraded co-translationally and the RMA1/Ubc6e E3 complex appears capable of mediating this process. Our interpretations of the data presented in Figures 3.8, 3.9 and 3.10 are consistent with the finding that CFTR can be ubiquitinated co-translationally (Sato et al., 1998). Yet, whether RMA1 and Ubc6e truly act co-translationally and ubiquitinate CFTR and CFTR $\Delta$ F508 while it is attached to the ribosome requires further study.

#### **Derlin-1 interacts with RMA1 and Ubc6e to facilitate CFTR degradation --**

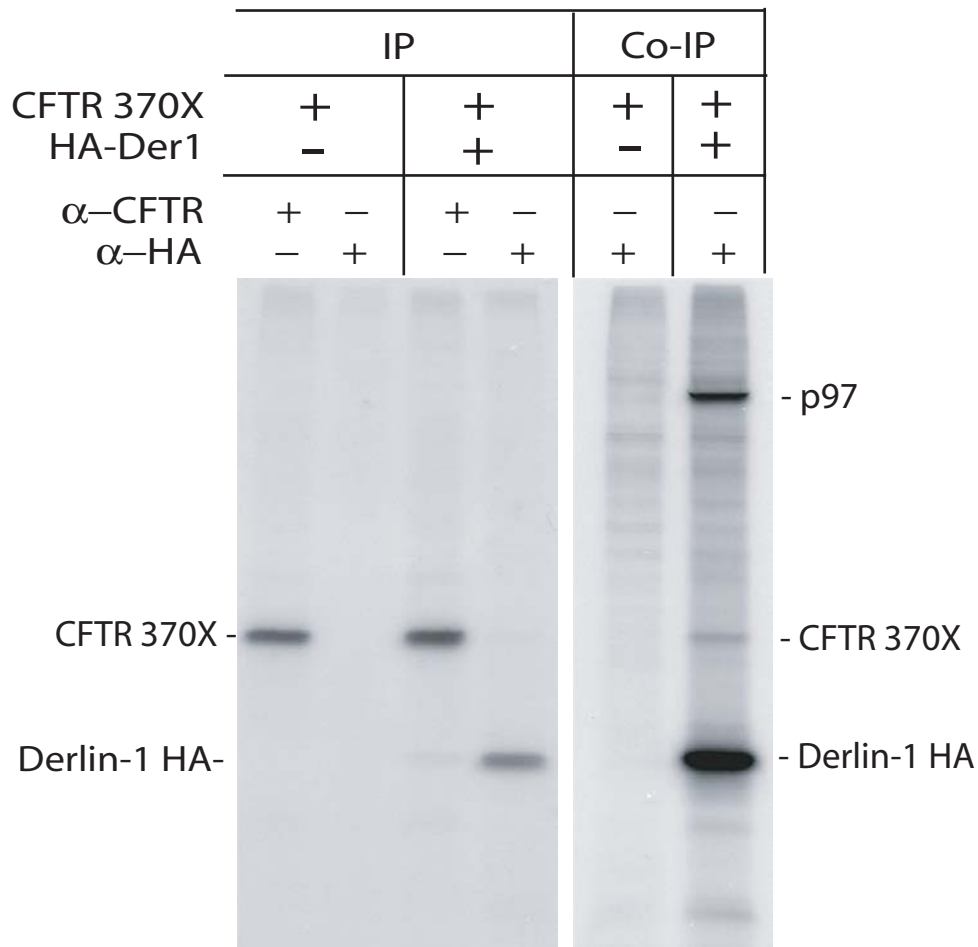
RING domain E3 ubiquitin ligases such as RMA1 are typically members of multi-subunit complexes in which they serve as scaffolds that link the ubiquitin conjugating activity of an E2 to the substrate binding activity of a specific effector molecule (Hershko and Ciechanover, 1998). Thus, it is plausible that RMA1 promotes CFTR degradation by functioning in a complex that contains a membrane inserted substrate selector that senses the folded state of CFTR (Figures 3.11, 3.12 and 3.13). 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 approximately 3-fold lead to the retention of the B-form of CFTR in the ER (Figure 3.11A and B). HA-Derlin-1 formed co-immunoprecipitable complexes

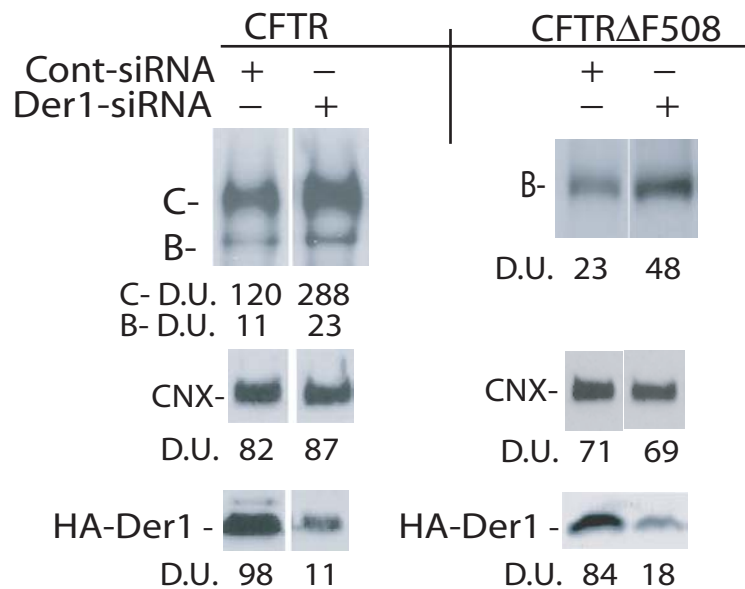


**Figure 3.11 Derlin-1 specifically interacts with B-form CFTR to reduce its level.**

(A) Pulse-chase analysis of CFTR biogenesis in the presence or absence of transiently expressed Derlin-1 (pcDNA3.1-HA-Derlin-1, 0.05 $\mu$ g). Cells were labeled for 20 min with 35S-translabel and then lysed under non-native conditions. Protein concentration in cell extracts was determined. CFTR was immunoprecipitated from 30 $\mu$ g of total extract protein. (B) Western blot to show expression level of endogenous Derlin-1 relative to overexpressed HA-Derlin-1. (C) B-form CFTR co-immunoprecipitates with HA-Derlin-1. CFTR was expressed alone or with HA-Derlin-1 (pcDNA3.1-HA-Derlin-1, 0.025 $\mu$ g) and extracts of 35S-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  $\alpha$ -CFTR. HA-Derlin-1 was immunoprecipitated from the other portion under native buffer conditions and the quantity of CFTR that co-precipitated was determined by reimmunoprecipitating CFTR from HA-Derlin-1 immunoprecipitates. A band marked with \* denotes a background band that precipitates with protein-G beads. Direct immunoprecipitations of CFTR were carried out with 30 $\mu$ g of cell extract protein as the starting material and HA-Derlin-1 co-immunoprecipitations were conducted with 120 $\mu$ g 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 non-specifically precipitate with  $\alpha$ -HA represents less than 1% of the total immunoprecipitable CFTR and the quantity of the B-form of CFTR that co-precipitated with Derlin-1-HA at T=0 and T=3 of the chase period represents around 12% of total immunoprecipitable CFTR at each respective time point.



**Figure 3.12 CFTR370X can be co-immunoprecipitated 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 non-native conditions. In panels labeled Co-IP; HA-Derlin-1 was precipitated with  $\alpha$ -HA antibody. p97 and CFTR370X were present in the co-precipitates and were identified by reimmunoprecipitation (data not shown). Direct immunoprecipitations were conducted from 30 $\mu$ g of cell extract and co-immunoprecipitations utilized 20 $\mu$ g of extract protein as starting material. Panels were exposed to X-Ray film for the same time period and CFTR370X that co-precipitated with HA-Derlin-1 was calculated to be equivalent to 8% of total CFTR370X.



**Figure 3.13 Decreased RMA1 level results in elevated CFTR and CFTR $\Delta$ F508 levels.**

Treatment of cells for 24 hrs with siRNAi (50nM) against Derlin-1 leads to increased expression of CFTR and CFTR $\Delta$ F508. 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. Quantification of bands on gels is expressed in raw densitometer units.

with the newly synthesized B-form of CFTR (Figure 3.11C). 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 around 50% (Figure 3.11C). 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 co-immunoprecipitable complexes with CFTR370X, which represents MSDI of CFTR (Figure 3.12). 8 percent of the total immunoprecipitable CFTR $\Delta$ F508 and 12 percent of the total immunoprecipitable CFTR370X present in cells was co-precipitated with HA-Derlin-1 (see the legend to Figures 3.11 and 3.12). The level of complex formation detected between Derlin-1 and either CFTR or CFTR370X is similar to the quantity of CFTR that co-precipitated 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 under estimation 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.

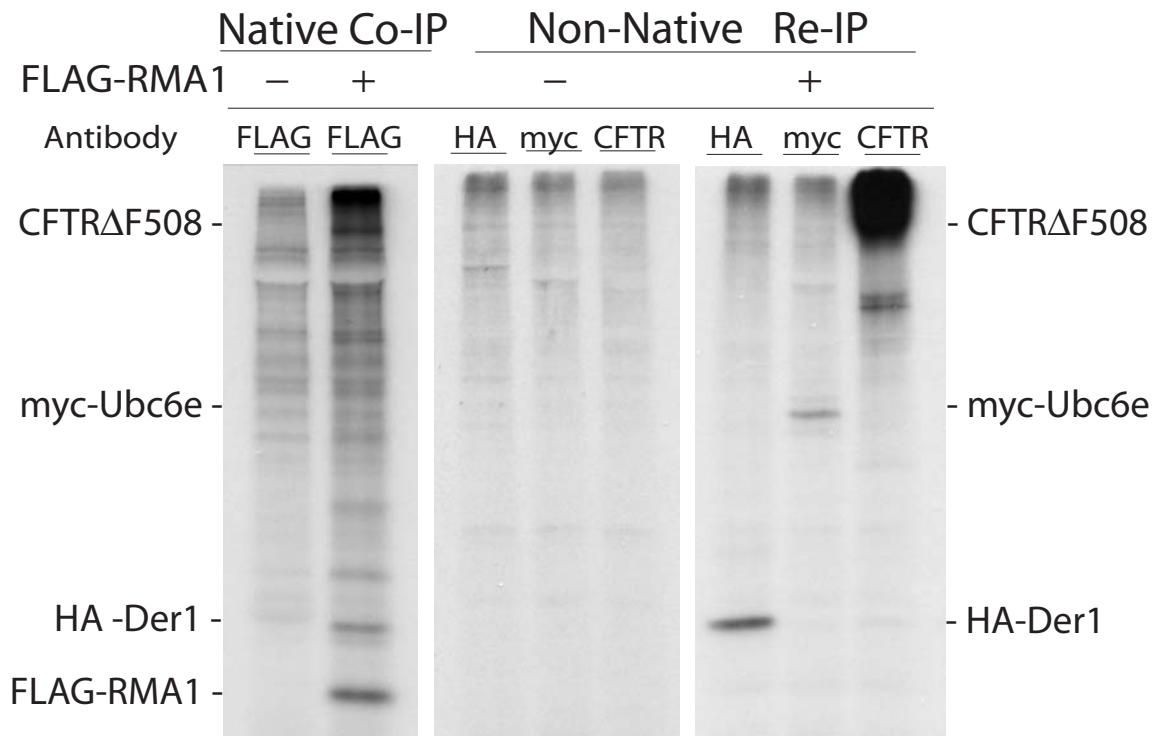
The retrotranslocation factor p97 is known to be present in complexes that contain Derlin-1 and E3 ubiquitin ligases (Ye et al., 2005; Lilley and Ploegh, 2005) and we observed it to co-precipitate with Derlin-1 and CFTR370X (Figure 3.12). The presence of p97 in a complex with Derlin-1 and a CFTR biogenic 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 non-native 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 around 2-fold more of the folded C-form of CFTR and the non-native B-form of CFTR $\Delta$ F508 to accumulate (Figure

3.11C). These collective data demonstrate that the positive and negative modulation of Derlin-1 activity reciprocally modulates the partitioning of 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.

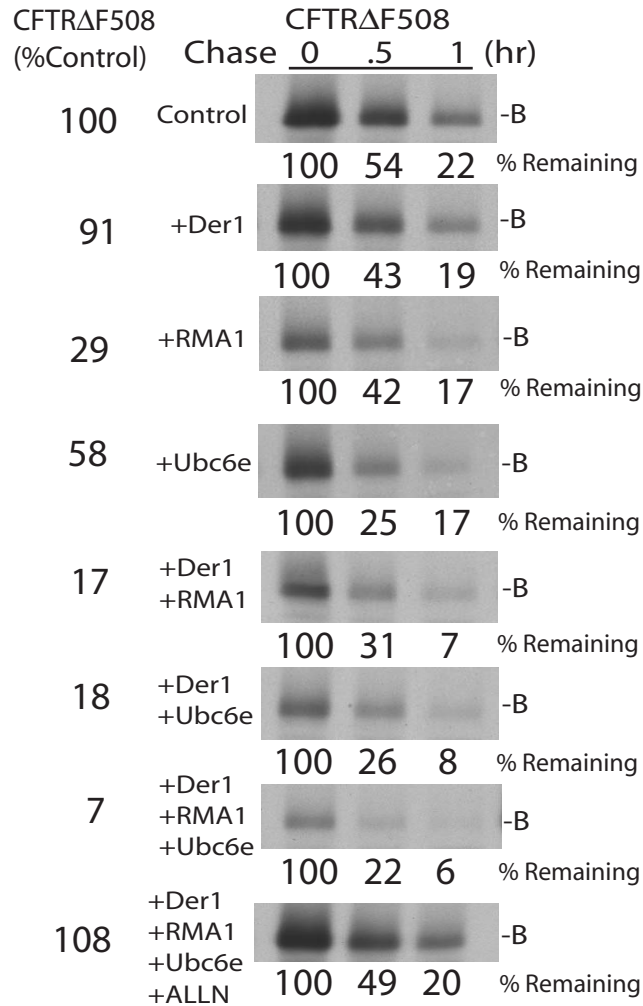
To determine whether Derlin-1 cooperates with RMA1 and Ubc6e to mediate QC of CFTR, the ability of these different ER membrane proteins to physically and functionally interact was examined (Figures 3.14, 3.15, 3.16 and 3.17). Extracts of cells were prepared from <sup>35</sup>S-labeled cells that co-expressed FLAG-RMA1 with myc-Ubc6e, HA-Derlin-1 and CFTR $\Delta$ F508 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 CFTR $\Delta$ F508 were identified as interaction partners of RMA1 via their re-immunoprecipitation from the RMA1 co-precipitate (Figure 3.14). 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 co-precipitation reactions (data not shown). Ubc6e and Derlin-1 also appeared to functionally interact to promote degradation of CFTR $\Delta$ F508 because their combined expression reduced the accumulation of <sup>35</sup>S-CFTR $\Delta$ F508 to levels that were around 90% lower than the control and increased CFTR $\Delta$ F508 turnover 2-fold (Figure 3.15). 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 CFTR $\Delta$ F508 synthesis that is resultant from its co-expression with multiple proteins. However, the following

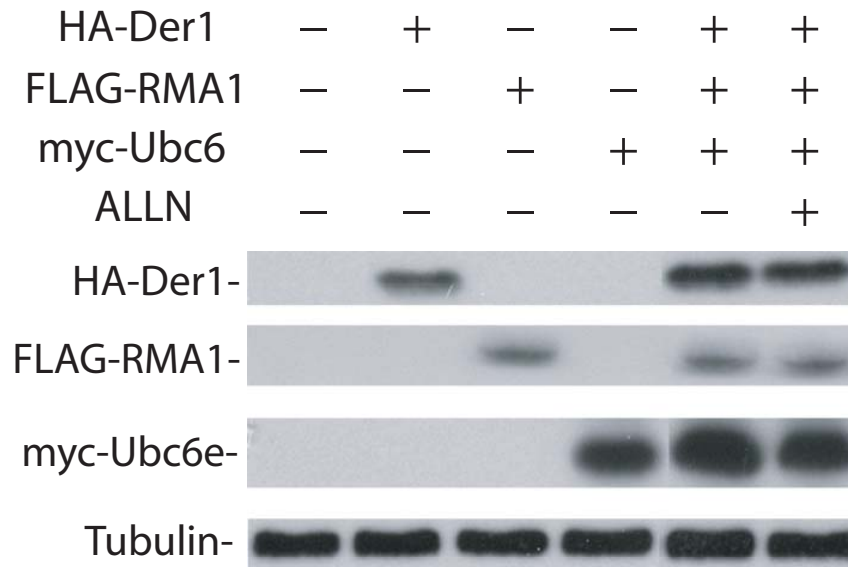


**Figure 3.14 Nascent CFTR F508, HA-Derlin-1, and myc-Ubc6e are present in a co-precipitable complex with FLAG-RMA1.** Cells were labeled with  $^{35}\text{S}$ -translabel for 1 hr and solubilized in a buffer containing 0.1% Triton X-100. Co-immunoprecipitations and re-immunoprecipitations were then carried out as described in the material and methods 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 re-immunoprecipitated from native precipitates under denaturing conditions.

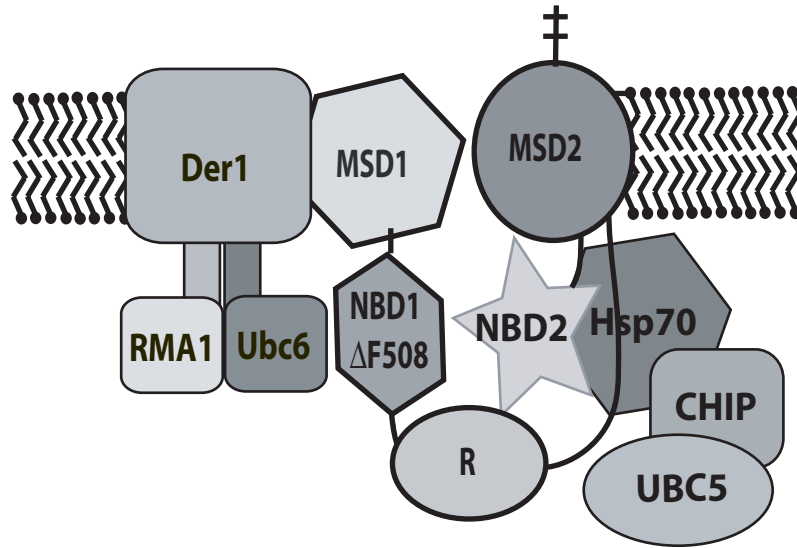




**Figure 3.15 Co-expression of RMA1 and Ubc6e in addition to Derlin-1 potentiates the degradation of 35S-CFTR $\Delta$ F508.** Total CFTR $\Delta$ F508 present at the end of the 20 min labeling period was quantified and is expressed as a % of the total signal in the control. Changes in the level of CFTR $\Delta$ F508 during the course of the indicated chase reaction were quantified and expressed as a % of the total signal at T=0.



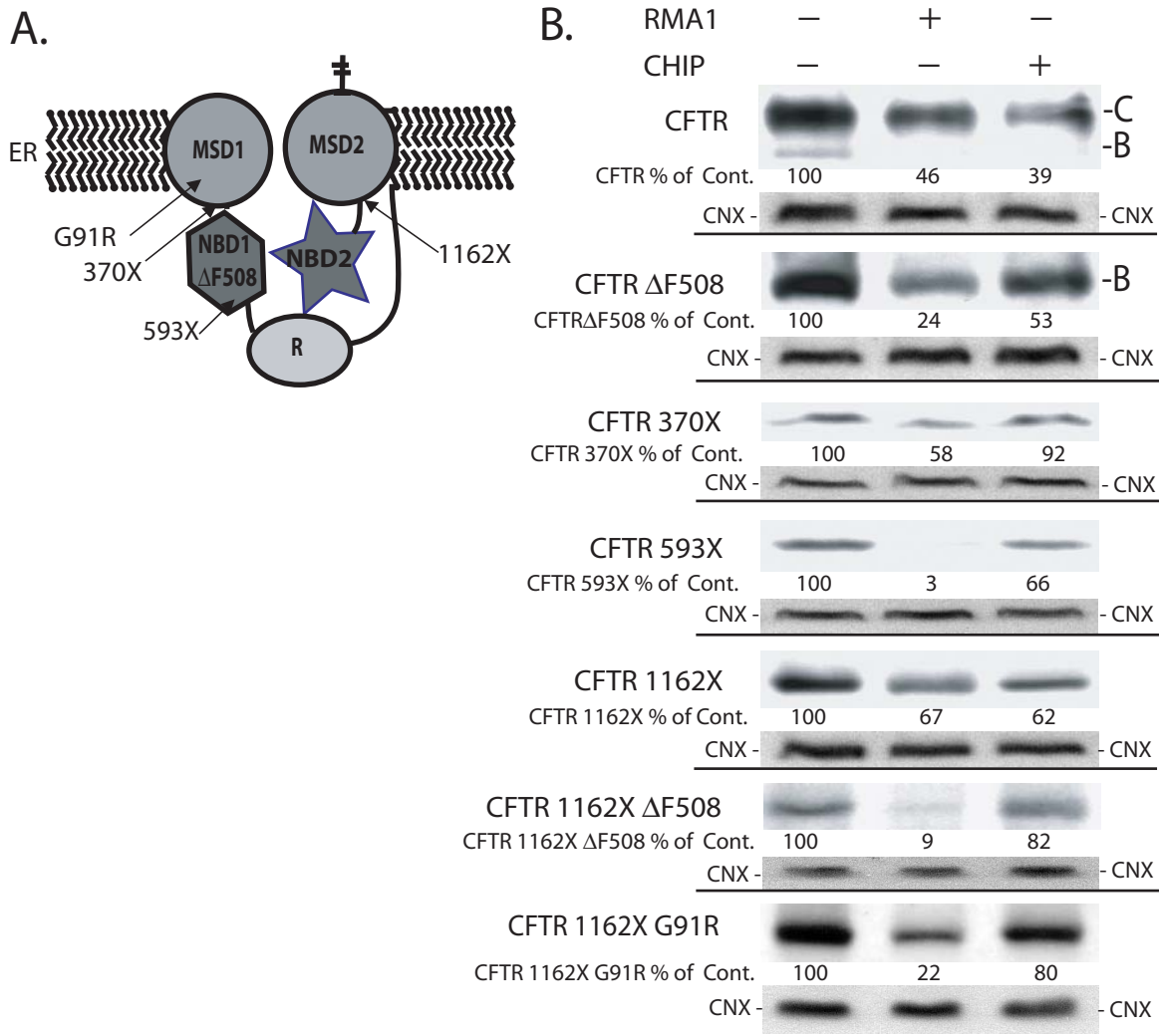
**Figure 3.16 Co-expression of components does not complicate expression profiles of individual components.** Western blot analysis of transiently expressed HA-Derlin-1, FLAG-RMA1 and myc-Ubc6e levels when these proteins are expressed individually and in concert with each other.



**Figure 3.17 A schematic diagram representing the E3 ubiquitin ligase complexes that function to select CFTR and CFTR $\Delta$ F508 for proteasomal degradation.** Provided is a model representing the hypothesized orientation of two quality control complexes interacting simultaneously with CFTR $\Delta$ F508.

observations indicate that elevation of the activity of the RMA1 E3 complex is the predominant cause for the reduced accumulation of CFTR $\Delta$ F508 when RMA1, Ubc6e and Derlin-1 are jointly expressed. First, when RMA1, Derlin-1 and Ubc6e were co-expressed, the cell is fully capable of synthesizing CFTR $\Delta$ F508 because it accumulated above control levels when the proteasome inhibitor ALLN was present during cell labeling (Figure 3.15). Second, the expression levels of individual forms of these proteins are not reduced by their joint co-expression (Figures 3.9A-B and 3.16). 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 CFTR $\Delta$ F508 for proteasomal degradation (Figure 3.17).

**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 sub-domains of CFTR. This question was investigated by determining whether there were differences in the stage of biogenesis at which different length CFTR and CFTR $\Delta$ F508 fragments that resemble translation intermediates became sensitive to RMA1 or CHIP (Figure 3.18A). Cells were transfected with quantities of CHIP and RMA1 expression plasmids that lead to a partial reduction in the steady-state level of CFTR and CFTR $\Delta$ F508 (Figure 3.18B). 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 around 30%. CFTR1162X is truncated after MSDII and appears to fold to a native-like conformation that is a poor substrate for chaperones (Meacham



**Figure 3.18 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. Quantification of CFTR fragment levels are expressed as % of control.

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 CFTR1162X $\Delta$ F508 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 sub-domains of CFTR.

### **3.4 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 CFTRs cytosolic domains, and CFTR biogenic intermediates are most sensitive to CHIP action after NBDII synthesis. Hence, the CHIP E3 appears to play a post-translational role in QC of CFTR.

Folding of CFTR and CFTR $\Delta$ F508 appear 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 CFTR $\Delta$ F508 than nascent CFTR to arrest in a conformation that is recognized by the RMA1 checkpoint. Yet, the folding defect in CFTR $\Delta$ F508 that is sensed by components of the RMA1 E3 complex is not insurmountable and forms of CFTR $\Delta$ F508 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 CFTR $\Delta$ F508 to pass through sequential QC checkpoints that are staffed by ER membrane inserted and cytosolic E3 ubiquitin ligases.

The exact nature of the non-native form(s) of CFTR and CFTR $\Delta$ F508 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 CFTR $\Delta$ F508<sup>1162X</sup> 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 CFTR $\Delta$ F508 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 synthesized and might be more accessible to the CHIP E3. In addition, the post-translational folding of NBDII appears to be the rate limiting step in CFTR folding and this process is defective in CFTR $\Delta$ F508 (Du et al., 2005). Thus, it is logical that Hsc70/CHIP E3 complex would recognize forms of CFTR and CFTR $\Delta$ F508 in which NBDII, and possibly other cytosolic domains, have failed to fold or assemble efficiently.

The ubiquitin ligase 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 non-native CFTR, but is masked in on-pathway biogenic intermediates. Since  $\Delta$ F508NBD1 is prone to misfolding (Thibodeau et al., 2005), is it possible that the binding of Derlin-1 to MSDI is coupled to ubiquitination of regions



in  $\Delta F508$ NBDI 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 CFTR $\Delta F508$  and it can also be co-immunoprecipitated 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 co-factors is an open question. Nonetheless, the ability of Derlin-1 to enter into complexes with non-native CFTR suggests that it 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 post-translational 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-1 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 co-factors are all implicated to function in either substrate ubiquitination or retrotranslocation, their interactions with Derlin-1 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 co-precipitate with Derlin-1, CFTR370X, CFTR and CFTR $\Delta$ F508 (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.

### **3.5 Materials and Methods**

**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).  $\alpha$ -RMA1 is a rabbit polyclonal antibody that was produced against Gst-RMA1 $\Delta$ TM purified from *E.coli*. The specificity of rabbit  $\alpha$ -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 1.0µg of pcDNA3.1(+)-CFTR and 1.5µg of pcDNA3.1(+)-CFTRΔF508. For co-expression experiments, RMA1, Ubc6e and UbcH5a were utilized at 0.25-0.5µg/well of a 6-well plate. pcDNA3.1(+)-HA-Derlin-1 was introduced into cells at 0.01-0.1µg/well. The details of the protocols for <sup>35</sup>S-labeling of cells, immunoprecipitations and Western blots are described elsewhere (Meacham and Cyr, 2002). When CFTR biogenesis was assessed by immunoprecipitation from <sup>35</sup>S-labeled cells, the protein concentration of lysates 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 co-localization 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 co-immunoprecipitation** -- Expression plasmids for CFTRΔF508, FLAG-RMA1, myc-Ubc6e and HA-Derlin-1 were co-transfected into HEK293 cells, which were labeled 18 hrs later with <sup>35</sup>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 500µl of 150mM NaCl, 50mM phosphate, 1mM PMSF (phenylmethanesulfonyl fluoride), 1X 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 co-immunoprecipitation reactions were analyzed by SDS-PAGE and fluorography. Verification of the identity of  $^{35}\text{S}$ -proteins in complexes isolated by co-immunoprecipitation was accomplished by reimmunoprecipitation of the indicated protein from co-immunoprecipitates (Meacham et al., 1999).

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## **Chapter Four**

### **Use of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) as a Model Substrate to Study ER Protein Quality Control in Mammalian Cells**

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#### **4.1 Abstract**

Components of the ubiquitin-proteasome system function on the surface of the endoplasmic reticulum (ER) to select misfolded proteins for degradation. Herein we describe methods that allow for the study of the pathway for proteasomal degradation of the cystic fibrosis transmembrane conductance regulator (CFTR). The experimental system described employs transiently transfected HEK-293 cells and is utilized to monitor the biogenesis of CFTR by western blot and pulse-chase analysis.

## 4.2 Introduction

Endoplasmic reticulum associated degradation (ERAD) is a process that involves the recognition and degradation of misfolded luminal and transmembrane proteins via the ubiquitin-proteasome system (McCracken and Brodsky, 2003). Model substrates that are utilized to study basic principles of the ERAD process include the T-cell receptor subunit alpha (TCR $\alpha$ ) (Lenk, 2002), 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, HMGR) (Hampton and Rine, 1994; Hampton and Bhakta 1997), a mutant form of carboxypeptidase Y (Hiller et al., 1996; Plemper et al, 1999) and CFTR (Cheng et al., 1990).

Misfolded forms of CFTR and  $\Delta$ F508 CFTR appear to be recognized by the CHIP/Hsc70 E3 ubiquitin ligase complex (Meacham et al., 1999; Meacham et al., 2001) and then degraded via a pathway that is blocked by proteasome inhibitors (Ward et al., 1995; Jensen et al., 1995). CFTR is a 1480 residue glycomembrane protein that contains two membrane spanning domains and cytoplasmic sub-domains that include nucleotide binding domain 1, nucleotide binding 2 and a regulatory domain. Folding of CFTR is slow and 60-70% of it is selected for ERAD prior to reaching the native state and escaping the ER.  $\Delta$ F508 CFTR contains a deletion of F508 in nucleotide binding domain 1 that causes 100% of it to be degraded (Ward and Kopito, 1994). Since CFTR contains transmembrane and cytoplasmic subdomains, the study of its ERAD has the potential to uncover how elements of the cytosolic protein quality control system cooperate with ER localized proteins to degrade polytopic proteins.

Since  $\Delta$ F508 CFTR misfolds and is completely degraded via ERAD it serves as an excellent model substrate for basic studies. However,  $\Delta$ F508 CFTR expression is very low in cultured epithelial cells. Thus, the study of its degradation requires the use of cell lines which stably express  $\Delta$ F508 CFTR from a strong promoter (Lukacs et al, 1994) or transient cell expression

systems that utilize Cos-7 or HEK-293 cell lines Meacham et al., 2001; Ward and Kopito, 1994).

Herein we describe an experimental system to study ERAD of  $\Delta F508$  CFTR in a cell expression system that employs HEK-293 cells that are transfected with pcDNA3.1- $\Delta F508$  CFTR. Methods to determine the steady state level and detergent solubility of  $\Delta F508$  CFTR by Western blot are described. In addition, we include a protocol to study the rate of  $\Delta F508$  CFTR degradation via pulse-chase analysis.

### **4.3 Materials**

#### **Cell Culture Materials.**

1. HEK-293 Cells are grown in Dulbecco's Modified Eagle Medium (Gibco, cat. no. 11995-065) supplemented with 10% Fetal Bovine Serum (Sigma, cat. no. F-2442) and 1% Penicillin-Streptomycin (Gibco, cat. no. 15140-122) (DMEM), at 37°C and 5% CO<sub>2</sub> to 85-90% confluency in 100-mm culture dishes. Each well contains approximately  $7.5 \times 10^6$  cells.
2. Citric Saline: 135mM KCl, 150mM Na-citrate, pH 7.4. Filter-sterilize and store at 4°C.
3. Minimum Essential Medium without L-methionine (Sigma, cat. no. M3911) (MEM-Met): Pre-warm media to 37°C prior to use. Cells are incubated in this media to deplete intracellular methionine prior to incubation with Trans <sup>35</sup>S-Label.
4. Trans <sup>35</sup>S-Label (1200Ci/mmol; ICN Biomedicals, cat. no. 51006). Trans <sup>35</sup>S-Label is a mixture of <sup>35</sup>S-methionine and <sup>35</sup>S-cysteine utilized for radiolabeling of cellular proteins. Supplement MEM-Met with Trans <sup>35</sup>S-Label to a final concentration of 200 $\mu$ Ci/ml.
5. Cyclohexamide: Make a 25mg/ml stock in 100% ethanol and store at 4°C.

6. Phosphate Buffered Saline pH 7.4 (PBS): 135mM NaCl, 2.7mM KCl, 10mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8mM KH<sub>2</sub>PO<sub>4</sub>.
7. ALLN (N-Acetyl-Leu-Leu-NorLeu-AL; Calbiochem cat. no. 208719), a proteasome inhibitor that is utilized to block degradation of CFTR. Prepare a 100mM stock of ALLN in ethanol and add it to cell culture media at a final concentration of 200μM.
8. Effectene Plasmid DNA Cell Transfection Reagent (Qiagen, cat. no. 301427)
9. Highly pure plasmid DNA: Purified DNA should be free of endotoxins and contain low levels of genomic DNA and RNA.

### **Reagents for Sample Preparation**

1. 10% BSA (Bovine Serum Album) in PBS.
2. PMSF (Phenylmethylsulfonyl Fluoride): Make a fresh 100mM stock of PMSF in 100% molecular grade ethanol. Maintain at room temperature.
3. Protease Inhibitor Cocktail (PI): Complete™ Protease inhibitor cocktail (Roche, cat. no. 1697498).
4. Pansorbin cells (Calbiochem, cat. no. 507861): Cells used to preclear cell extracts of radiolabeled proteins that precipitate non-specifically in immunoprecipitations. Wash Pansorbin cells 3 times with PBS and resuspend the cells in PBS as a 50% slurry. Store at 4°C.
5. CFTR antibody: clone MM13-4 (Upstate Biotechnology, cat. no. 05-581).
6. Protein G-Agarose (P.G. beads): (Roche, cat. no. 1 243 233) Resuspend P.G. beads in PBS supplemented with 1% BSA. Incubate P.G. beads at 4°C on a rotator for 24 hours to block nonspecific binding sites on the beads. Pellet the beads with a microcentrifuge and resuspend them as 50% volume/volume slurry in PBS supplemented with 0.2%

BSA. Store at 4°C.

7. Cell Lysis buffers:

- a. Radio-immunoprecipitation analysis (RIPA) buffer: 150mM NaCl, 1% NP-40 (IGEPAL™), 0.5% Deoxycholic acid, 0.2% SDS, 50mM Hepes pH 7.4. RIPA can be stored at room temperature, but must be ice cold when used to lyse cells. RIPA should be supplemented with 1mM PMSF, PI and with 0.2% BSA just prior to use in cell lysis.
- b. Detergent Soluble Fraction (DSF) buffer: 10mM Tris-HCl, 1% Triton-X100, 5mM EDTA, pH 7.5. Store stock solution at room temperature, but use ice cold. Supplement with 1mM PMSF, and PI, just prior to use.
- c. Detergent Insoluble Fraction (DIF) buffer: 10mM Tris-HCl, 1% SDS, pH 7.5. Store stock solution at room temperature. Use DIF buffer at room temperature to prevent precipitation of the SDS. Supplement with 1mM PMSF, and PI just prior to use.

**SDS-PAGE gel reagents.**

1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 4X Sample Buffer: 250mM Tris-HCl, pH 6.8, 8% SDS, 8mM [Ethylenedinitrilo]-tetraacetic acid disodium salt (EDTA) pH 8.0, 40% Glycerol, 40µg/ml Coomassie Blue, and distilled water. This solution can be stored at room temperature for several months. Prior to use, add 80µl of 98% β-mercaptoethanol, 1mM PMSF, and PI to 1ml of SDS-PAGE 4X Sample Buffer. Mix well before use, and discard any material not used within several hours.
2. SDS-PAGE Electrode Buffer pH 8.3: 10mM Tris-HCl, 75mM Glycine and 0.1% SDS.

3. 4X SDS-PAGE Resolving Gel Buffer, pH 8.8: 1.5M Tris-HCl, 8mM EDTA, and 0.4% SDS. Adjust to pH 8.8. Store at room temperature.
4. 4X SDS-PAGE Stacking Gel Buffer, pH 6.8: 0.5M Tris-HCl, 8mM EDTA, and 0.4% SDS. Adjust to pH 6.8. Store at room temperature.
5. Acrylamide-Bis Solution: 30% Acrylamide, 0.8% N,N methylene bisacrylamide. Store in a dark or aluminum-foil covered glass bottle at 4°C.
6. 10% Ammonium persulfate (10% APS).
7. TEMED (N,N,N',N'-Tetramethylethylenediamine) (Fisher Scientific, cat. no. BP150-100)
8. 7% SDS-Page Gels are made by combining gel components with the following volumes in this order: 2.35ml Acrylamide-Bis Solution, 5.15ml water, 2.5ml 4X SDS-PAGE Resolving Gel Buffer pH 8.8, 100µl 10% APS, 7.5µl TEMED. This 7% Gel mixture is cooled on ice prior to adding the TEMED, then mixed well without forming bubbles, and poured between two glass plates of a mini gel apparatus (BioRad). Gently add a layer of water to the top surface of the gel to obtain a flat smooth surface. Complete polymerization should occur within 20 minutes. When the Resolving Gel is polymerized, pour off the top water layer and add the Stacking Gel mixture. The Stacking Gel mixture is composed of the following: 0.6ml Acrylamide-Bis Solution, 2.35ml water, 1ml 4X SDS-PAGE Stacking Gel Buffer, pH 6.8, 75µl 10% APS, 5µl TEMED. This Stacking Gel mixture should be cooled on ice prior to adding TEMED, mixed well, then quickly added to the top of the 7% Resolving Gel. Well combs should be inserted immediately and Stacking Gel polymerization should occur within 10 minutes.

9. SDS-PAGE Gel Stain (Stain): 25% Methanol, 10% Glacial Acetic Acid, 2.5g/l Coomassie Blue. Use distilled H<sub>2</sub>O to prepare this solution and thereby minimize the formation of precipitates.
10. SDS-PAGE Gel Destain: 10% Methanol, 10% Acetic Acid, distilled water.
11. Photo Enhancer: 0.5M Sodium Salicylate, pH 7.4.

#### **Western Blot Reagents.**

1. Western Blot Transfer Buffer: 20% Methanol, 0.02% SDS, 20mM Tris-base, 150mM Glycine-HCl. Store at room temperature.
2. Ponceau S Protein Stain (Ponceau S): Dissolve 1g Ponceau S. in 2ml Glacial Acetic Acid and 198ml water. Store at room temperature.
3. PBSTr-X100: PBS supplemented with 0.1% Triton-X100
4. Western Blot Blocking Solution: PBSTr-X100, 4% non-fat dry milk, 0.8% BSA.
5. Antibody Solution: Antibody diluted into Western Blot Blocking Solution.
6. Nitropure, cast, pure, 0.45 micron Nitrocellulose membrane. (Osmonics)
7. Whatman type filter paper cut to the approximate size of the transfer apparatus spacer sponges.

#### **4.4 Methods**

##### **Preparation of HEK- 293 cells for transfection.**

1. Grow HEK-293 cells to 90% confluency and then detach them by rinsing with 3ml and incubating for 5 minutes with 1 ml room temperature Citric Saline.
2. After cells have detached, dilute the Citric Saline with 9ml of DMEM. Pipette several times to break-up large clumps of cells.
3. A near confluent 100-mm culture dish will provide sufficient cells for 10-12, 35-mm



wells or culture dishes. To ensure even distribution of cells in the wells, dilute the cells suspended in DMEM so that 3ml can be added to each well. Allow these cells to adhere 12-14 hours, and replace the DMEM with 2ml of fresh DMEM.

4. Allow the cells to grow to 70% confluency ( $\sim 1 \times 10^6$  cells per well). Replace DMEM with 1ml of fresh DMEM about two hours prior to transfection.

#### **Transfection of HEK-293 cells.**

1. We have found that Qiagen's Effectene transfection reagent yields a transfection efficiency of  $\geq 70\%$ . Transfection efficiency is best if HEK-293 cells are maintained by splitting cells every 3 days.
2. The mammalian expression plasmid pcDNA 3.1-CFTR or  $\Delta F508$ CFTR is introduced into cells with the Effectene Transfection Reagent according to the manufactures instructions. Briefly, for each 35-mm well of cells add 0-3.0 $\mu$ g of pcDNA 3.1-CFTR to 200 $\mu$ l EC Buffer and mix well; add 4 $\mu$ l of Enhancer reagent/ $\mu$ g of pcDNA3.1 DNA. Mix the cocktail and incubate for 10 minutes at room temperature. Add 5 $\mu$ l of Effectene/ $\mu$ g pcDNA 3.1-CFTR, mix and incubate for 10 minutes. Dilute the transfection mixture with 800 $\mu$ l of DMEM.
3. Aspirate the DMEM from the HEK-293 cells and gently add the transfection cocktail. Incubate the cells for 3-4 hours at 37°C and 5% CO<sub>2</sub>. Replace the transfection mixture with fresh DMEM. Incubate for about 20 hours.
4. CFTR and  $\Delta F508$ -CFTR expression levels in HEK-293 cells are a function of post transfection incubation time and the amount of DNA used to transfect the cells. We typically harvest the cells 20 hours after the transfection and utilize 1 $\mu$ g of pcDNA 3.1-CFTR or  $\Delta F508$  CFTR.

5. Optimal experimental conditions for CFTR and  $\Delta$ F508 CFTR expression are those that allow the cell to efficiently insert nascent protein into the ER membrane and generate the immaturely glycosylated species that migrates on SDS-PAGE gels with an apparent molecular weight of 140kDa termed the B-form. Upon folding and escaping the ER, CFTR, if modified further and converted to a maturely glycosylated species that migrates with an apparent molecular weight of 160kDa, is termed the C-form. Care must be taken to assure that expression levels of CFTR and  $\Delta$ F508 CFTR do not exceed the cells capacity to insert it into the ER.
6. The quality of the expression plasmid utilized in transfections is a source of variation in the level of CFTR and  $\Delta$ F508 CFTR. Therefore, it is wise to determine the level of CFTR and  $\Delta$ F508 CFTR expression, derived from new preparations of DNA and adjust quantities utilized in transfections accordingly.
7. The age of the cultured HEK-293 cells utilized in transfections has an influence on the efficiency of CFTR and  $\Delta$ F508 CFTR expression. HEK-293 cells with a passage number in excess of 50 passes yield low levels of CFTR and  $\Delta$ F508 CFTR expression.

#### **Harvest of Transfected HEK-293 cells.**

Harvesting HEK-293 cells is convenient because detachment from the growth surface only requires that cells be bathed in citric saline. This permits cell lysis to be conducted in a microfuge tube and minimizes variability in the protein content of extracts made from different cell preparations.

1. To harvest HEK-293 cells remove the DMEM and incubate cells in 1ml ice cold Citric Saline for 5-7 minutes.
2. Detachment the cells by repeatedly pipetting the citric saline over the cells. Once the

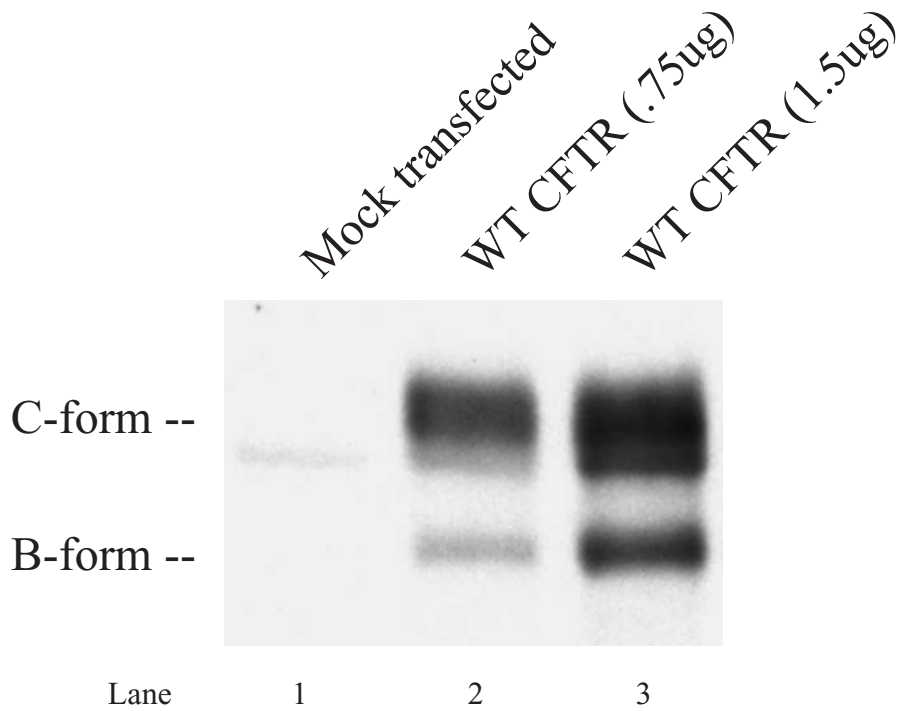
cells are detached, transfer them from each 35-mm culture well to a micro-centrifuge tube.

3. Pellet the cells by centrifugation 800 x g for 3 minutes at 4°C. Cell pellets should be kept on ice prior to lysis, or frozen in liquid nitrogen for storage and later lysis.

#### **Western Blot analysis of CFTR and $\Delta F508$ CFTR expression.**

Steady-state levels of CFTR and  $\Delta F508$  CFTR expression are determined in Western blots of cell extracts produced from transfected HEK-293 cells (Figure 4.1).

1. Lyse cell pellets by the addition of 100 $\mu$ l of 2X SB.
2. Incubate the samples at 37°C for 15 minutes.
3. Centrifuge the samples at 16,000 x g for 20 minutes.
4. To remove the viscous pellet, carefully place the pipette tip near the bottom of the tube and suck a portion of the pellet into the tip. Drag the pellet out of each tube leaving behind the supernatant.
5. The volumes of individual supernatants will vary, so it is important to denote the volume of the supernatant for each sample. Load an equal volume from each supernatant to a previously prepared 7% SDS-PAGE gel. Electrophoreis samples by applying a constant voltage of 100V to gels for 115 min.
6. To prepare for the wet transfer of proteins in the SDS-PAGE gel to nitrocellulose with a BioRad mini-gels apparatus.
  - a. Soak a piece of Nitrocellulose membrane that is cut to the size of the mini-gel in Western Blot Transfer Buffer for 10 minutes.
  - b. Soak the transfer apparatus sponges in Western Blot Transfer Buffer.
  - c. Cut 3M Whatman filter paper to the same size as the apparatus sponges.



**Figure 4.1 Western blot analysis of CFTR expression in transfected HEK-293 cells.** HEK-293 cells were transiently transfected with the indicated amounts of pcDNA3.1 or pcDNA3.1-CFTR. Western blots were then performed as described in the text. The B-form denotes the immaturely glycosylated ER localized form of CFTR. The C-form denotes the maturely glycosylated plasma membrane form of CFTR.

7. Upon completion of the electrophoresis, soak the SDS-page gel in Western Blot Transfer Buffer for 5 minutes. Then assemble the gel into the transfer apparatus with a nitrocellulose membrane.
8. Transfer the proteins in the gel to the nitrocellulose by applying a constant voltage of 80V for three hours to the apparatus at 4°C.
9. Once the transfer is complete, remove the nitrocellulose membrane from the transfer apparatus and briefly rinse it with distilled water. Stain the proteins on the membrane with Ponceau S for 5 minutes and destain with water. This step is important for comparison of the protein loads in each lane.
10. Block sites on the nitrocellulose membrane that bind antibodies non-specifically via incubation of the membrane on a shaker with Western Blot Blocking Solution at room temperature for 3 hours, or over night at 4°C.
11. Incubate the membrane with  $\alpha$ CFTR MM13-4 primary antibody at a 1:500 dilution in Western Blot Blocking Solution.
12. The Nitrocellulose membrane and the primary antibody solution should be placed in a heat sealed plastic bag, and rocked for 2-3 hours at room temperature or overnight at 4°C.
13. Wash the Nitrocellulose with PBSTr 3 x 10 minutes.
14. Block the Nitrocellulose again with Western Blot Blocking Solution for 15 minutes. Incubate the membrane with goat anti mouse sera conjugated to Horse Radish Peroxidase (Biorad) that is diluted 1:3000 with Western Blot Blocking Solution for 40-60 minutes at room temperature.
15. Wash the nitrocellulose membrane 5 x 5 minutes with PBSTr on a shaker.

16. Incubate the nitrocellulose membrane with Enhanced Chemiluminescent Reagent from Pierce. Wrap the membrane in plastic wrap, and utilize it to expose X-ray film for 30 seconds to 2 minutes. Develop the film and visualize the position of CFTR and  $\Delta F508$  CFTR.

### **Analysis of the solubility of CFTR and $\Delta F508$ CFTR**

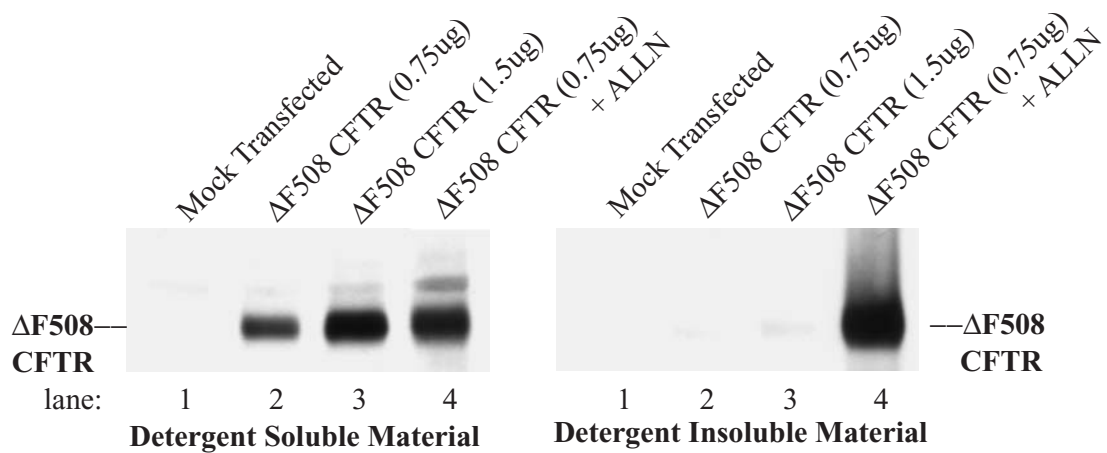
Misfolded CFTR and  $\Delta F508$  CFTR whose degradation is blocked often accumulates in cells as an insoluble aggregate (Ward and Kopito, 1995). The formation of insoluble CFTR and  $\Delta F508$  CFTR aggregates can be detected via the protocol listed below (Figure 4.2).

### **Preparation of Detergent Soluble and Detergent Insoluble Fractions of Cell Extracts.**

1. HEK-293 cell pellets are lysed via incubation in 150 $\mu$ l of DSF buffer on a rotator for 1 hour at 4 °C.
2. Spin the samples in a centrifuge at 16,000 x g for 15 minutes at 4°C.
3. Transfer 40 $\mu$ l of supernatant, which represents the 1% Triton-X100 detergent soluble fraction (DSF), to a pre-cooled microfuge tube. Then, carefully remove the remaining supernatant. Rinse the pellet with 50 $\mu$ l DSF buffer and spin the sample in a centrifuge at 13,000 x g for 5 minutes at 4°C. This pellet represents the Triton-X100 insoluble fraction (DIF).

### **Solubilization of the Detergent Insoluble Material.**

1. To solubilize the DIF pellet add 30 $\mu$ l of room temp DIF buffer and incubate for 15 minutes. Light agitation helps expose the pellet(s) to the buffer.
2. Add 120 $\mu$ l of DSF buffer, do not mix the sample, and return the tubes to ice for 2 minutes.
3. Sonicate the samples (intensity 6) for 7 sec x 2, allowing samples to cool between



**Figure 4.2 Western blot analysis of the solubility of  $\Delta F508$  CFTR.** HEK-293 cells were transfected with pcDNA3.1 or pcDNA3.1- $\Delta F508$  CFTR. Where indicated, 200 $\mu$ M ALLN was added to cell growth media 12 hours prior to harvest. Cells were lysed and prepared for Western blot analysis as described above. The band labeled  $\Delta F508$  CFTR corresponds to the ER localized B-form of  $\Delta F508$  CFTR.

assaults. Importantly, samples must be maintained on ice while sonicating to prevent samples from overheating.

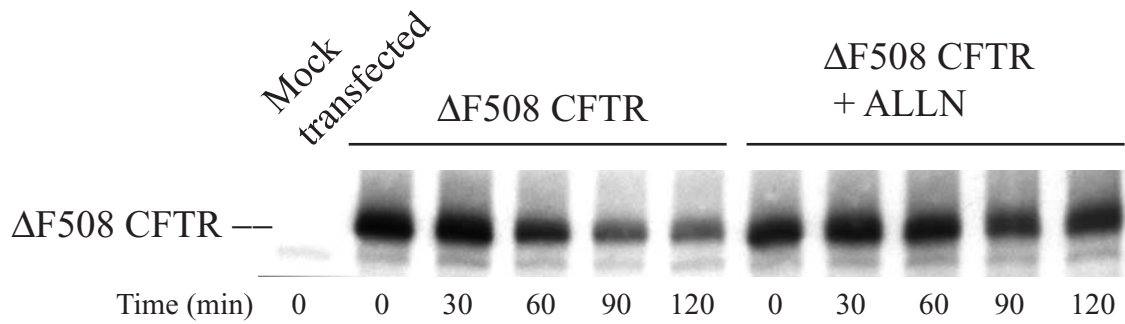
4. Pellet any remaining insoluble material by centrifuging at 16,000 x g at 4°C for 5 minutes.
5. Transfer 40µl of supernatant to a new pre-cooled tube and store on ice.
6. Add 15µl of SDS-PAGE 4X Sample Buffer to each tube containing 40µl of either DSF or DIF. Mix gently, and incubate at 37°C for 15 minutes.
7. Load samples to a SDS-PAGE gel and follow steps 5-16 in section 3.4. above for Western blot analysis.

#### **Kinetic Analysis of ΔF508-CFTR degradation in HEK-293 cells.**

To study the rate of ΔF508 CFTR degradation, cellular proteins are labeled with <sup>35</sup>S and the kinetics of the destruction of <sup>35</sup>S -ΔF508 CFTR are determined (Figure 4.3).

1. Transfect HEK-293 cells with pcDNA3.1-ΔF508 CFTR as described in section 3.1 and 3.2.
2. After a 24 hour post transfection period remove the media and wash the cells with 1 ml of pre-warmed PBS.
3. Replace the PBS with 2ml MEM minus methionine and methionine-starve the cells for 30-40 minutes in a 5% CO<sub>2</sub> atmosphere at 37°C.
4. Remove the MEM minus methionine and replace it with 500µl MEM minus methionine supplemented with 100µCi Trans <sup>35</sup>S-Label. Incubate the cells for 20 minutes at 37°C and 5% CO<sub>2</sub>.
5. Remove labeling media and wash the cells with 500µl of DMEM. To the cells add 2 ml of DMEM supplemented with 25µg/ml cyclohexamide. Incubate the radiolabeled cells





**Figure 4.3 Kinetics of  $\Delta F508$  CFTR degradation.** HEK-293 cells were transfected with  $\Delta F508$  CFTR and where indicated were treated with 200 $\mu$ M ALLN for 1 hour prior to the labeling of cells with 35S-translabel. Immunoprecipitation and autoradiography of 35S- $\Delta F508$  CFTR was carried out as described in the text.

for 0-3 hours at 37°C and 5% CO<sub>2</sub>.

6. To harvest cells, place the 35-mm culture wells on ice, remove the media and add 1 ml of ice cold Citric Saline.
7. Collect the detach cells and transfer them into pre-cooled 1.5ml microfuge tubes. Pellet the cells and remove the supernatant. Cell pellets that are harvested at different time points can be stored on ice for the duration of the chase.
8. When cells incubated for different time periods have all been harvested, they can be lysed via the addition 500µl of ice cold RIPA buffer that is supplemented with 0.2% BSA, 1mM PMSF and PI. Cells are resuspended in the RIPA buffer by mixing with a pipette. If the samples are too viscous, additional RIPA buffer can be added to each tube. Incubate samples for 1 hour at 4°C on a rocker.
9. Clear the sample material that might stick to Protein A-sepharose non-specifically by adding 20µl of a 50% Pansorbin slurry to each 500µl of extract. Incubate samples for 15 minutes at 4°C on a rotator. Centrifuge samples >16,000 x g for 15 minutes at 4°C. Transfer 80% of the pre-cleared supernatant to a new pre-cooled tube and discard the pellet.
10. Add 3µl CFTR MM13-4 antibody to the pre-cleared supernatant and incubate for 1 hour on a rotator at 4°C.
11. Add 20µl of a 50% Protein-G Agarose slurry to each sample and incubate for 1 hour on a rotator at 4°C.
12. Pellet the Protein-G Agarose beads by centrifugation for 1 minute at 800 x g and 4°C.
13. Remove the supernatant, resuspend and wash the pelleted beads 3 times with ice cold RIPA buffer.

14. To each pellet add 15 $\mu$ l of 2X SDS-PAGE Sample Buffer that is pre-warmed to 37°C and immediately incubate the tubes at 37°C for 10-15 minutes. Do not boil the samples because this leads to CFTR aggregation.
15. Pellet the agarose beads by centrifugation at 13,000 x g at room temperature for 1 min. Remove 12 $\mu$ l of the supernatant and load it onto a 7% SDS-PAGE gel.
16. Electrophoreis the samples via the application of 100V to the gel for 115 minutes.

**Detecting immunoprecipitated and radiolabeled  $\Delta$ F508 CFTR by autoradiography.**

1. Remove the SDS-PAGE gel from the glass plates and rinse with dH<sub>2</sub>O.
2. Fix and stain the protein in the gel by incubation in stain for 10 minutes.
3. Soak the gel in destain until the protein bands on the gel are clearly visible.
4. Rinse the gel with dH<sub>2</sub>O for at least 5 minutes to remove destain from the gel.
5. Soak the gel in 0.5M Sodium Salicylate for 10 minutes.
6. Briefly rinse the gel with dH<sub>2</sub>O and place it on Whatman paper to be dried on a slab type gel dryer for 1 hour at 80°C.
7. Expose X-ray film to the dried SDS-PAGE gel for 12-18 hours at -80°C.

Develop the film with a processor and observe the location of the <sup>35</sup>S- $\Delta$ F508 CFTR.

## 4.5 References

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