A Dissertation

entitled

Role of Hsp105 in CFTR Biogenesis

by

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Cystic fibrosis (CF) is a life-threatening genetic disease arising from mutations in the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR). The mutant ΔF508 CFTR accounts for more than 70% of the CF causing alleles and exhibits temperature sensitive export defect from the endoplasmic reticulum (ER). Hsp105/110 facilitates the nucleotide exchange of Hsp70 and possesses independent chaperone activity (holdase activity) in vitro. Human Hsp105α, the constitutively expressed isoform, has been shown to associate with CFTR in the ER but its role in CFTR biogenesis is as yet unclear. We show that Hsp105 plays versatile roles in CFTR biogenesis in the ER affecting synthesis, maturation and degradation. The nucleotide exchange activity of Hsp105 is necessary for the degradation of CFTR in the ER. Its holdase activity reduces synthesis and stabilizes CFTR in the ER. Hsp105 stabilizes CFTR in the ER and proportionally promotes its processing. Hsp105 preferentially associates with ΔF508 CFTR and promotes its stabilization and rescue at both reduced and physiological temperatures. In addition, Hsp105 escorts the mutant CFTR in post-ER compartments.
and prolongs its half-life. Hsp105’s versatile functions and its high affinity for misfolded ΔF508 CFTR make it a useful molecular target for the rescue of protein misfolding in CF. Hsp70 and Hsp90 regulate CFTR biogenesis but their functional relationship remains unknown. Using the temperature-dependent rescue of ΔF508 CFTR as a model system we provide evidence for functional cooperation between Hsp70 and Hsp90 mediated by cochaperones Hop and Hsp105 in conformational maturation in the ER. Hsp70 associates with ΔF508 CFTR earlier than Hsp90. Hsp90 directly promotes its maturation and the integrity of Hsp70-Hsp90 chaperone network is essential for maturation of ΔF508 CFTR at reduced temperature. Our data support Hsp105 as an integral player in the Hsp70-Hsp90 chaperone network mediated CFTR conformational maturation in the ER.
For my daughter Adishree, and my entire family.
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List of Abbreviations

ABC      ATP-binding cassette
apoB     apolipoprotein B
CF       Cystic fibrosis
CFTR     Cystic fibrosis transmembrane regulator
CHIP     Carboxy terminal of Hsc70 interacting protein
COP II   Coatamer complex II
ER       Endoplasmic Reticulum
ERAD     ER-associated degradation
Hip      Hsc70 interacting protein
Hop      Hsp70-Hsp90 organizing protein
MSD      Membrane spanning domain
NBD      Nucleotide binding domain
NEF      Nucleotide exchange factor
SBD      Substrate binding domain
SHR      Steroid hormone receptor
TPR      Tetratricopeptide repeat
VCP      Valosin containing protein
Introduction

Hsp105/110 belongs to the Hsp70 superfamily of molecular chaperones and consists of an amino terminal ATPase domain and a carboxy terminal substrate binding domain (SBD) (Lee-Yoon et al., 1995). In spite of being long recognized as a major heat shock protein Hsp105 has not been studied intensively until recently and its specific cellular functions are still not clear. After Hsp70 and Hsp90, Hsp105 is the most abundant heat shock protein in most mammalian tissues and is highly abundant in brain (Easton et al., 2000). Hsp105α and Hsp105β are mammalian members of the HSP105 family (Lee-Yoon et al., 1995). Hsp105α is a 105-kDa protein constitutively expressed and can be further induced in response to various forms of stress, whereas Hsp105β is an alternatively spliced form of Hsp105α (Yasuda et al., 1995) that is specifically produced following heat shock at 42°C (Ishihara et al., 1999; Yasuda et al., 1999). Hsp105 is known to form high molecular mass complexes with Hsc70 in the cytosol, (Hatayama et al., 1998) and is known to functionally relate to Hsc70 (Yamagishi et al., 2004; Shaner et al., 2005) as well as Hsp90 (Liu et al., 1999). Hsp105 is also known to refold denatured proteins more efficiently than Hsc70 in vitro (Oh et al., 1997), and its Hsc70-independent chaperone activity (or holdase activity) does not require its ATPase domain (Oh et al., 1999). Hsp105 facilitates the nucleotide exchange of Hsc70 (Dragovic et al., 2006; Raviol et al., 2006b). Yeast Hsp105 Sse1 collaborates with Hsp70 Ssb or Ssa in regulating the co-translational or post-translational folding of cellular proteins, respectively (Yam et al., 2005).

Cystic fibrosis (CF) is considered as one of the most prevalent lethal, autosomal-recessive genetic diseases (Tsui, 1992). It is caused by different mutations in the gene
encoding cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan, 1993). CFTR is a member of the ATP-binding cassette (ABC) family of transporters (Kartner et al., 1991; Collins, 1992). It regulates the chloride ion conductance in many tissues including lungs, intestines, pancreas and cardiac muscle (Riordan, 1993; Amaral, 2005). CF is a multi-system disease but lung disease is the major cause of mortality. The deletion of the phenylalanine at residue 508 (ΔF508) represents about 70% of all the CF causing alleles. The ΔF508 mutation impairs the export of CFTR from the endoplasmic reticulum (ER) and thereby leads to its ER-associated degradation (ERAD) (Jensen et al., 1995; Ward et al., 1995).

Folding of newly synthesized, large, multidomain transmembrane proteins like CFTR is a complex process and molecular chaperones have been shown to recognize unfolded or improperly folded proteins and help them acquire their native conformation (Frydman, 2001). CFTR has been known to interact with a number of factors/proteins that regulate the various stages involved in its processing. Hsp70 mediates the maturation as well as quality control of nascent CFTR (Yang et al., 1993; Meacham et al., 2001) whereas inhibiting Hsp90 activity has been shown to prevent CFTR maturation and promote its degradation (Loo et al., 1998).

Since Hsp70 has such important role in CFTR biogenesis and Hsp105 has been identified as a nucleotide exchange factor (NEF) for Hsp70, Hsp105 is likely to have an important role in regulating CFTR maturation and quality control. Hsp105 was recently shown to associate with apolipoprotein B (apoB). It stabilized apoB, and enhanced its secretion, suggesting a pro-folding and pro-maturation role for Hsp105 (Hrizo et al., 2007). In this study, we used a combination of different approaches to analyse various
roles of Hsp105 in CFTR biogenesis. We show that the Hsp105 holdase activity reduces CFTR synthesis, inhibits its ERAD and promotes its maturation whereas its NEF activity enhances the ERAD of CFTR. The interplay between these two activities within the ER produces a dose-dependent effect on CFTR biogenesis. We also observe that Hsp105 preferentially associates with ΔF508 CFTR in the ER, which protects the mutant protein from ERAD. Interestingly, Hsp105 shows extensive association with ΔF508 CFTR in post-ER compartments and prolong its half life. Furthermore, Hsp70 and Hsp90α also have much greater association with the mature form of ΔF508 CFTR, suggesting a role for cytoplasmic chaperone machinery in escorting misfolded cargo molecules in post-ER compartments. These findings suggest that Hsp105 might serve as a potential chaperone target for the rescue of protein misfolding in CF and other protein misfolding diseases.

ΔF508 is a temperature sensitive mutant (Denning et al., 1992) and can be transported to the plasma membrane at reduced temperature (Kopito, 1999). It has been recognized that the ΔF508 CFTR channel is functional in vivo (Dalemans et al., 1991; Drumm et al., 1991; Pasyk and Foskett, 1995) and after reconstituting it into the phospholipid bilayer (Li et al., 1993). The temperature-dependent export of ΔF508 CFTR involves changes in chaperone recruitment (Wang et al., 2008). We tried to understand the mechanism behind maturation of ΔF508 CFTR at reduced temperature by examining the roles of Hsp70 and Hsp90 and their functional relationship through their multiple cochaperones. Our data indicate the involvement of an Hsp70-Hsp90 chaperone network containing at least Hsp70, Hsp90, Hop and Hsp105 in the temperature-dependent maturation of ΔF508 CFTR, and suggest that this phenomenon is highly dependent on the interplay between major cellular chaperones and cochaperones. A deeper understanding of this would be of
great value in the potential rescue of ΔF508 CFTR by manipulating these molecular chaperones. Our data are consistent with the existence of an Hsp70-Hsp90 relay system within the cytoplasmic side of the ER similar to the one that mediates the activation of SHRs in the cytosol. The coordinated actions of such a chaperone system mediates the maturation of CFTR in the ER. Hsp105 is an integral player in the system.
Molecular chaperones are ubiquitous proteins responsible for maintaining protein homeostasis in the cell. The cellular functions of the chaperones include proper folding of the nascent polypeptide chains, protein translocation across the membranes of various cellular compartments and degradation of damaged proteins. Some of the molecular chaperones are heat- and/or stress-inducible and are thus known as heat shock proteins. However, most of these proteins are also expressed in the absence of stress and have been shown to perform critical functions in the cell. The heat shock proteins of mammalian cells can be classified into different families of sequence-related proteins, which are designated by their molecular weight in kilodaltons. The major families are: the Hsp25/ Hsp27/ Hsp28 family, the Hsp40 family, the Hsp70 family, the Hsp90 family and the Hsp105 family. Chaperones may hamper or facilitate the degradation of a given substrate, or can even perform both functions. Also, chaperones that are pro-folding or pro-degradation for one substrate may switch their roles or have no effect on another substrate. For example, it was shown in vitro that both Hsp70 and Hsp90 are required for the degradation of apoB (Gusarova et al., 2001). In contrast, the Hsp105, a chaperone which functionally associates with Hsp70 and Hsp90, facilitates the degradation of a soluble human protein von Hippel-Lindau (VHL) in yeast (McClellan et al., 2005), stabilizes apoB (Hrizo et al., 2007). Together, these observations indicate that chaperones can perform a diverse number of functions depending on the family to which they belong, on the identity and conformation of the bound substrate and on cochaperone partners.
**Structure, Function and Regulation of Hsp70:**

Hsp70 whose bacterial ortholog is DnaK, is perhaps the best-studied class of molecular chaperones. It is induced when the cellular concentration of unfolded proteins rises (Morimoto, 1998). Hsp70 has a wide range of functions in the cell which include the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of organellar and secretory proteins, and controlling the activity of regulatory proteins (Mayer et al., 2000; Pratt and Toft, 2003; Young et al., 2003). Hsp70 is composed of a ~44-kDa amino terminal ATPase domain (NBD) and a ~25-kDa substrate binding domain (SBD). The SBD is further subdivided into a $\beta$-sandwich subdomain of ~15 kDa and a carboxy terminal $\alpha$-helical lid which opens when ATP is bound, but closes when ADP is bound (Flaherty et al., 1990; Zhu et al., 1996; Jiang et al., 2005) (Figure 1).

**Figure 1:** Schematic representation of Hsp70 domain structure.

Hsp70 exhibits two functional states. When bound to ADP, it exhibits high affinity for its substrates, whereas when bound to ATP, it exhibits low substrate affinity and increased rate of peptide release. Since ATP hydrolysis is critical to trap a peptide, and ADP-ATP exchange is required for substrate release, Hsp70 has cochaperones which catalyse individual steps in the Hsp70 ATP hydrolytic cycle which include ATPase activators such as Hsp40s and nucleotide exchange factors such as BAG-1 (Hohfeld and Jentsch, 2006).
HspBP1 (Kabani et al., 2002) and Hsp105 (Dragovic et al., 2006; Raviol et al., 2006b) (Figure 2).

**Figure 2:** The ATPase cycle of Hsp70.

**Hsp40:**

Initial work with *Escherichia coli* identified DnaJ as one of the key regulatory cochaperones of DnaK. The DnaJ/Hsp40 family of proteins which have Hsp70 interacting J-domain stimulate the Hsp70-ATP hydrolysis favoring tight substrate binding (Liberek et al., 1991). The J-domain folds into a four-α-helical bundle and most likely interacts with Hsp70’s ATPase domain (Qiu et al., 2006). In addition, DnaJ is known to bind to unfolded polypeptides itself although it is not an ATPase and it does not have a regulated substrate binding cycle. It is thought that the DnaJ binds the substrate before/during the activation of DnaK ATP hydrolysis and transfers the substrate to DnaK upon DnaK ATPase activation (Schroder et al., 1993; Szabo et al., 1996). Following the J
domain, DnaJ has a glycine/phenylalanine rich linker, a central domain having two zinc-finger motifs and a carboxy terminal homodimerization domain. Hsp40 proteins having this architecture are classified as type I and include two major cytosolic Hsp40s: DJA1 (DnaJ1/Hdj2) and DJA2 (DnaJ2/Hdj3) in humans and Ydj1 in yeast. The main human type II Hsp40 in the cytosol, DJB1 (DnajB1/Hdj1) have the J domain and glycine/phenylalanine rich linker but diverge in the rest of the protein (Qiu et al., 2006). The type III members are homologous only in J domain (Young et al., 2003; Walsh et al., 2004).

**Figure 3:** Schematic representation of domain structure of Hsp40s.

**Hsp70 NEFs:**

NEFs recharge the nucleotide binding domain of Hsp70 with ATP and facilitate substrate release (Szabo et al., 1994). There are four evolutionary unrelated groups of NEFs: homologs of the E.coli protein GrpE, occurring in eubacteria and organelles of...
prokaryotes, BAG domain proteins, HspBP1/Fes1p homologs, and Hsp105/Grp170 family proteins (Hohfeld and Jentsch, 1997; Kabani et al., 2002; Steel et al., 2004). GrpE together with DnaJ is essential for the normal functioning of DnaK (Liberek et al., 1991; Szabo et al., 1996). GrpE is a homodimer that binds to the NBD of DnaK, opening its lobes. Dissociation of GrpE results in binding of ATP causing nucleotide exchange (Harrison et al., 1997). There are six different homologs of BAG proteins in human, BAG-1, BAG-2, BAG-3, BAG-4, BAG-5 and BAG-6 (Takayama and Reed, 2001). All these homologs contain one BAG domain except BAG-5 which has five BAG domains. However, NEF activity has only been demonstrated for the BAG domain of BAG-1. BAG-1 has NEF activity for human Hsc70 through a mechanism similar to that of GrpE with DnaK (Sondermann et al., 2001). However, recently human BAG-2 interaction with Hsc70 has been shown but with a different mechanism (Xu et al., 2008). BAG-1 has been shown to stimulate the carboxy terminal of the Hsc70-interacting protein (CHIP)-mediated degradation of the glucocorticoid hormone receptor (Demand et al., 2001). Also, BAG-1 possesses a ubiquitin-like domain that is utilized for associating with the proteasome (Luders et al., 2000; Alberti et al., 2002). Domain arrangement of BAG-1 helps it recruit Hsc70 complexes to the proteasome, leading to proteasomal degradation of molecular clients. Importantly, cochaperone cooperation not only provides a means to stimulate chaperone-assisted degradation but also interferes with it.

HspBP1 and Hsp105 proteins coexist in the cytosol and the ER of most of the eukaryots. A crystallographic study shows that HspBP1 and its yeast homolog Fes1 follow entirely different mechanism to carry out their NEF activity (Shomura et al., 2005). HspBP1 has been identified as an inhibitor of the CHIP and was found to be
present in ternary complexes with Hsc70 and CHIP. While other NEFs of Hsp70 consist of an Hsp70-binding domain, Hsp105/Grp170 itself belongs to the Hsp70 superfamily and was reported to bind and stabilize unfolded proteins (Hohfeld and Jentsch, 1997; Kalin et al., 1999; Goeckeler et al., 2002). The NEF mechanism of Hsp105 has recently been analyzed in great detail (Polier et al., 2008; Schuermann et al., 2008).

Hsc70 interacting protein (Hip), Hsp70-Hsp90 organizing protein (Hop) and CHIP are some of the other major cochaperones of Hsp70. Hip is an ~43 kDa protein which interacts with the ATPase domain of human Hsc70 (Hohfeld et al., 1995). Hip stabilizes the ADP state of Hsp70 and stimulates the chaperone activity of Hsp70 by preventing premature substrate release (Hohfeld and Jentsch, 1997). Hip antagonizes the substrate discharging function of BAG-1 by competing with BAG-1 for binding to the ATPase domain of Hsc70 (Kanelakis et al., 2000). However, BAG-independent functions of Hip were found recently. A tetratricopeptide repeat (TPR) deletion variant of Hip and a point mutant with considerably reduced affinity to Hsp70 was found to have significant stimulatory effect on glucocorticoid receptor activation, indicating an Hsp70-independent function of Hip in this process in the yeast system (Nelson et al., 2004). Hop (yeast Sti1) is an ~60kDa protein and is a component of the progesterone receptor complex (Smith, 1993). Hop interacts with the carboxy terminal EEVD motifs of Hsp70 and Hsp90 proteins via its three TPR domains (Scheufler et al., 2000; Odunuga et al., 2003). Hop enhances the maturation rate of the progesterone receptor (Morishima et al., 2000). Recently yeast Sti1 but not the mammalian Hop was found to stimulate the ATPase activity of the yeast Hsp70 (Wegele et al., 2003). CHIP forms a dimer in solution and competes with Hop for binding to the C-terminus of Hsc70 and Hsp90 (Nikolay et al., 2009).
2004). It is known to act as an E3-ubiquitin ligase and ubiquitinates Hsc70 substrates in vitro and in vivo, promoting their degradation by the proteasome (Connell et al., 2001; Meacham et al., 2001).

**Hsp90:**

Hsp90 is a highly conserved, abundant molecular chaperone constitutively expressed in eukaryotic cells. This protein accounts for nearly 1% of the total cellular protein in eukaryotic cells (Welch and Feramisco, 1982). Cells exposed to heat shock and other stress conditions such as in the case of cancer, overexpress Hsp90 (Chiosis et al., 2004). Hsp90 family of chaperones play an essential role in conformational maturation of nascent polypeptides and the refolding of denatured proteins (Terasawa et al., 2005). Hsp90 mediated stabilization effect was noticed in the 1970s and 1980s well before the formal identification of the 90-kDa component as Hsp90 (Pratt and Toft, 1997). Hsp90 distinguishes itself from other chaperones by having most of its known client molecules as signal transduction proteins, classical examples being steroid hormone receptors (SHRs) and signaling kinases (Picard et al., 1990; Xu and Lindquist, 1993). Hsp90 is essential for the activity of numerous signaling proteins and hence plays a key role in cellular signal transduction networks. Hsp90 binds to client molecules at a late stage of folding (Jakob et al., 1995) and is activated by ligand binding or interaction of the client with other factors. Hsp90 operates as part of a multichaperone machinery in the cytosol, which includes Hsp70, peptidyl-prolyl isomerases (PPIase) and other cochaperones (Bose et al., 1996; Freeman et al., 1996). The Hsp90 chaperones are observed to exhibit more restricted substrate specificity than Hsp70s. On the basis of its role in regulating cancer
cell growth and its unusual ATP-binding pocket, specific inhibitors for the chaperone have been developed (Chiosis et al., 2004; Whitesell and Lindquist, 2005). There is evidence to support that Hsp90 can switch between pro-folding and pro-degradation, and thus may decide a nascent polypeptide’s ultimate fate. A selected number of secreted proteins have been shown to require Hsp90 for either folding (e.g. Ste6p*) (Huyer et al., 2004) or degradation (e.g. a mutant form of the insulin receptor and apoB) (Imamura et al., 1998; Gusarova et al., 2001).

In mammalian cells there are two genes encoding cytosolic Hsp90, Hsp90α and Hsp90β. Hsp90α has ~85% sequence identity with Hsp90β (Hickey et al., 1989). In human, Hsp90 exists as a homodimer. It has three highly conserved domains, a 25-kDa amino terminal ATP-binding domain, a 35-kDa middle domain, and a 12-kDa carboxy terminal dimerization domain (Figure 4). The extreme carboxy terminal end has an MEEVD motif which is a binding site for the TPR domains found in several cochaperones (Pratt et al., 2004). The function of Hsp90 depends upon the ability of the amino terminal domain to bind and hydrolyze ATP (Obermann et al., 1998; Panaretou et al., 1998; Dragovic et al., 2006).

Figure 4: Domain structure of Hsp90.
Recently a kinetic model was proposed based on the real-time detection of the conformational changes of Hsp90 by fluorescence resonance energy transfer (FRET) (Hessling et al., 2009). It shows five intermediate states which might represent the various stages of the Hsp90 ATPase cycle suggested from previous structural and biochemical studies (Hahn, 2009). In summary, ATP binding to the Hsp90 homodimer promotes association of the amino terminal domains leading to a compact complex. ATP hydrolysis restores the open conformation of Hsp90 (Figure 5).

In the early 1990s, a number of additional proteins that co-purified with Hsp90 in complex with steroid receptors or protein kinases were identified (Pratt and Toft, 2003; Wegele et al., 2004; Pearl and Prodromou, 2006). The majority of the cochaperones of Hsp90 identified so far contain the TPR domain which can bind to the carboxy terminal end of Hsp90 or Hsp70 (Pratt et al., 2004; Wandinger et al., 2008). The TPR-containing cochaperones such as Hop/Sti1, TPR immunophilins (FKBP51, FKBP52, and Cyp40),

**Figure 5.** The ATPase cycle of the Hsp90
PP5/Ppt1 and CHIP have specific functional domains involved in the regulation of Hsp90 client proteins or the Hsp90 chaperone machinery itself. Hop/Sti1 has three TPR domains through which it binds simultaneously to Hsp70 and Hsp90, mediating the transfer of the client protein from Hsp70 to Hsp90 (Loo et al., 1998). Hop/Sti1 also inhibits Hsp90 ATPase activity which is thought to be advantageous for the loading of the client protein to the Hsp90 chaperone machinery (Richter et al., 2003). Immunophilins harboring the PPIase domain have different roles in the regulation of steroid receptors (Ratajczak et al., 2003). Although FKBP51 and FKBP52 have 70% similarity between them, FKBP51 inhibits the glucocorticoid receptor activity (Denny et al., 2000), whereas FKBP52 potentiates the glucocorticoid receptor, as well as the androgen receptor and progesterone receptor (Yong et al., 2007). Yeast Ppt1 activates Hsp90 by directly dephosphorylating it (Wandinger et al., 2006). PP5 has been shown to dephosphorylate Cdc37, leading to the release of Cdc37 from Hsp90 (Vaughan et al., 2008). Therefore, PP5 seems to regulate the dynamic assembly of the Hsp90 machinery during the folding of kinase client proteins recruited by Cdc37. CHIP is a TPR-containing cochaperone having E3 ubiquitin ligase activity (McDonough and Patterson, 2003). It has been suggested that CHIP might be involved in the ubiquitin-mediated degradation of Hsp90 client proteins which failed to fold properly.

**Hsp70-Hsp90 Chaperone relay system in conformational maturation:**

Protein folding is a spontaneous process. In eukaryotes there are different chaperones which help in the conformational maturation of partially or completely unfolded proteins. Hsp70 and Hsp90 are two of the most prominent heat shock proteins in the eukaryotic
cytosol. They are known to cooperate in the folding and maturation of key regulatory proteins (Picard, 2002; Pratt and Toft, 2003; Wegele et al., 2004). Hsp90 is frequently described as a “molecular chaperone machine” but there is no evidence that Hsp90 exists as one large complex containing Hsp90 and all cochaperones. Instead, Hsp90 and cochaperone proteins have been observed to interact with client proteins in an ordered pathway that involves sequential ATP-dependent interactions of the client protein with Hsp70 and Hsp90. However, there is evidence that some proteins, after being processed by Hsp70, are transferred to Hsp90 although there is evidence that Hsp90 can act independently of Hsp70 as well and each of these pathways can lead to folded, functional proteins or degradation as shown in Figure 6 (Wegele et al., 2004).

![Substrate](#)

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<tr>
<th>Substrate</th>
<th>Hsp70</th>
<th>Hsp90</th>
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<td>Folding</td>
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Figure 6: Pathways of Hsp70 and/or Hsp90 mediated folding.

It was found using ultracentrifugation experiments that ligand-free SHRs are part of large multiprotein complexes (Wilson et al., 1977; Pratt and Toft, 1997) and that Hsp90 is a component of these complexes (Ziemiecki et al., 1986; Sanchez et al., 1987). Later, two additional factors Hsp70 and Hop (Perdew and Whitelaw, 1991; Smith et al., 1993) were
also identified. Hop was shown to bind to both Hsp70 and Hsp90 (Perdew and Whitelaw, 1991; Smith et al., 1993; Scheufler et al., 2000).

According to the current model, a client protein first binds to Hsp40 (Schroder et al., 1993; Hernandez et al., 2002). This complex then associates with Hsp70/ATP and the client protein is locked in by the subsequent Hsp40-stimulated hydrolysis of ATP. The substrate protein bound to Hsp70 is brought into contact with Hsp90 via Hop, the mammalian counterpart of yeast Sti1 (Smith, 1993; Chang et al., 1997). Hop and Hsp70 which belong to the “intermediate complex” are subsequently replaced with TPR immunophilins and p23 to yield the “final complex.” During the chaperone pathway, the client has to be transferred from Hsp70 to Hsp90 (Figure 7).
**Figure 7: Hsp70-Hsp90 relay system in SHR activation.** SHR is delivered to the Hsp70 complex via Hsp40. Hop connects elements of the Hsp70 and Hsp90 machineries to form the “intermediate complex”. Hop, Hsp70 and Hsp40 are then replaced by p23 and Immunophilins to form “final complex”. IMM, TPR Immunophilins.

Recently it was demonstrated using luciferase as a substrate protein (Wegele *et al.*, 2006) that Hsp90 exhibits a specific positive effect only in the presence of Ydj1. In the absence of Ydj1, the transferred luciferase was trapped in Hsp90 in an inactive conformation. Identical results were observed for the yeast and the human chaperone systems although the regulatory function of human Hop is completely different from that of yeast Sti1.

Hsp70 and its cofactors have been known to collaborate with Hsp90 to promote the maturation of a subset of cellular proteins, most notably protein kinases and transcription factors (Picard, 2002; Wandinger *et al.*, 2008). There are a number of key cellular regulatory proteins which are implicated in human diseases and are Hsp90 clients. Efforts are underway to understand in detail how these proteins are recognized and processed. Interruption of the Hsp70/Hsp90 folding cycle through genetic or pharmacological means, such as by the specific inhibitor geldanamycin (GA), is known to result in ubiquitinylation and degradation of the client protein (Whitesell and Lindquist, 2005). Ydj1 has been identified as an important regulator of Hsp70, suggesting that Hsp70 is a major nexus of client fate evaluation and that other Hsp70-regulating partners might influence this decision (Mandal *et al.*, 2008). Sse1 has been physically and genetically linked to the Hsp90 chaperone complex in yeast, although the mechanism remains unclear (Liu and DeFranco, 1999; Goeckeler *et al.*, 2002).
It has been shown recently that Sse1 plays a role in client maturation through the Hsp70/Hsp90 chaperone system (Mandal et al.). They show that upon inhibition of Hsp90, Sse1 is required for the degradation of the model protein kinase, Ste11ΔN. Also, consistent with the NEF activity of Hsp105, Hsp70 binding and nucleotide exchange activity of Sse1 was required for both GR and Ste11 activity. It is also shown that Sse1 is required for the ubiquitinylation of Ste11ΔN upon Hsp90 inhibition. These new findings suggest that Hsp105 chaperones can control the fate of some client proteins by influencing the decision to fold or degrade as dictated by the Hsp70/Hsp90 chaperone environment.

**Hsp105:**

The Hsp105 subfamily of Hsp70 superfamily is a relatively poorly understood group. In the 1990s, cloning of Hsp105 had been described from a variety of species such as hamster, mouse and yeast etc. (Foltz et al., 1993; Mukai et al., 1993; Lee-Yoon et al., 1995; Yasuda et al., 1995; Storozhenko et al., 1996; Kaneko et al., 1997; Mauk et al., 1997). In spite of being long recognized as a major heat shock protein Hsp105 has not been studied very intensively and its specific functions in the cell were still not known. Yeast possesses two highly similar Hsp105 homologues encoded by the genes SSE1 and SSE2. Cells lacking Sse1 are temperature sensitive and slow growing, whereas loss of Sse2 has no functional consequences (Mukai et al., 1993; Shirayama et al., 1993). It was also demonstrated that deletion of both the genes results in lethality, showing that Hsp105 chaperones play a critical role in cellular physiology (Shaner et al., 2004; Trott et al., 2005; Raviol et al., 2006a). Sse1 is found in association with Ssa and Ssb, the two groups
of cytosolic Hsp70 proteins found in yeast (Shaner et al., 2005; Yam et al., 2005), and activates the ATPase activity of Ssa1 in a synergistic manner with the yeast Hsp40 Ydj1.

After Hsp70 and Hsp90, Hsp105 is the most abundant heat shock protein in most mammalian cell lines and tissues and is the most abundant in brain (Easton et al., 2000). Hsp105α and Hsp105β are mammalian members of the HSP105 family (Lee-Yoon et al., 1995). Hsp105α is a 105-kDa protein constitutively expressed and inducible in response to various forms of stress, whereas Hsp105β is an alternatively spliced form of Hsp Hsp105α (Yasuda et al., 1995) that is specifically produced following heat shock at 42ºC (Ishihara et al., 1999; Yasuda et al., 1999). Hsp105 is mainly localized in the cytoplasm of mammalian cells under non-stress and stress conditions (Hatayama et al., 1994), and exists in a complex associated with Hsc70/Hsp70 in mammalian cells (Hatayama et al., 1998). Studies have indicated that both rodent and yeast Hsp105s are efficient holdases in vitro, binding to and preventing aggregation of denatured proteins (Brotsky and McCracken, 1999; Oh et al., 1999). In fact, Hsp105 might serve as a better chaperone in this regard than Hsc70/Hsp70 itself (Oh et al., 1997). It is only with very recent studies that functional interaction between Hsp105 and Hsc70/Hsp70 has been established (Wang et al., 2004a; Yamagishi et al., 2004; Shaner et al., 2005; Yam et al., 2005). Hsp105 is shown to act as an NEF for Hsp70 (Dragovic et al., 2006; Raviol et al., 2006b). It is also reported that the NEF activity of Hsp105 requires a functional ATPase domain and an intact carboxy terminus to carry out this function (Dragovic et al., 2006). A recent report also successfully describes that Hsp105 exhibits intrinsic ATPase activity (Raviol et al., 2006a). Binding of ATP to Hsp105 is required for its binding to Hsc70/Hsp70 (Shaner et al., 2006).
Hsp105 is composed of amino terminal ATP binding domain (NBD) and carboxy terminal substrate binding domain (SBD). The SBD consists of a central $\beta$-sheet domain, a loop, and an $\alpha$-helix domain similar to that of the HSP70 family proteins (Figure 8) (Yasuda et al., 1995; Ishihara et al., 1999).

![Figure 8: Domain structure of Hsp105.](image)

Although cytosolic Hsp105 shows unmistakable NBD similarity to Hsp70s, it has a larger size due to acidic SBD insertions and carboxy terminal extensions.

Two recent reports (Polier et al., 2008; Schuermann et al., 2008) describe structures of bovine and human Hsp70 in complex with the yeast Hsp105, Sse1. These new structures shed light on the mechanism by which its NEF activity regulates Hsp70 activity. Schuermann et al. determined the crystal structure of yeast Sse1 complexed with bovine Hsc70 (Figure 9) and found that the most extensive interactions are between the NBDs of the two proteins, especially aa 1-384 of each molecule. Also, the Hsp105 SBD$\alpha$ embraces Hsc70.
Figure 9: Ribbon Models of the Hsp105-Hsc70 Nucleotide Exchange Complex. Hsc70 is colored in blue tones, and Hsp105 is colored in autumn tones (red, orange, yellow, brown). NBDs of Hsp105 and Hsc70 are colored yellow and light cyan, respectively. The interdomain linkers of Hsp105 and Hsc70 are red and blue, respectively. The Hsc70 SBD is dark cyan, and the Hsp105 SBDβ and SBDα are orange and brown, respectively. Subdomains IA–IIB of the NBDs are labeled in (A) and (D), and individual domains are labeled in (B); different views of the complex are related by the indicated rotations (Schuermann et al., 2008).
When comparing with the other Hsp70 NEFs, Sse1 is unique in that it contains a SBD, and its α-SBD makes extensive contacts with lobe II of the Hsp70 NBD (Cyr, 2008). Such interactions can account for the high stability reported for Hsp70:Sse1 complexes (Shaner and Morano, 2007). In the Hsp70:Sse1 structure, the Sse1 SBD is located in close proximity to the Hsp70 SBD (Schuermann et al., 2008). Thus, Hsp70 and Sse1 might be able to bind simultaneously to certain substrates. It is proposed that the ability of Sse1 to bind polypeptide may be important for the folding of large proteins with multiple domains (Polier et al., 2008).

It is also shown using two independent approaches that nucleotides are required for the formation of Ssa1-Sse1 complex and that the NEF activity of Sse1 is controlled by nucleotide (Andreasson et al., 2008). Amide exchange was used in combination with mass spectrometry to analyze the effects of nucleotides on Sse1, and it was shown that nucleotide binding results in formation of a stable conformation of Sse1 that is required for association with the Ssa1. The interaction between Sse1 and Ssa1 releases bound ADP from Ssa1 but nucleotide remains bound to Sse1. After verifying the complete removal of nucleotide, the nucleotide containing and nucleotide-free protein samples were incubated with Ni-NTA matrix to isolate Ssa1 and the amount of Sse1 remaining in complex with Ssa1 was assessed. Further removal of the nucleotide did not affect the integrity of the complex. It was also shown that nucleotide binding to Ssa1 results in dissociation of the Sse1-Hsp70 complex.

Hsp105 was identified to associate with CFTR in a recent study using a global proteomic approach (Figure 10) (Wang et al., 2006). Since Hsp70 plays an important role in CFTR biogenesis and Hsp105 acts as an NEF for Hsp70, Hsp105 might have an
important role in conformational maturation and quality control of CFTR. Hsp105 associates with apoB, stabilize it, and enhance its secretion, suggesting a role for Hsp105 in protein folding and maturation (Hrizo et al., 2007).

Figure 10: Chaperone network associated with CFTR in the ER (Wang et al., 2006).

CF:

CF is a life-threatening autosomal recessive genetic disease most commonly found in Caucasian population (Tsui, 1992). It is caused by the mutations in the gene encoding a protein known as CFTR. CF occurs in about 1 in 3,200 Caucasian newborns. However it affects only 1 in 15,000 African Americans newborns and about 1 in 31,000 Asian
American newborns. (http://ghr.nlm.nih.gov/condition=cysticfibrosis). Also, about 1 in every 20 Americans is a carrier for CF.

CF affects epithelial cells of multiple organs in the body. Clinical manifestations of CF include lung disease, abnormal digestive functions, elevated sweat salt concentration, infertility, heart enlargement, diabetes, pancreatic inflammation liver disease and gallstones. However, lung disease is the major cause of morbidity and mortality. Defective CFTR causes salt water imbalance in the airway epithelia causing dehydrated and thick mucus which collects in the small airways of the lungs. This mucus leads to the formation of bacterial infections and/or colonization predominantly by *Pseudomonas aeruginosa* leading to respiratory failure.

CFTR:

CFTR is a glycoprotein that functions as a chloride ion channel at the apical surface of many epithelial cells (Riordan *et al.*, 1989b; Rommens *et al.*, 1989). It is a member of ATP-binding cassette (ABC) family of proteins. This ion channel is regulated by phosphorylation and intracellular nucleotide binding (Welsh *et al.*, 1992; Riordan, 1993; Jilling and Kirk, 1997). The gene that encodes for human CFTR is located on the chromosome number 7 in region q31.2. CFTR regulates the conductance of other ion channels as well, such as epithelial sodium channel (ENac). It is composed of five domains: two membrane-spanning domains (MSD1 and MSD2) each composed of six transmembrane segments, two nucleotide-binding domains (NBD1 and NBD2) that bind and hydrolyze ATP, and a regulatory (R) domain (Riordan *et al.*, 1989b; Riordan, 1993). R domain contains several consensus sites for phosphorylation by protein kinase A and
protein kinase C. Modification of the R domain, either through the addition or removal of chemical phosphate groups, regulates the movement of chloride ions across the membrane.

**CFTR Trafficking:**

CFTR is core-glycosylated in the ER (band B). Once checked for correct folding it moves to the Golgi complex, where it is complex glycosylated (band C). Therefore, glycosylation of CFTR is an excellent marker to follow its intracellular trafficking. Since ΔF508 CFTR cannot move out of the ER, it displays only band ‘B’ (Figure 11).

![Figure 11. Wild-type and ΔF508 CFTR processing.](image)

There are about 1400 different CFTR mutations described (Guggino and Stanton, 2006). Nearly 70% of all disease-causing alleles lead to the deletion of phenylalanine at position 508 (ΔF508). Over 90% of the patients carry at least one ΔF508 allele. CFTR is co-translationally translocated in the ER and transported to the Golgi apparatus for additional processing such as glycosylation before it is transported to the cell membrane. But CFTR protein with the ΔF508 mutation resides the ER. The quality-control machinery of the ER recognizes that the protein is misfolded and marks the defective
protein for degradation. As a result, ΔF508 CFTR cannot reach the cell membrane (Gelman et al., 2002).

ΔF508 mutant was found to be temperature sensitive (Denning et al., 1992) and it was also shown that some degree of maturation and transport to the plasma membrane occurs in cells grown at reduced temperature (Kopito, 1999) or in presence of certain chemical such as glycerol (Sato et al., 1996). However, chloride channel activity of the mutant CFTR was detected in *Xenopus* Oocytes (Drumm et al., 1991), Vero cells (Dalemans et al., 1991), Sf9 insect cells (Bear et al., 1992) which are grown at reduced temperature and after reconstituting it into the phospholipid bilayer (Li et al., 1993). This gives an indication that the CF phenotype can be alleviated by directing the mutant CFTR from the ER to the plasma membrane.

**CFTR Biogenesis:**

Polytopic integral membrane proteins are known to acquire proper transmembrane orientation through the action of discrete topogenic determinants such as signal, stop transfer, and signal anchor sequences which are encoded within transmembrane segments and their flanking residues (Blobel, 1980; Wickner and Lodish, 1985). The biogenesis of the CFTR initiates on cytosolic ribosomes that are targeted through the signal recognition particle (SRP) to the ER membrane translocon (Sec61 complex) via the SRP receptor (Lu et al., 1998; Skach, 2000). It has been shown that CFTR and other polytopic proteins utilize multiple translocation mechanism to generate similar topologies for pairs and groups of TM (Sadlish and Skach, 2004). It was shown that CFTR amino terminal topology is determined by co- and post-translational events. TM1 exhibits very inefficient
signal sequence activity and is able to direct correct topology in ~25% of nascent CFTR chains. By contrast, TM2 signal sequence activity is efficient and even in the absence of a functional TM1 signal sequence, TM2 is able to direct CFTR amino terminus topology via a ribosome dependent, post-translational pathway.

It has been determined recently using protease susceptibility that CFTR predominantly folds co-translationally (Kleizen et al., 2005). Limited proteolysis was used in combination with in vitro translation in semipermeabilized cells to examine folding of CFTR and it was observed that all CFTR domains reach a protease-resistant structure during nascent chain elongation, suggesting predominantly co-translational folding. It is shown both in vitro and in vivo that CFTR does not undergo extensive post-translational folding. Carboxy terminally truncated constructs have been used to show that individual CFTR domains form well-defined structures independent of carboxy terminal parts and hence the multidomain protein CFTR folds mostly co-translationally, domain by domain. It has been proposed that individual CFTR modules first fold fast and independently, after which they interact with preceding domains co-translationally to generate a more stable fold. This suggests that most of CFTR folding is over by the time of nascent chain termination. However, full-length CFTR domains reside longer in the ER than their synthesis time, implying the possibility of additional conformational maturation steps before exit. Post-translational folding is shown to be related to MSD1 and MSD2, possibly because of increasing compactness and interaction with each other (Kleizen et al., 2005).

The nascent CFTR undergoes inefficient maturation, which is mediated by various molecular chaperones and powered by the hydrolysis of cytoplasmic ATP (Lukacs et al.,
Pulse chase experiments have indicated that the synthesis of wild-type or ΔF508 nascent core glycosylated polypeptide is linear for 15 minutes and half of that time is required for completion of a single chain (Ward and Kopito, 1994; Loo et al., 1998). The newly synthesized CFTR has a half-life of approximately 30 minutes in both wild-type CFTR and ΔF508 CFTR.

After co-translational maturation, CFTR moves to the Gogi. The transport from ER to Golgi is mediated by COPII vesicles (Kuehn et al., 1998; Antonny and Schekman, 2001; Wang et al., 2004b; Mancias and Goldberg, 2005; Tang et al., 2005). During COPII vesicle formation, Sec12 activates Sar1 by replacing GDP with GTP. Sar1-GTP together with the cargo molecule recruits Sec23/24 cargo selection protein complex. CFTR binds to the Sec24 which remains in complex with Sec23. Assembly of vesicle is then carried out by the Sec13/31- coat assembly complex. Hydrolysis of GTP attached to Sar1 results in the vesicle uncoating, and the vesicle membrane fuses with pre Golgi intermediate complex (Mancias and Goldberg, 2005). CFTR finally moves to the plasma membrane and functions as an ion channel.

**CFTR Degradation:**

It has been shown using pulse-chase studies that CFTR is an inefficiently folded protein and based on the cell type ~50%–70% of newly synthesized CFTR is degraded (Lukacs et al., 1994; Ward and Kopito, 1994) by the proteasome (Jensen et al., 1995; Ward et al., 1995). However in lung epithelial cells that express endogenous CFTR, folding and maturation have been shown to reach close to 100% efficiency (Varga et al., 2004).
There are several lines of evidence suggesting that the CFTR undergoes degradation in the ER via ubiquitin proteasome pathway. Treatment of cells expressing wild-type CFTR or ΔF508 CFTR with proteasome inhibitors causes the accumulation of multiubiquitinated forms of CFTR detected as a characteristic "ladder," suggesting that the degradation is mediated by the proteasome. It has also been demonstrated that undegraded CFTR is modified by ubiquitin (Ward et al., 1995). It has been shown that CHIP functions with Hsc70 to sense the folded state of CFTR and targets aberrant forms of CFTR for proteasomal degradation by promoting their ubiquitination (Meacham et al., 2001). Although ubiquitin-proteasome-mediated degradation is the dominant pathway for disposal of misfolded CFTR in mammalian cells, the folding state of CFTR is monitored not only during the early stage of its biogenesis in the ER but is also shown to be surveyed in post-Golgi compartments. It has been shown using ubiquitinated adducts of CFTR that ubiquitinated channels are selectively retrieved from recycling and are redirected for degradation into lysosomes (Sharma et al., 2004).

**Molecular chaperones in CFTR biogenesis:**

Folding of newly synthesized large multidomain transmembrane proteins like CFTR is a complex process. Molecular chaperones can recognize unfolded or improperly folded proteins/domains and help them acquire their functional native state (Frydman, 2001). Nascent or damaged polypeptides are unstable and their interaction with these chaperones stabilizes them and prevents their aggregation and allows them to fold properly. The interaction of CFTR with several molecular chaperones in assisting the productive folding of CFTR nascent polypeptides in the cytoplasm has been shown.
**Hsp70:**

The Hsc70/Hsp70 (Yang *et al.*, 1993; Meacham *et al.*, 1999; Farinha *et al.*, 2002) and Hsp90 (Loo *et al.*, 1998) facilitate early steps in CFTR biogenesis and its stabilization in the ER. Depending on its cochaperones, Hsc70/Hsp70 can either perform a folding function or a degradative role for their substrate protein. When co-acting with Hdj-2/Hdj-1, Hsc70/Hsp70 promotes CFTR stabilization (Farinha *et al.*, 2002), when co-acting with CHIP, Hsc70/Hsp70 promote proteasome mediated degradation of CFTR (Meacham *et al.*, 2001; Goldfarb *et al.*, 2006). The major difference between Hsp70 and Hsc70 was thought to be their mode of expression. However, recent work has shown that Hsp70 and Hsc70 have different effects on the biogenesis of membrane proteins. Hsp70 was observed to promote the surface expression of the ENaC, whereas Hsc70 decreased the functional expression of the channel (Goldfarb *et al.*, 2006).

**Hsp40:**

Members of the Hsp40 family stimulate Hsp70 ATP hydrolysis. It has been reported that two cytoplasmic Hsp40s, Hdj1 and Hdj2, interact with CFTR. Hdj2 binds to the channel only when NBD1 emerges from the ribosome whereas Hdj1 binds to CFTR throughout its synthesis (Meacham *et al.*, 1999). It has been shown that ~2-fold more Hdj2 binds to ΔF508 CFTR than to wild-type CFTR. Hence, Hdj2 may be involved in the selection of misfolded CFTR for degradation by the Hsp70/CHIP E3 ubiquitin ligase complex (Meacham *et al.*, 2001). The Hsc70-Hdj2 complex also has the ability to maintain the solubility of a large, cytoplasmic nucleotide binding domain in CFTR during the translation of the carboxy terminal portion of the protein, indicating its role in folding.
the other hand, it was found that overexpression of the cysteine string protein (Csp), a cytoplasmic Hsp40 previously implicated in vesicle trafficking (Chamberlain and Burgoyne, 1998), prevented the maturation of wild-type CFTR (Zhang et al., 2002). Interestingly, co-expression of Csp and Hdj2 led to an additive increase in ER-localized CFTR that was independent of Hsp70 association, and Csp on its own retained NBD1 in solution and prevented its aggregation (Zhang et al., 2006). These data suggest that Csp can protect and retain CFTR in the ER during its synthesis.

**CHIP:**

CHIP is shown to be directly involved in the selection of CFTR biogenic intermediates for degradation and forms complexes with the B form of both wild-type CFTR and ΔF508 CFTR (Meacham et al., 2001). CHIP interacts with Hsp90 and affects the fate of its substrates (Ballinger et al., 1999). It interacts with Hsc70 through a set of TPR motifs (Ballinger et al., 1999). The function of CHIP in membrane protein biogenesis was suggested by localization studies that showed that a portion of this cochaperone co-localizes with CFTR and Hsc70 at the ER (Meacham et al., 2001). Also, exogenous Myc–CHIP expression prevents the cell-surface localization of CFTR and causes it to display a perinuclear location (Moyer et al., 1998).

**NEFs of Hsp70:**

Recently, functional interaction of CHIP with other cochaperones of Hsc70 has been observed (Demand et al., 2001; Alberti et al., 2002; Alberti et al., 2004; Westhoff et al., 2005). The cochaperone BAG-1 stimulates the CHIP-mediated degradation of the glucocorticoid hormone receptor. The two cochaperones can bind simultaneously to Hsc70 (Demand et al., 2001). BAG-1 interacts with the amino terminal ATPase domain
of Hsc70 through its BAG domain and CHIP with the carboxy terminus (Hohfeld and Jentsch, 1997; Ballinger et al., 1999; Demand et al., 2001). The Hsc70 cochaperone HspBP1 has been identified as an inhibitor of CHIP (Alberti et al., 2004). Like BAG-1, HspBP1 binds to the ATPase domain of Hsc70 and is present in ternary complexes with Hsc70 and CHIP. It inhibits the ubiquitin ligase activity of CHIP and interferes with the CHIP-induced degradation of CFTR (Alberti et al., 2004). Also, BAG-2 was identified as a main component in CHIP complexes which stimulates the maturation of CFTR in conjunction with Hsc70. BAG-2 was shown to inhibit the ubiquitin ligase activity of CHIP by abrogating the CHIP/E2 cooperation. The activity of BAG-2 resembles that of the HspBP1 (Arndt et al., 2005).

**Valosin-containing protein (VCP):**

Mammalian p97/VCP and its yeast counterpart Cdc48 are known to participate in retrotranslocation of misfolded proteins from the ER for degradation by the cytosolic proteasomes (Wang et al., 2004a). It has been shown that p97/VCP and its cofactors (Ufd1, Npl4, and p47) interact with misfolded ubiquitinated substrates to dislodge them from the ER to the cytosol for proteasome degradation (Ye et al., 2003). It has also been reported recently that p97/VCP physically interacts with gp78 to couple ubiquitination, retro translocation, and proteasome degradation (Zhong et al., 2004). Recently, it was shown that p97/VCP and gp78 form complexes with CFTR during retro-translocation from the ER for degradation by the cytosolic proteasome. Interference with the VCP-CFTR complex promotes accumulation of immature CFTR in the ER and partially rescues functional chloride conductance of the cell surface (Vij et al., 2006). Hence it can
be said that VCP is directly associated with CFTR and is required for ERAD of CFTR, but it is still not clear if the VCP- and CHIP-mediated pathways operate in parallel to each other.

**Hsp90:**

Hsp90 is required for CFTR biogenesis (Loo *et al.*, 1998), and cellular depletion of the Hsp90 cochaperone Aha1 has been shown to stabilize CFTR (Wang *et al.*, 2006; Kadota *et al.*, 2008). However, the step where Hsp90 acts in the CFTR folding pathway is not clear. Administering an Hsp90 inhibitor has been shown to affect either the folding or degradation of CFTR, depending on how the experiment was performed. In vitro study shows that the Hsp90 inhibitor, GA, liberates Hsp90 from full-length CFTR and prevents its degradation (Fuller and Cuthbert, 2000). On the other hand, GA enhanced the degradation of CFTR in mammalian cells (Loo *et al.*, 1998) suggesting instead that chaperone release destabilizes CFTR and leads to its subsequent degradation. It is, therefore, possible that Hsp90 is involved in both folding and degradation of CFTR. A more folded conformation may allow Hsp90 to protect the protein until the final conformation is achieved, whereas a less mature form may be selected by Hsp90 for proteasome mediated degradation. There is a recent report that Hsp90 cochaperones can play either pro-folding or pro-degradation roles during ΔF508 CFTR maturation in mammalian cells (Wang *et al.*, 2006).
Calnexin:

The role of quality-control lectins on CFTR protein biogenesis has also been examined. The ER lumenal chaperone calnexin forms transient complexes with the ER localized and immaturely glycosylated B-form of CFTR (Pind et al., 1994). CFTR degradation is unaffected in calnexin knock out yeast (Zhang et al., 2001). In mammalian cells, the impact of calnexin on wild-type CFTR and ΔF508 CFTR biogenesis has been examined. The degradation of ΔF508 CFTR is unaffected when cells were treated with glucosidase inhibitors castanospermine and deoxynojirimycin, or mannosidase inhibitors kifunensine, but the degradation of wild-type CFTR requires mannose trimming (Farinha and Amaral, 2005). Calnexin overexpression is shown to have no effect on wild-type CFTR biogenesis, but slightly accelerates the degradation of ΔF508 CFTR. It is also reported that calnexin overexpression slows the ERAD of ΔF508 CFTR and wild-type CFTR (Okiyoneda et al., 2004), and that the overexpression of a calnexin fragment leads to the trafficking of a small amount of ΔF508 CFTR to the plasma membrane (Okiyoneda et al., 2002). Calnexin, and possibly calreticulin, may function as pre-protein ‘stabilizers’ for only the most aggregation-prone substrates (Svedine et al., 2004).
Hsp105α Promotes the Rescue of CFTR Misprocessing by Enhancing Folding in the ER and Escorting in Post-ER Compartments

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Abstract

Hsp105/110 facilitates the nucleotide exchange of Hsp70 and possesses holdase activity. We investigated the role of human Hsp105α, the constitutively expressed isoform, in the biogenesis of the cystic fibrosis transmembrane conductance regulator (CFTR). We show that Hsp105α regulates CFTR synthesis, maturation and degradation in the ER. The Hsp105α nucleotide exchange activity is necessary for the degradation of CFTR in the ER whereas its holdase activity reduces CFTR synthesis, stabilizes it in the ER and promotes its maturation. The combination of these activities leads to a dose-dependent effects on CFTR biogenesis. Misfolding of ΔF508 CFTR is the most common cause of cystic fibrosis. Hsp105α preferentially associates with ΔF508 CFTR, protects it from degradation in the ER and escorts the misfolded cargo protein in post-ER compartments. Hsp105α overexpression promotes ΔF508 CFTR rescue at both reduced and physiological temperatures. We conclude that Hsp105α is a versatile player in CFTR biogenesis. The functional interplay of its nucleotide exchange and holdase activities and its unique affinity for the misfolded ΔF508 CFTR make it a good molecular target for the rescue of CFTR misprocessing.
Introduction

Hsp105/110 belongs to the Hsp70 family of molecular chaperones and consists of an amino terminal ATPase domain and a carboxy terminal substrate binding domain (SBD) (Lee-Yoon et al., 1995). Hsp105 refolds denatured proteins more efficiently than Hsc70 in vitro (Oh et al., 1997), and its Hsc70-independent chaperone activity (or holdase activity) does not require its ATPase domain (Oh et al., 1999). In the cytosol, Hsp105 forms high molecular weight complexes with Hsc70 (Hatayama et al., 1998) and functionally relates to Hsc70 (Yamagishi et al., 2004; Shaner et al., 2005) as well as Hsp90 (Liu et al., 1999). Hsp105 facilitates the nucleotide exchange of Hsc70 (Dragovic et al., 2006; Raviol et al., 2006b). Yeast Hsp105 Sse1 collaborates with Hsp70 Ssb or Ssa in regulating the co-translational or post-translational folding of cellular proteins, respectively (Yam et al., 2005). There are two mammalian Hsp105 isoforms: Hsp105α and Hsp105β (Yasuda et al., 1995). Hsp105α is constitutively expressed and is further inducible by heat shock or stress. Hsp105β is strictly heat induced and is an alternatively spliced form of Hsp105α.

The cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP-binding cassette transporter whose deficiency leads to cystic fibrosis (CF) (Riordan et al., 1989a). CFTR is highly susceptible to misfolding due to mutations, which in turn leads to the retention of the nascent channel protein in the endoplasmic reticulum (ER) (Cheng et al., 1990; Smit et al., 1995; Seibert et al., 1996; Seibert et al., 1997; Vankeerberghen et al., 1998; Ostedgaard et al., 1999; Cormet-Boyaka et al., 2004; Clain et al., 2005) and its subsequent ER-associated degradation (ERAD) (Jensen et al., 1995; Ward et al., 1995). Most strikingly, the deletion of the phenylalanine at residue 508 (ΔF508) accounts for
70% of all CF-causing alleles and is seen in over 90% of CF patients (Cheng et al., 1990). Cytoplasmic chaperone machinery plays an important role in the biogenesis of CFTR. Hsp70 mediates the maturation as well as quality control of nascent CFTR (Yang et al., 1993; Meacham et al., 2001). Inhibiting Hsp90 activity prevents CFTR maturation and promotes its degradation (Loo et al., 1998). A global proteomic profiling of CFTR-associated proteins revealed an extensive cytoplasmic chaperone network containing Hsp70, Hsp90 and multiple cochaperones including Hsp105α (Wang et al., 2006). Given the importance of Hsp70 in CFTR biogenesis and the identified role for Hsp105 as a nucleotide exchange factor (NEF) for Hsp70, Hsp105α is likely to have an important role in regulating CFTR maturation and quality control. Hsp110 has been shown to associate with the cytoplasmic exposed apolipoprotein B peptide during its co-translational translocation across the ER membrane, stabilize it, and enhance its secretion, suggesting its stabilizing, pro-folding and pro-maturation roles (Hrizo et al., 2007).

In the current study, we conducted a systematic analysis of Hsp105α’s roles in CFTR biogenesis. We found that Hsp105α impacts three distinct aspects of the ER-associated folding of CFTR: synthesis, maturation and ERAD. The Hsp105α NEF activity is necessary for the ERAD of CFTR whereas its holdase activity reduces CFTR synthesis, inhibits its ERAD and promotes its maturation. The interplay of these two activities within the ER produces a dose-dependent effect on CFTR biogenesis. Hsp105α preferentially associates with ΔF508 CFTR in the ER, which provides extra protection to the mutant protein from ERAD. Interestingly, Hsp105α preferentially associates with ΔF508 CFTR in post-ER compartments and prolongs its half life. Furthermore, Hsp70 and Hsp90α also have much greater association with the mature form of ΔF508 CFTR,
suggesting a role for cytoplasmic chaperone machinery in escorting misfolded cargo molecules in post-ER compartments. These findings suggest that Hsp105α is a unique chaperone target custom designed for the rescue of protein misfolding in CF and perhaps other protein misfolding diseases.
Material and methods

Antibodies and Chemicals

CFTR monoclonal antibodies (mAbs) used in this study include MM13-4, M3A7, 13-1 and 24-1. Other antibodies used include anti-Hsp105 mAb (Novoceastra, Newcastle upon Tyne, UK), anti-Hsp70, anti-Hsp90α and anti-Hsp90β mAbs (Stressgen, Ann Arbor, MI), anti-Hsp70 polyclonal antibodies (Stressgen), anti-actin mAb (Millipore, Temecula, CA), and anti-FLAG mAb (Sigma, St. Louis, MO). Restriction enzymes, mung bean nuclease and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA). Protein G beads were purchased from GE Healthcare (Piscataway, NJ) or East Coast Bio (North Berwick, ME), and CHX and BFA from Sigma.

Plasmids

The Hsp105α shRNA expression plasmid was constructed using IMG-800-1 vector (Imgenex, San Diego, CA) according to the manufacturer’s protocol. The Hsp105α target sequence used is 5’–CAGCCATGTTGTTGACTAAGC–3’. The control plasmid contains a non-targeting sequence 5’–TCAGTCACGTTAATGGTCGTT–3’.

The pCMV-SPORT6 harboring human full-length Hsp105α coding sequence (pCMV-SPORT6-Hsp105α) was obtained from ATCC (Manassas, VA) and was the basis for constructing Hsp105α mutants. Amino acid sequence alignment was performed using the Sequence Manipulation Suite software (Stothard, 2000).

The pCMV-SPORT6-Hsp105α was digested with Xho I and Not I. Blunt ends were generated by treatment with mung bean nuclease, and the plasmid was re-
circularized by ligation to produce pCMV-SPORT6-Hsp105α-MB. This removes the extra Xba I site in the multiple cloning sites region so that the Xba I site within Hsp105α coding sequence becomes unique. The amino terminal Kpn I-Xba I fragment was used as a cassette to introduce the G232D substitution by overlapping polymerase chain reactions (PCR) from pCMV-SPORT6-Hsp105α-MB. To construct the ΔATP (Met-384-858) mutant, the carboxy terminal Sal I-Pst I fragment was used as a cassette. The methionine was added before residue 384 to serve as a start codon to generate pCMV-SPORT6-Hsp105α-ΔATP.

The shRNA-refractory Hsp105α expression plasmid was constructed by introducing 3 silent mutations in the shRNA target sequence of pCMV-SPORT6-Hsp105α-MB to yield 5’-CAGCCATGCTGCTCTACTAAGC-3’. The mutations were introduced by overlapping PCR and the DNA fragment was ligated back into the Sal I-Xba I cassette of the same plasmid.

To construct the carboxy terminal FLAG-tagged Hsp105α, a 1.2 kb carboxy terminal fragment of Hsp105α starting from the native Xba I site and ending before the stop codon was amplified from pCMV-SPORT6-Hsp105α-MB and inserted between the Xba I and Sma I sites of pFLAG-CMV vector (Sigma) to yield pFLAG-CMV-Hsp105α-C. The junction was so designed that the carboxy terminal FLAG tag is in frame with Hsp105α. Then the amino terminal fragment of Hsp105α was subcloned from pCMV-SPORT6-Hsp105α-MB into pFLAG-CMV-Hsp105α-C using the Kpn I-Xba I cassette to yield pFLAG-CMV-Hsp105α. The FLAG-tagged Hsp105α G232D expression plasmid was constructed by cloning the amino terminal Kpn I-Xba I fragment from the pCMV-SPORT6-Hsp105α-G232D into the pFLAG-CMV-Hsp105α-C. The FLAG-tagged
Hsp105α ΔATP expression plasmid was constructed by cloning the amino terminal Sal I-Xba I fragment from pCMV-SPORT6-Hsp105α-ΔATP into pFLAG-CMV-Hsp105α-C. All plasmids containing PCR-generated fragments were sequence confirmed.

To construct a plasmid suitable for Hsp105α stable expression, we subcloned the coding sequence of Hsp105α from pCMV-SPORT6-Hsp105α into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) through Kpn I and Not I sites. The pcDNA3-EGFP was obtained from Addgene (Cambridge, MA), and the EGFP coding sequence was subcloned into pcDNA3.1(+) through Xho I and Xba I sites to generate pcDNA3.1(+-EGFP. The CFTR expression plasmids pcDNA3.1(+-CFTR-WT, pcDNA3.1(+-CFTR-ΔF508 and pcDNA3.1(+-CFTR-DAA have been described previously (Roy et al., 2010). The pcDNA3.1(-)-Myc-Rab3A expressing Myc-tagged wild-type Rab3A was provided by Dr. William Balch (The Scripps Research Institute, La Jolla, CA).

**Cell Culture and Transfection**

Human embryonic kidney 293 (HEK) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS) and 100 units/mL each of penicillin and streptomycin. The HEK cells stably expressing ΔF508 (HEK-ΔF) or wild-type CFTR (HEK-WT) (Silvis et al., 2003; Roy et al., 2010) were maintained in the above medium supplemented with 150 μg/mL hygromycin B (EMD Chemicals, Gibbstown, NJ) as described previously (Roy et al., 2010). Cell transfection was performed using Lipofectamine 2000 (Invitrogen). HEK cells were transfected with Hsp105α shRNA or control shRNA construct and stable cell lines were generated by clonal selection with 400 μg/mL G418. To produce HEK-ΔF cells stably overexpressing
Hsp105α or vector control, HEK-ΔF cells were transfected with pcDNA3.1(+)–Hsp105α or pcDNA3.1(+), and selected with 400 μg/mL G418. Once generated, the stable cell lines were maintained in appropriate media supplemented with 200 μg/mL G418.

Cell Lysis and Quantitative Immunoblotting

Cells were lysed on ice in Triton X-100 lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 (v/v) and the Complete™ protease inhibitor cocktail (Roche Diagnostics GmbH, Mannhem, Germany) for 30 min. Insoluble material was removed by centrifugation at 16,000 g for 20 min. Protein concentration was determined by Bradford Assay. Cell lysates were separated by SDS-PAGE, immunoblotted with relevant antibodies and visualized by ECL. Protein loading was adjusted according to its intracellular abundance. Multiple exposures were taken to ensure that the intensity of bands is within the dynamic range. The protein bands are quantified by densitometry using ImageJ software (National Institutes of Health).

Pulse-chase Analysis

Equal number of cells were seeded in 60 mm cell culture dishes. The cells were transfected with CFTR alone or in combination with other expression plasmids. After 16-24 hours, the CFTR-expressing cells were pulse labeled with EasyTag Express 35S Protein Labeling Mix (Perkin Elmer, Waltham, MA) for 30 min and chased for the indicated time. Cells were lysed as described above and CFTR was immunoprecipitated.
The radio-labeled CFTR were visualized by autoradiography and quantified by phosphorimaging using a Typhoon TRIO Variable Mode Imager (GE Healthcare).

**Quantitative Co-immunoprecipitation**

HEK cells transiently transfected with plasmids encoding different forms of CFTR were lysed as described above. CFTR was immunoprecipitated with Protein G Sepharose beads coated with CFTR mAb as described previously (Wang et al., 2008). For co-immunoprecipitation of FLAG-tagged Hsp105α proteins, HEK cells were transiently transfected with FLAG-tagged Hsp105α constructs. The cell lysates were incubated with anti-FLAG mAb on ice for 1 hour before Protein G Sepharose beads were added. The mixture was rocked at 4°C for 1 hour or overnight before the beads were recovered, washed and eluted. For all co-immunoprecipitation experiments, HEK cells mock transfected were included to control for non-specific binding of proteins to the Protein G Sepharose beads.
Results

**Hsp105α regulates the synthesis, maturation and ERAD of CFTR.**

To test the role of Hsp105α in CFTR biogenesis, we co-transfected HEK cells with a fixed dose of wild-type CFTR and increasing doses of Hsp105α or Myc-tagged Rab3A (control), and assessed the steady state levels of CFTR in the ER-localized core-glycoform (bands B) and the Golgi-processed complex glycoform (band C) (Figure 1A). To facilitate comparison, we normalized the levels of band B, band C and the C/B ratio in Hsp105α-overexpressing cells to those in the control cells. We observed a dose-dependent decrease in the fold of change in band B (Figure 1D) but the reduction in the fold of change in band C is not obvious at lower Hsp105α dosage (up to 0.5 μg) (Figure 1C). As a result, the change in the C/B ratio increases at doses of up to 2 μg (Figure 1E), suggesting an underlying increase in CFTR processing. With the increase in Hsp105α dosage, the level of Hsp105α was elevated by up to 15 fold (Figure 1B and 1F).

Changes in the steady state levels of CFTR in bands B and C can result from changes in synthesis, processing, degradation or the combinations thereof. We conducted pulse-chase analysis at three representative Hsp105α doses, and observed a dose-dependent reduction in the amount of radioisotope incorporation into CFTR after the 30-minute pulse (Figure 1G-I, upper), suggesting that Hsp105α overexpression inhibits CFTR synthesis in a dose-dependent manner.

The post-translational turnover of CFTR band B and its processing to band C during the chase period also show dose-dependent changes (Figure 1G-I). At 0.2 μg (~4-fold increase in Hsp105α based on Figure 1F), we observed post-translational destabilization of band B (Figure 1G, middle), indicating a role for Hsp105α in the
ERAD of CFTR. The slight decrease in the rate of appearance of band C (Figure 1G, lower) most likely results from the enhanced degradation of band B, suggesting that the processing efficiency per se was not compromised. At 1 μg (~11-fold increase in Hsp105α), we observed a slight stabilization of band B, and a dramatic increase in processing (Figure 1H), suggesting that, at higher expression level, Hsp105α reduces CFTR ERAD and promotes its maturation. At 4 μg (~15 fold increase in Hsp105α), no major change in the ERAD or processing of CFTR was observed (Figure 1I). Therefore, the decrease in the steady state level of CFTR at 4 μg of Hsp105α (Figure 1A) is solely attributable to the massive reduction in CFTR synthesis (Figure 1 I).

We conducted similar dose response analysis on ΔF508 CFTR which is defective in maturation due to conformational defects (Cheng et al., 1990; Lukacs et al., 1994). A dose-dependent decrease in the steady state level of ΔF508 CFTR band B is apparent except that such decrease appears to be less sensitive to the increase in Hsp105α dosage (Figure 2A-C). Pulse-chase analysis revealed a dose-dependent decrease in CFTR synthesis (Figure 2D-F). The destabilization of band B at low dosage is less pronounced (Figure 2D). The stabilization of band B at intermediate dosage might have partially compensated for the reduction in synthesis (Figure 2E) so that the steady state level of band B is not dramatically reduced (Figure 2B). Again, at high dosage, the reduction in synthesis (Figure 2F) is the major cause for the decrease in the steady state level of CFTR (Figure 2C).

To confirm the above roles for Hsp105α in CFTR biogenesis, we knocked down its expression using shRNA. Due to the high abundance of the protein (Easton et al., 2000), significant knockdown was observed only after stable expression of the shRNA,
which led to a decrease in Hsp105α level by 80% (Figure 3A, middle and lower). This decrease in Hsp105α was not accompanied by significant changes in major cytoplasmic chaperones such as Hsp70, Hsc70, Hsp90α and Hsp90β (Figure 3A, middle and lower).

Surprisingly, knocking down Hsp105α does not dramatically alter the steady state level of wild-type CFTR in band B or C (Figure 3A, upper). We only observed a mild but statistically insignificant increase in the level of band B. Pulse-chase analysis revealed a 20% increase in CFTR synthesis in the Hsp105α-knockdown cells with minimal changes in band B turnover or processing (Figure 3B). When shRNA-refractory Hsp105α was introduced into the knockdown cells, CFTR synthesis was decreased by 33% and its maturation was increased with minimal increase in band B stability (Figure 3C). These phenotypes parallel those of Hsp105α overexpression at 1 μg (Figure 1H) except to a lesser extent.

Hsp105α knockdown increases the steady state level of ΔF508 CFTR and its rescue reduces it in a dose-dependent manner (Figure 3D). As revealed by pulse-chase analysis, Hsp105α knockdown increases ΔF508 CFTR synthesis by 52% and slightly destabilizes its band B post-translationally (Figure 3E). In contrast, its rescue decreases ΔF508 synthesis by 40% and stabilizes the band B (Figure 3F). In both the knockdown and rescue, Hsp105α’s effect on CFTR synthesis overpowered its effect on band B stability, leading to corresponding changes in the steady state level of ΔF508 CFTR (Figure 3D). The enhancement of the stability of ΔF508 CFTR by Hsp105α rescue (Figure 3F) parallels the stabilization of the band B of both wild-type (Figure 1H) and ΔF508 (Figure 2E) CFTR by Hsp105α overexpression at 1 μg.
For Hsp105α overexpression experiments (Figures 1 and 2), Myc-tagged Rab3A, a small G-protein specializing in regulated exocytosis in neuroendocrine cells (Schluter et al., 2002), was used as the control for Hsp105α. To verify that such effects were not produced by Myc-Rab3A, we have used EGFP as the control for Hsp105α rescue in knockdown cells (Figure 3C and 3F). In both cases, we consistently observed a role for Hsp105α in reducing CFTR synthesis, inhibiting the ERAD of ΔF508 CFTR and promoting the maturation of wild-type CFTR. We further evaluated the impact of different control plasmids on the steady state level of wild-type CFTR after co-transfection with CFTR. We found that, at 4 μg, the empty vector, Myc-Rab3A and EGFP have similar effects on the steady state level of CFTR whereas Hsp105α dramatically reduces the levels of both bands B and C (Figure S1A).

Taken together, our data indicate that Hsp105α reduces the synthesis and regulates the ERAD and maturation of CFTR in a dose-dependent manner.

**Hsp105α promotes the rescue of CFTR misprocessing.**

Given that Hsp105α regulates multiple aspects of CFTR biogenesis, we explored its potential role in ΔF508 CFTR processing. As ΔF508 CFTR displays a temperature-sensitive processing phenotype (Denning et al., 1992), we first tested if Hsp105α overexpression promotes ΔF508 maturation at reduced temperature, and the answer is yes. We observed an increase in the steady state level of band C but not band B (Figure 4A). Surprisingly, under identical condition, the same fold of increase in Hsp105α led to reduced steady state levels of wild-type CFTR in bands B and C (Figure 4B), presumably due to inhibition of synthesis (Figure 1H and 1I). This is consistent with the higher
sensitivity of the steady state level of wild-type CFTR to the increase in Hsp105α dosage than ΔF508 (Figures 1 and 2). To confirm the effect of Hsp105α on the temperature dependent rescue of ΔF508 CFTR, we stably overexpressed Hsp105α in HEK-ΔF cells (Silvis et al., 2003; Roy et al., 2010), and subjected the cells to low temperature time course (Wang et al., 2008). We observed an increase in the kinetic conversion from band B to C as reflected in an elevated rate of increase in C/B ratio (Figure 4C).

To probe the mechanism underlying the phenotypic difference between wild-type and ΔF508 CFTR upon Hsp105α overexpression at reduced temperature (Figure 4A and 4B), we assessed the Hsp105α association with wild-type, an ER exit code mutant (DAA) (Wang et al., 2004b) and ΔF508 CFTR within the ER by quantitative co-immunoprecipitation. Cells expressing wild-type, DAA and ΔF508 CFTR were treated with brefeldin A (BFA) to block the ER-to-Golgi trafficking so that the vast majority of the CFTR molecules are retained in the ER. We found that ΔF508 CFTR has a much greater association with Hsp105α than wild-type or DAA CFTR (Figure 4D). As ΔF508 CFTR is known to have more extensive association with Hsp70s (Yang et al., 1993; Roy et al., 2010) and Hdj-2 (Meacham et al., 1999) than wild-type CFTR, our data suggest that an Hsp70 chaperone complex containing both Hdj-2 and Hsp105α is extensively involved in ΔF508 CFTR folding, and this may contribute to the observed difference in the response of wild-type and ΔF508 CFTR toward the same changes in the level of Hsp105α.

We further tested if Hsp105α overexpression enhances ΔF508 processing at physiological temperature, and the answer is yes as well. In HEK cells, a partially processed complex glycosylated species of ΔF508 CFTR with a slightly lower apparent
molecular weight than the normal band C has been consistently observed at 37°C. This band arises from the residual processing of ΔF508 CFTR and was named as band I (Wang et al., 2008). Overexpressing Hsp105α not only increases the total level of complex glycosylated CFTR (bands I + C) but also leads to a dramatic shift from band I to the fully processed band C (Figure 5A). This indicates a qualitative improvement in ΔF508 CFTR processing. The same shift from band I to C was also observed during the low temperature rescue of ΔF508 CFTR in HEK cells (Wang et al., 2008). Interestingly, the observed qualitative and quantitative enhancement in ΔF508 CFTR processing is not accompanied by an increase in the level of band B in HEK cells (Figure 5A), suggesting no increase in the ER accumulation of the mutant CFTR. Under the same condition, we have observed an even greater increase in band C of the exit code mutant DAA and its level of band B is unchanged either (Figure 5B). This suggests that the enhancement of rescue of CFTR misprocessing by Hsp105α overexpression is not restricted to conformational mutant such as ΔF508 CFTR but extends to the DAA mutant which has minimal conformational defect (Roy et al., 2010). The greater increase in the mature form of DAA CFTR might be partially attributable to its higher post-ER stability (Roy et al., 2010).

**Contributions of Hsp105α NEF and holdase activities to CFTR biogenesis.**

Hsp105α accelerates the nucleotide exchange of Hsp70 (Dragovic et al., 2006; Raviol et al., 2006b) and has holdase activity (Oh et al., 1999). To assess the contributions of these activities to CFTR biogenesis, we took advantage of two well-characterized Hsp105α mutants: the G233D mutant (Shaner et al., 2004) and the ATPase domain deletion mutant
(ΔATP) (Oh et al., 1999). The G233D substitution in yeast Sse1 blocks its ATP-binding, impairs its Hsp70 interaction, dramatically diminishes its NEF activity, and abolishes Sse1 biological activity (Shaner et al., 2004; Shaner et al., 2005; Dragovic et al., 2006). Based on sequence alignment (Figure 6A), we constructed the corresponding mutant in human Hsp105α (G232D, or G-D). Deletion of the ATPase domain of Hsp110 is known to abolish its NEF activity (Dragovic et al., 2006). Both the G-D and the ΔATP mutants retain the holdase activity at a level comparable to the wild-type protein in vitro (Oh et al., 1999; Goeckeler et al., 2002).

Overexpressing Hsp105α at 4 μg dramatically reduces the steady state level of wild-type CFTR (Figure 1A, 1C and 1D), and this is attributable to its inhibition of CFTR synthesis (Figure 1 I). We co-expressed wild-type CFTR with 4 μg of the G-D or ΔATP mutant and measured the steady state level of CFTR in bands B and C (Figure 6B). Expression of the G-D mutant partially reverses the dramatic decrease in band B but only slightly increases the level of band C (Figure 6B and 6C). In contrast, the expression of the ΔATP mutant increases the steady state levels of both band B and band C (Figure 6B and 6C). At the same dosage, the ΔATP mutant has much lower protein level than the full-length Hsp105α (Figure 6B, arrowhead), and the same has been observed for the FLAG-tagged Hsp105α (Figure 7C). Interestingly, such a low level of ΔATP mutant produces a dramatic increase in CFTR band B with concomitant increase in band C (Figure 6B and 6C).

At the same dosage when co-expressed with ΔF508 CFTR, Hsp105α increases the level of bands I + C of ΔF508 CFTR without significant change in the level of band B (Figure 6E and 6F). The G-D mutant reverses such an effect and the ΔATP mutant
increases both band B and band C (Figure 6E and 6F). Unlike wild-type Hsp105α, overexpressing the G-D mutant which lacks the NEF activity but retains the holdase activity failed to rescue ΔF508 CFTR (Figure 6E and 6F), suggesting a potential role for Hsp105α NEF activity in ΔF508 rescue. The increase in the level of ΔF508 band B by ΔATP expression (Figure 6E and 6F) parallels its effect on wild-type CFTR (Figure 6B and 6C).

At 6 μg, further increase in Hsp105α dosage reduced the steady state level of ΔF508 CFTR (Figures 2C and 6H), which is attributable to reduction in synthesis (Figure 2F). The G-D mutant not only reverses such an effect but further increases the steady state levels of both band B and band C (Figure 6H and 6I). The expression of the ΔATP mutant had an even greater effect (Figure 6H and 6I). Given that the level of the ΔATP mutant is much lower than that of the G-D mutant (Figure 6B, 6E and 6H), it is clear that ΔATP is more effective in increasing the steady state level of CFTR band B. The overexpression of Hsp105α or its mutants has minimal effect on the level of major cytoplasmic chaperones Hsp70, Hsc70, Hsp90α and Hsp90β (Figure 6D, 6G and 6J), suggesting that such effects are largely attributable to changes in Hsp105α.

To further define the role of the G-D and ΔATP mutants in CFTR biogenesis, we first co-expressed 4 μg of wild-type CFTR with 4 μg of the G-D mutant in HEK cells and performed pulse-chase analysis. The G-D mutant decreases CFTR synthesis by 54%, stabilizes band B and promotes its processing (Figure 7A), the combination of which might have led to a modest decrease in band B and a more dramatic decrease in band C at the steady state when compared with the non-overexpression control (Figure 6 B and C, Cntrl). Then, we co-expressed 4 μg of ΔF508 CFTR with 4 μg of the ΔATP mutant, and
found that the ΔATP mutant reduces CFTR synthesis by 35% but dramatically stabilized ΔF508 CFTR in the ER (Figure 7B), the combination of which might have led to the increase in band B and corresponding increase in band C at the steady state when compared with the non-overexpression control (Figure 6E and 6F, Cntrl). Consistent with the similar activities of the G-D and ΔATP mutants in vitro (i.e. the lack of NEF activity and presence of full holdase activity), both mutants reduce CFTR synthesis and ERAD, and promote its maturation. As overexpressing wild-type Hsp105α at 4 μg reduces CFTR synthesis but has minimal effect on its ERAD or processing (Figure 1I), the NEF activity of Hsp105α must be essential for the ERAD of CFTR and may be involved in the regulation of its maturation.

Given the striking difference in the steady state level of G-D and ΔATP, the ΔATP mutant is more potent in inhibiting the ERAD of CFTR. We tested if the G-D mutant is able to associate with Hsp70 by co-immunoprecipitation, and found that the FLAG-tagged G-D mutant associates with Hsc70 to a similar degree as the FLAG-tagged wild-type Hsp105α (Figure 7D). Since the ATPase domain of Hsp105α is necessary and sufficient for its interaction with Hsp70 (Shaner et al., 2006), the ΔATP mutant is not expected to associate with Hsp70. Due to the extremely low expression level of the ΔATP mutant (Figures 6B, 6E, 6H and 7C), we tested its Hsp70 association in a separate and scaled-up experiment, and found that indeed the ΔATP mutant failed to associate with Hsc70 above the mock level (Figure 7E). Therefore, unlike the ΔATP mutant, the G-D mutant is fully capable of being incorporated into the Hsp70-containing chaperone complex. This in turn explains the different steady state levels of the two mutants despite

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the same dosage of transfection. The ΔATP mutant that fails to associate with Hsp70 might be more susceptible to degradation.

Taken together, the above data support a major role for Hsp105α holdase activity in reducing the synthesis, decreasing the ERAD, and promoting the maturation of CFTR. In contrast, its NEF activity is necessary for promoting the ERAD, and possibly regulating the maturation of CFTR.

**Hsp105α escorts ΔF508 CFTR in post-ER compartments.**

ΔF508 mutation negatively impacts the ER export (Cheng *et al.*, 1990) and post-ER stability of CFTR at physiological temperature (Lukacs *et al.*, 1993), leading to severe misprocessing at the steady state (Roy *et al.*, 2010). The rescue of ΔF508 CFTR misprocessing by Hsp105α overexpression at 37°C is striking, especially in the absence of an increase in ER accumulation of the mutant protein (Figures 5A and 6E and 6F). As increase in the level of complex glycosylated ΔF508 CFTR can be produced by enhanced export, elevated post-ER stability, or both, we measured the post-ER stability of ΔF508 CFTR in the absence and presence of Hsp105α overexpression by cycloheximide (CHX) chase. As shown in Figure 8A, Hsp105α overexpression dramatically improves the post-ER stability of ΔF508 CFTR. In this experiment, Myc-Rab3A was used as the control. To exclude the rare possibility that Rab3A might have caused such difference, we repeated the experiment using EGFP as the control, and the same effect was observed (Figure S1B).

To probe the potential mechanism, we assessed the degree of association of Hsp105α with the immature and mature form of wild-type and ΔF508 CFTR. At 37°C,
the vast majority of ΔF508 CFTR exists in band B. To obtain the band C of ΔF508 CFTR, we incubated HEK-ΔF cells at reduced temperature to allow processing to occur and then treated the cells with CHX to remove band B. We then assessed the association of either the band B or band C of ΔF508 CFTR with Hsp105α by quantitative co-immunoprecipitation using a CFTR mAb. HEK cells expressing wild-type CFTR (HEK-WT) (Silvis et al., 2003; Roy et al., 2010) were treated in the same manner as a control. Since both bands B and C are present in wild-type CFTR at 37°C, we quantitatively compared the Hsp105α’s association with bands B+C with its association with band C alone.

Strikingly, we observed a much greater association of Hsp105α with the post-ER form of ΔF508 CFTR than with its ER form (Figure 8B and 8C). This is not caused by the lowering of the temperature and/or the treatment with CHX as, under the same condition, less association of Hsp105α was observed with the band C of the wild-type CFTR at 30°C than with the combination of bands B and C at 37°C, which suggests that the band B of wild-type CFTR associates more extensively with Hsp105α than its band C (Figure 8D). Overall, we have observed much lower Hsp105α association with wild-type CFTR than with ΔF508 CFTR, and this is true for both band B and band C (Figure 8C and 8D). These findings suggest a potential role for Hsp105α in escorting ΔF508 CFTR in post-ER compartments.

To test the association of CFTR with other cytoplasmic chaperones, we extended the analysis to include Hsp70, Hsc70, Hsp90α and Hsp90β, and found that Hsp70 and Hsp90α have dramatically higher association with the band C than with band B of ΔF508 CFTR (Figure 8C). Hsc70 only showed a moderate increase in association with ΔF508
band C as compared with band B, and the association of Hsp90β with post-ER ΔF508 CFTR was hardly detectable (Figure 8C). Again, the association of Hsp70s and Hsp90s with wild-type CFTR is generally much lower than with ΔF508 CFTR for both bands B and C (Figure 8B-D). Furthermore, with wild-type CFTR, slightly lower association of these chaperones with band C was observed than with the combined bands B and C (Figure 8D), suggesting that these cytoplasmic chaperones have slightly greater association with wild-type CFTR in the ER than in post-ER compartments. These data point to the possibility that cytoplasmic Hsp70s, Hsp90s, and perhaps their cochaperones regulate the post-ER stability of ΔF508 CFTR by specifically escorting the mutant cargo protein in post-ER compartments. It remains to be determined where and how such escorting occurs, and whether these chaperones function as a team or individually.
Discussion

**Hsp105α is a versatile player in the ER-associated folding of CFTR.**

Cytoplasmic chaperone machinery plays a critical role in the biogenesis of integral membrane cargo proteins. Major cytoplasmic chaperones such as Hsp70 and Hsp90 have multiple roles in distinct stages of membrane protein biogenesis. Understanding their specific roles in each stage is an important but challenging task. In the current study, we examined the roles of Hsp105α, an Hsp70 NEF and a holdase, in CFTR biogenesis. Using a combination of steady state and pulse-chase analyses, we found that Hsp105α regulates CFTR biogenesis in the ER at three levels: synthesis, post-translational maturation and ERAD.

Hsp105α knockdown, rescue, overexpression and dose response analyses (Figures 1-3) revealed that Hsp105α reduces the synthetic yield of both wild-type and ΔF508 CFTR in a dose-dependent manner. The reduction in synthesis is likely due to Hsp105α’s impact on the co-translational folding of CFTR as both Hsp70 and Hsp110 have been implicated in co-translational folding of membrane proteins in the ER (Oberdorf *et al.*, 2005; Yam *et al.*, 2005). This effect is attributable to its holdase rather than its NEF activity as both the G-D and ΔATP mutants retain such an effect (Figure 7). Therefore the reduction in CFTR synthesis is likely independent of Hsp70. As Hsp110 has been shown to inhibit the co-translational degradation of apolipoprotein B during its translocation across the ER membrane (Hrizo *et al.*, 2007), the reduction of CFTR synthesis by Hsp105α might result from inefficient co-translational folding.

A pro-maturation role has been identified for Hsp105α. This effect is apparent on wild-type CFTR (Figures 1 and 3), on ΔF508 CFTR at reduced temperature (Figure 4),
and even on the DAA mutant (Figure 5B). In the case of wild-type CFTR, such a role is contributed by its holdase activity (Figure 7A) but appears to be inhibited by the presence of its NEF activity at high Hsp105α dosage (Figure 1I). On the other hand, the rescue of ΔF508 CFTR appears to require its NEF activity as the G-D mutant fails to do so at the same expression level (Figure 6E and 6F). The mechanism by which the Hsp105α NEF activity regulates CFTR maturation remains unclear. Hsp105α may promote Hsp70-mediated folding post-translationally by regulating its ATPase cycle. Yeast Sse1 has been shown to be an Hsp90 cochaperone (Liu et al., 1999), and plays an important role in the regulation of client protein maturation and degradation in the cytosol (Mandal et al., 2010). It is likely that an analogous mechanism exists on the cytoplasmic face of the ER membrane, where the Hsp70-Hsp90 chaperone system (Wang et al., 2006) actively regulates the conformational maturation of CFTR. It is worth noting that such a pro-maturation effect only occurs within a specific concentration range (Figure 1), suggesting that a proper ratio between Hsp105α and Hsp70 or its cochaperones such as Hdj-2 is essential in providing an optimal chaperone environment for the promotion of CFTR maturation. Understanding the parameters of such an environment will help maximize the cell’s ability to process CFTR.

The effect of Hsp105α on the post-translational ERAD of CFTR is less straightforward. Mild overexpression of Hsp105α promotes the ERAD of wild-type CFTR (Figure 1G) whereas further increase in Hsp105α dosage slightly improves CFTR stability in the ER (Figure 1H). A similar trend has been observed with ΔF508 CFTR except to a lesser extent (Figure 2D and 2E). In fact, the stabilization effect of Hsp105α on the ER-localized ΔF508 CFTR is the major contributing force counteracting the
reduction in its synthesis at intermediate dosage, which maintains the steady state level of band B relatively constant (Figures 2B, 2E, 5A, 6E and 6F). The promotion of the ERAD of CFTR by Hsp105α is consistent with Hsp70’s role in CHIP-mediated ER quality control (Connell et al., 2001; Meacham et al., 2001). Such a role for Hsp105α is dependent upon its NEF activity as the G-D mutant which is specifically deficient in NEF activity reduced the ERAD of wild-type CFTR at high dosage (Figure 7A) but not wild-type Hsp105α at the same dosage (Figure 1I). The stabilization of CFTR in the ER at 1 μg of Hsp105α (Figures 1H and 2E) might be attributed to its holdase activity. Therefore, it is likely that the NEF and holdase activities of Hsp105α represent two competing forces in the regulation of the ERAD of CFTR. At low level of overexpression, the NEF-mediated pro-degradation effect dominates (Figures 1G, and 2D). At intermediate level, the holdase activity overpowers by a narrow margin (Figures 1H and 2E). At high level, the two forces are cancelled out (Figures 1I and 2F). On top of the above regulation, Hsp105α’s effect on CFTR synthesis weighs in to finally dictate the steady state level of CFTR (Figures 1A, and 2A-C).

**Differential impact of Hsp105α on the ER-localized wild-type and ΔF508 CFTR.**

At close-to physiological levels, Hsp105α inhibits the ERAD of ΔF508 but not wild-type CFTR (Figure 3B, 3C, 3E and 3F). We also observed a greater increase in the synthesis of ΔF508 than wild-type CFTR upon Hsp105α knockdown (Figure 3B and 3E). Moreover, ΔF508 CFTR is more resistant to the dose-dependent reduction in the steady state level of band B than wild-type CFTR (Figures 1, 2, 4 and 6). These differences are attributable to the different extent of association of Hsp105α with the two forms of CFTR.
in the ER (Figure 4D). Unlike wild-type CFTR, ΔF508 is trapped in a chaperone-
protected folding intermediate (Lukacs et al., 1994). Furthermore, the kinetic differences
in their conformational maturation might also be part of the equation.

Interestingly, the Hsp105α dose response curve for CFTR band C level (Figure
1C) shows striking resemblance to the dose response curve for in vitro folding activity of
Hsp110 in combination with Hsc70 and DJA2 (Tzankov et al., 2008). This suggests that,
in living cells, Hsp105α collaborates with Hsp70 and Hsp40 in mediating CFTR
maturation, and that the disproportional increase in Hsp105α level limits the ability of the
chaperone complex to fold its client molecules efficiently. In the case of CFTR, it may
lead to impaired co-translational folding and reduced synthesis. However, within certain
dosage range, overexpressing Hsp105α can have beneficial effect on CFTR processing
and ΔF508 CFTR rescue.

**A prominent role for Hsp105α holdase activity in CFTR biogenesis.**

The most essential cellular functions of Hsp105 depend on its NEF activity
(Dragovic et al., 2006; Raviol et al., 2006b). This is demonstrated by the rescue of the
defective growth phenotype of the sse1,2Δ cells by other nucleotide exchange factors in
yeast (Sadlish et al., 2008). On the other hand, Hsp105 possesses holdase activity that
prevents aggregation and facilitates refolding of denatured proteins (Oh et al., 1997).
Consistent with this, Hsp105 stabilizes apolipoprotein B from co-translational ERAD
when the rate of translocation falls behind the rate of translation (Hrizo et al., 2007).

Our data underscore a prominent role for Hsp105α holdase activity in CFTR
biogenesis. Both the G-D and ΔATP mutants stabilizes CFTR in the ER, enhances its
maturation and reduces its synthesis (Figure 7A and 7B). In contrast, its NEF activity is important for CFTR ERAD (Figures 1I and 7A) and may regulate CFTR maturation through an as yet poorly defined mechanism (Figures 1I, 6E, 6F and 7A). Despite its low expression level (Figure 6B, 6E and 6H), the ΔATP mutant potently inhibits the ERAD of CFTR (Figure 7B). The absence of the NEF-mediated pro-ERAD effect combined with the stabilizing effect conferred by its holdase activity greatly increases the foldable pool of ΔF508 CFTR in the ER (Younger et al., 2004), and this more than compensates for the effect produced by the reduction of synthesis, leading to a significant increase in the steady state level of band B and corresponding increase in the level of band C (Figure 6B, 6C, 6E, 6F, 6H and 6I).

By contrast, the G-D mutant is less potent in stabilizing band B (Figure 7A) despite its higher expression level. The G-D mutant associates with Hsc70 to a similar extent as wild-type Hsp105α (Figure 7D), suggesting that the G-D mutant is still retained in the cytoplasmic chaperone complex in mammalian cells. The association of the G-D mutant with Hsp70s has been observed in yeast but to a much lesser extent (Shaner et al., 2005). The cause of such difference is not entirely clear. In the yeast study, the epitope-tagged Sse1 proteins were reintroduced into the Sse1 knockout strain (Shaner et al., 2005), whereas, in the current study, we expressed the tagged Hsp105α in cells containing endogenous level of Hsp105α. It is possible that Sse1 knockout has severely perturbed chaperone complex formation and that the adding-back of the mutant Sse1 was unable to fully restore the functionality of the Hsp70-containing chaperone complex. The presence of the “NEF-dead” G-D mutant in the chaperone complex may have secondary
effects on the functionality of the Hsp70 chaperone complex and therefore contribute to the different phenotypes of the G-D and ΔATP mutants.

**Implication in the treatment of CF and other protein misfolding diseases.**

The current study reveals multiple roles for Hsp105α in CFTR biogenesis that may be exploited to enhance the rescue of ΔF508 CFTR (Figure 9A). The maturation of the ΔF508 CFTR in the ER is arrested in a chaperone-protected, early folding intermediate (Figure 9B). Enhanced Hsp105α activity reduces ΔF508 synthesis to prevent its excessive ER accumulation. In the meantime, its holdase activity curbs the ERAD and promotes the refolding and maturation of ΔF508 CFTR. Through its NEF activity, terminally misfolded ΔF508 CFTR is cleared away. Through its association with Hsp70 and potentially other cytoplasmic folding components, Hsp105α might functionally integrate into a larger cytoplasmic chaperone complex and further facilitate ΔF508 CFTR maturation. An in-depth understanding of the functional regulation of Hsp105α in relation to its chaperone partners will reveal the molecular mechanism of chaperone-mediated CFTR maturation on the cytoplasmic side of the ER membrane. This in turn will facilitate the development of novel therapeutics for the rescue of ΔF508 misprocessing.

The ΔF508 CFTR is severely impaired in post-ER stability at physiological temperature (Lukacs et al., 1993) and this results from its accelerated endocytosis (Swiatecka-Urban et al., 2005). We identified a potential role for Hsp105α in escorting the misfolded ΔF508 CFTR in post-ER compartments (Figure 9B). Both Hsc70 and Hsp110 have been implicated in clathrin basket dissociation (Schmid and Rothman, 2002).
1985; Chappell et al., 1986; Schuermann et al., 2008). However, a general impact of Hsp105α on clathrin-mediated endocytosis cannot account for the dramatic difference in Hsp105α association with wild-type and ΔF508 CFTR in post-ER compartments (Figure 8B-D). Furthermore, our data indicate that, aside from Hsp105α and Hsp70, the association of Hsp90α with mature ΔF508 was also dramatically increased. It is highly likely that the cell can utilize a similar Hsp70-Hsp90 chaperone complex on the cytoplasmic face of post-ER compartments to escort and regulate the turnover of misfolded cargo molecules to sustain their biological function at the cell surface. A recent study indicate several well-characterized small molecule correctors rescue ΔF508 CFTR from ER retention but hardly restore its cell surface stability (Cholon et al., 2010). A better understanding of this process will enable us to design effective therapeutics aimed at reducing the rate of turnover of misfolded cargo molecules at the cell surface and maximize their cellular functions.

Many chaperone components impact ΔF508 CFTR rescue by regulating its ERAD. Reducing CHIP stabilizes CFTR in the ER and proportionally increases its cell surface localization (Meacham et al., 2001). Knocking down Hsp90 cochaperone Aha1 (Wang et al., 2006) or p97/VCP (Vij et al., 2006) has a similar effect. Histone deacetylase inhibitors such as 4-phenylbutyrate and suberoylanilide hydroxamic acid, and endogenous bronchodilator S-nitrosoglutathione enhance cell surface localization of ΔF508 CFTR by increasing its expression and altering cellular chaperone environment (Rubenstein et al., 1997; Choo-Kang and Zeitlin, 2001; Zaman et al., 2006; Hutt et al., 2010). Hsp105α is unique in that it enhances ΔF508 CFTR processing without dramatically increasing its ER accumulation. This is highly desirable for ΔF508 rescue as
massive accumulation of misfolded cargo proteins in the ER can induce the unfolded protein response and adversely affect CFTR expression and/or cell survival (Bartoszewski et al., 2008). This feature is largely attributable to Hsp105α’s ability to reduce CFTR synthesis, and is further enhanced by its ability to enhance ΔF508 post-ER stability.

Hsp105α is physiologically induced by heat or stress to convey cytoprotection. Enhancing its activity is unlikely to produce detrimental effects on the cell. Small molecule compounds that modulate its activities can be designed, which will significantly improve the cell surface expression of ΔF508 CFTR, and this can be used either alone or in combination with existing ΔF508 correctors to maximize therapeutic efficacy.

Given its uniquely high affinity to misfolded proteins, Hsp105α can serve as a potential molecular target for the treatment of other protein misfolding diseases such as congenital long QT syndrome (Zhou et al., 1998), diabetes and neurodegenerative diseases (Aridor and Hannan, 2000). Hsp105α holdase activity can prevent aggregation and degradation of misfolded proteins and allow them to refold. Its NEF activity may curb excessive accumulation through CHIP-mediated degradation and promote productive refolding through Hsp70 and other folding components. A systematic functional characterization of Hsp105α in the context of the disease-related misfolding and rescue will provide valuable information for the successful design of relevant molecular interventions.
Acknowledgements

We would like to thank Dr. William Balch for providing reagents. We thank Elaine Chalfin for technical assistance. We thank the Department of Biological Sciences at University of Toledo Main Campus for allowing us to use their phosphorimager. This work was supported by the Cystic Fibrosis Foundation and American Heart Association (to X.W.).


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Figure legends

Figure 1. Hsp105α overexpression impacts the synthesis, ERAD and maturation of wild-type CFTR. (A) HEK cells were co-transfected with 4 μg of wild-type CFTR and increasing doses of Myc-Rab3A (Cntrl) or Hsp105α (105). After 24 hours, cells were lysed and the lysates were immunoblotted for CFTR and actin. Results shown are representative of two independent experiments. (B) Equal amounts of cell lysates were immunoblotted for Hsp105 except that 4 fold higher loading was used for Cntrl at high dosage as indicated. (C-F) CFTR levels were quantified by densitometry and normalized to the levels of actin. To facilitate comparison, fold of changes in CFTR band C (C), band B (D), the C/B ratio (E) and Hsp105α (F) are shown. In all figures, the plasmid dosage is based on 35 mm culture dishes. (G-I) HEK cells were co-transfected with 4 μg wild-type CFTR and the indicated doses of 105 or Cntrl. Pulse-chase analysis was performed. In all pulse-chase analysis, the levels of CFTR in band B at 0 hour chase was normalized to that of the control and indicated below the autoradiograph. The levels of CFTR in bands B and C at all time points were normalized to the level of band B at 0 hour chase in the same set.

Figure 2. Hsp105α overexpression affects the synthesis and ERAD of ΔF508 CFTR. Dose response analysis was performed on ΔF508 CFTR as described in Figure 1. Both steady state (A-C) and pulse-chase (D-F) analyses were performed at the indicated Hsp105α dosage.
Figure 3. Hsp105α knockdown increases the synthesis of CFTR. (A) Upper, HEK cells stably expressing a non-targeting shRNA (Cntrl) or Hsp105α shRNA (105i) were transfected with wild-type CFTR. After 24 hours, cells were lysed and equal amounts of lysates were immunoblotted for CFTR. Representative CFTR immunoblots are shown in the inset, n=8. In all figures, the means and the standard errors of the mean (SEMs) are shown in the chart. Unpaired, two-tailed t-test was performed for all experiments with n≥3. Where indicated, * and ** denotes p ≤ 0.05 and 0.01, respectively. Lower, equal amounts of cell lysates were immunoblotted for the proteins indicated. All values were normalized to actin and then to the values of the mock to facilitate comparison, n=3. (B) The Cntrl and 105i cells were transfected with wild-type CFTR, and subject to pulse-chase analysis. (C) The 105i cells were co-transfected with 4 μg of wild-type CFTR and 4 μg of EGFP (GFP) or the shRNA-refractory Hsp105α expression plasmid (105r), and subject to pulse-chase analysis. (D) The Cntrl cells were transfected with 4 μg of ΔF508 CFTR (no KD). The 105i cells were co-transfected with 4 μg of ΔF508 CFTR and increasing doses of 105r. After 24 hours, the cells were lysed and equal amounts of lysates were immunoblotted for CFTR, Hsp105 and calnexin (calnxn). The levels of CFTR band B and Hsp105α were normalized to those of “no KD”. (E) The Cntrl and 105i cells were transfected with ΔF508 CFTR, and subject to pulse-chase analysis. (F) The 105i cells were co-transfected with 4 μg of ΔF508 CFTR and 4 μg of GFP or 105r, and subject to pulse-chase analysis.

Figure 4. Hsp105α promotes the processing of ΔF508 CFTR at reduced temperature. (A and B) HEK cells were co-transfected with 4 μg of Hsp105α (105) and
4 μg of ΔF508 (A) or wild-type (B) CFTR. HEK cells co-transfected with the same doses of Myc-Rab3A and CFTR served as control (Cntrl). After 24 hours, cells were shifted to 30°C and incubated for additional 16 hours. Equal amount of cell lysates were immunoblotted as indicated, n=3. (C) HEK-ΔF cells stably overexpressing Hsp105α (105) or pcDNA3.1(+) (Cntrl) were subjected to 30°C time course. Equal amount of cell lysates were immunoblotted as indicated. The C0 and C21 represent lysates from the Cntrl cell line at 0 and 21 hour after the temperature downshift, respectively. (D) HEK cells were transfected with wild-type (WT), DAA or ΔF508 (ΔF) CFTR. After 16 hours, 10 μg/mL BFA was added to the medium, and the cells were cultured for additional 24 hours. The mock transfected cells served as control (mock). The cells were lysed and CFTR co-immunoprecipitation was performed. The precipitated proteins were immunoblotted as indicated. The levels of associated Hsp105α were subtracted by the mock, normalized to the level of CFTR in band B, and then normalized to the value of ΔF, n=2.

Figure 5. Hsp105α promotes the maturation of CFTR processing mutants at 37°C. HEK cells were co-transfected with Hsp105α (105) and ΔF508 (A) or DAA (B) CFTR. HEK cells co-transfected with the same doses of Myc-Rab3A and CFTR served as control (Cntrl). After 24 hours, the cells were lysed and lysates were immunoblotted as indicated, n = 3.

Figure 6. The impact of the Hsp105α NEF and holdase activities on the steady state level of CFTR. (A) Sequence alignment of a segment of the ATPase domain of human
Hsp105α and yeast Sse1. (B, E and H) HEK cells were co-transfected with 4 μg of wild-type (B) or ΔF508 (E and H) CFTR together with the indicated doses of Myc-Rab3A (Cntrl), wild-type Hsp105α (105), the G-D or ΔATP mutant. After 24 hours, the cells were lysed and equal amounts of the lysates were immunoblotted as indicated. Two different exposures of the same immunoblot are shown for wild-type CFTR (B). The ΔATP mutant is labeled by arrowheads. (C, F and I) The levels of CFTR in bands B and C were normalized to the levels of the Cntrl to facilitate comparison, n=3. (D, G and J) The levels of the indicated chaperones were quantified, normalized to the levels of actin, and then to the levels of the Cntrl.

**Figure 7. The roles of Hsp105α G-D and ΔATP mutants in CFTR biogenesis.** (A) HEK cells were co-transfected with 4 μg of wild-type CFTR and 4 μg of Myc-Rab3A (Cntrl) or Hsp105α G-D mutant. Pulse-chase analysis was performed. (B) HEK cells were co-transfected with 4 μg of ΔF508 CFTR and 4 μg of Myc-Rab3A or Hsp105α ΔATP mutant. Pulse-chase analysis was performed. (C) HEK cells were transfected with FLAG-tagged Hsp105α, its G-D or ΔATP mutant. Equal amounts of cell lysates were immunoblotted for FLAG epitope. (D and E) HEK cells were transfected with the indicated forms of FLAG-tagged Hsp105α or mock transfected. After 24 hours, FLAG co-immunoprecipitation was performed, n=2 (D) and n=1 (E).

**Figure 8. Hsp105α escorts ΔF508 CFTR in post-ER compartments.** (A) HEK cells were co-transfected with 4 μg of ΔF508 CFTR and 1 μg of Myc-Rab3A (Cntrl) or Hsp105α (105). After 24 hours, 100 μg/mL CHX was added to the culture and the cells
were further incubated at 37°C as indicated. Equal amounts of the cell lysates were immunoblotted for CFTR and actin. The level of CFTR bands I + C were normalized to actin and expressed as the fraction of the value at the 0 time point, n=2. (B) HEK-ΔF or HEK-WT cells were incubated at 37°C (37), or incubated at 30°C for 21 hours followed by treatment with CHX for additional 12 hours (30). Cells were lysed, and CFTR co-immunoprecipitation was performed. The precipitated proteins were immunoblotted as indicated. HEK cells not expressing CFTR served as the mock. (C and D) The level of associated chaperones were subtracted by the level of the mock, normalized to the total level of CFTR, and then to the value of ΔF at 37.

**Figure 9. Hsp105α is a versatile player in CFTR biogenesis.** (A) The versatile roles of Hsp105α in the ER-associated folding and quality control of CFTR. 70, Hsp70. NBD, nucleotide binding domain. (B) Impact of Hsp105α on the biogenesis of wild-type and ΔF508 CFTR. The question mark denotes additional cytoplasmic folding components involved.
Hsp105α Promotes the Rescue of CFTR Misprocessing by Enhancing Folding in the ER and Escorting in Post-ER Compartments

Anita Saxena, Yeshavanth Kumar Banasavadi-Siddegowda, Gargi Bhrigu and Xiaodong Wang

Department of Physiology and Pharmacology, University of Toledo College of Medicine, Toledo, OH 43614
SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Impact of different control plasmids on CFTR biogenesis and ΔF508 post-ER stability. (A) HEK cells were co-transfected with 4 μg of wild-type CFTR and 4 μg of pcDNA3.1(+) (vector), Myc-Rab3A (Rab3A), EGFP (GFP) or Hsp105α (105). After 24 hours, cells were lysed and equal amounts of cell lysates were immunoblotted for CFTR and actin. (B) HEK cells were co-transfected with 4 μg of ΔF508 CFTR and 1 μg of GFP or 105. Twenty four hours post-transfection, CHX chase was performed and the data analyzed as described in Figure 8 A.
Figure 1

A

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Actin

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C

Change in C

D

Change in B

E

Change in C

F

Change in 105

G

Chase 0 1 2 3 0 1 2 3 h

CFTR in B

H

Chase 0 1 2 3 0 1 2 3 h

CFTR in C

I

Chase 0 1 2 3 0 1 2 3 h

CFTR in C

0.2 µg

0.1 µg

4 µg

Chase time (h)
Figure 6

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△ATPase (△ATP)

B  

WT CFTR, 4 µg 105

Cntrl 105  | G-D  | △ATP |

CFTR  
Hsp 105  
Hsp 70  
Hsp 90α  
Hsc 70  
Hsp 90β  
actin

E  

ΔF CFTR, 4 µg 105

Cntrl 105  | G-D  | △ATP |

CFTR  
Hsp 90β  
actin

Hsp 105  
Hsp 70  
Hsp 90α  
actin

Hsc 70  
Hsp 90β  
actin

G  

Chaperone level

Hsp 105  
Hsp 70  
Hsp 90α  
Hsp 90β
Figure 7

A

B

C

D

E
Figure 8

A

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CFTR

actin

105

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I/C remaining

CHX chase (h)

0.0 0.3 0.6 0.9 1.2 1.5

B

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CFTR

Hsp105

Hsp70

Hsc70

Hsp90α

Hsp90β

C

D

chaperone/CFTR

Hsp105

Hsp70

Hsc70

Hsp90α

Hsp90β

B+C

C

WT

ΔF
Figure 9

A

Maturation → Holdase → Hsp70 → NEF → CHIP → ERAD → Maturation

Synthesis → ERAD

W.T. CFTR → Hsp70 → Hsp105 → lumen

ΔF508 CFTR → Hsp70 → Hsp105 → lumen

B

ER

post-ER

export

co-translational

post-translational

Hsp105

Hsp70

ERAD

CHIP

NEF

Synthesis

Holdase

Maturation

Lumen

Maturation

Rescue
Figure S1

A

![Western blot of CFTR and actin](image1)

B

![Western blot of CFTR and actin](image2)

I/C remaining vs. CHX chase (h)

- GFP
- 105

[Graph showing I/C remaining with time for GFP and 105]
Hsp70-Hsp90 Chaperone System Mediates the Temperature-dependent Maturation of ΔF508 CFTR

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Running Title: Hsp70 and Hsp90 Cooperate in ΔF508 Maturation

Keywords: CFTR, cystic fibrosis, endoplasmic reticulum, Hsp70, Hsp90
ABSTRACT

Both Hsp70 and Hsp90 are involved in the biogenesis of the cystic fibrosis transmembrane conductance regulator (CFTR) but their functional relationship remains unknown. The deletion of phenylalanine at position 508 (ΔF508) blocks CFTR maturation in the endoplasmic reticulum (ER) but such blockage is released at reduced temperature in a chaperone-dependent manner. Using the temperature-dependent maturation of ΔF508 CFTR to understand the chaperone-mediated folding of CFTR, we provide evidence for functional cooperation between Hsp70 and Hsp90 through cochaperones Hop and Hsp105. Hsp90 is essential for the maturation of ΔF508 at reduced temperature. Sequential recruitment of Hsp70 and Hsp90 occurs shortly after the temperature downshift. Hsp70 and Hdj-2 associate with CFTR earlier than Hsp90 and Hsp105. The integrity of Hsp70-Hsp90 chaperone network is essential for the dynamic chaperone-CFTR association necessary for the maturation of ΔF508 CFTR at reduced temperature. Our data suggest that an Hsp70-chap90 chaperone network mediates CFTR forward folding on the cytoplasmic face of the ER membrane.
Hsp70 and Hsp90 play important roles in the biogenesis of cell surface ion channels such as the cystic fibrosis transmembrane conductance regulator (CFTR) (Yang et al., 1993; Loo et al., 1998) and the Kv11.1 potassium ion channel (Ficker et al., 2003). Hsp70 mediates the co-translational folding (Meacham et al., 1999; Oberdorf et al., 2005) and post-translational degradation (Meacham et al., 2001) of CFTR in the endoplasmic reticulum (ER). Hsp90 on the other hand is involved in the maturation and stabilization of nascent CFTR (Loo et al., 1998). Hsp70 and Hsp90 is known to team up in a chaperone relay system in the activation of steroid hormone receptors (SHRs) and signaling kinases, where multiple cochaperones mediate the functional connection between the two (Pratt, 1997; Pratt and Toft, 2003). But whether such a chaperone system functions on the cytoplasmic side of the ER membrane in mediating the biogenesis of CFTR remains unknown. A global proteomic analysis on CFTR-associated proteins in the ER revealed most of the cochaperones involved in Hsp70-Hsp90-mediated SHR activation (Wang et al., 2006). However, the functional link between Hsp70 and Hsp90 in the maturation of CFTR in the ER remains missing.

The functional deficiency of CFTR at the apical membrane is the major cause of cystic fibrosis (CF) (Riordan et al., 1989a). The ΔF508 mutation accounts for 70% of CF-causing alleles and is present in over 90% of CF patients among the Caucasian population. Such a mutation impairs the conformational maturation (Lukacs et al., 1994) and exocytic trafficking (Cheng et al., 1990). Interestingly, ΔF508 CFTR display a temperature-sensitive processing phenotype where the ER retention of the mutant protein is reversed at reduced temperature (Denning et al., 1992). Recent work indicates that the
ΔF508 temperature-sensitive processing relies on the cellular chaperone machinery (Wang et al., 2006; Wang et al., 2008) and is mediated through global conformational reversion (Roy et al., 2010). A picture start to emerge where the thermal stability offered by the reduced temperature cooperates with chaperone action allowing the otherwise misfolded ΔF508 CFTR to overcome the kinetic folding barrier (Qu and Thomas, 1996), pass the ER quality control, and couple to coatamer complex II (COPII) for ER export (Wang et al., 2004b). The essential role for the cellular chaperone machinery in this process is underscored by the identification of a specific cell line that is incapable of supporting the temperature-dependent export of ΔF508 CFTR (Wang et al., 2008; Roy et al., 2010). Furthermore, dynamic chaperone recruitment to ΔF508 CFTR shortly after the temperature downshift appears to be essential as such recruitment is absent in the impermissible cell line (Wang et al., 2008).

In the current study, we seek to understand the mechanism underlying the chaperone-mediated maturation of ΔF508 CFTR at reduced temperature in human embryonic kidney 293 (HEK) cells by examining the roles of Hsp70 and Hsp90, and by probing their functional relationship through multiple cochaperones. Our data provide evidence to support the involvement of a cytoplasmic Hsp70-Hsp90 chaperone network containing at least Hsc70, Hsp90, Hop and Hsp105 in the temperature-dependent conformational maturation of ΔF508 CFTR. Hsp70 appear to associate with ΔF508 CFTR earlier than Hsp90. The recruitment and action of Hsp90 is essential for the maturation of ΔF508 CFTR. Cochaperone Hop and Hsp105 are important in functionally connecting Hsp70 and Hsp90 in the process. These conclusions, although drawn from the temperature-dependent export of ΔF508 CFTR, may reflect a common process used by
the cell to mediate the conformational maturation of polytopic transmembrane proteins in the ER.
RESULTS

Hsp105 depletion down-regulates Hsc70 and Hsp90β and inhibits the temperature-dependent export of ΔF508 CFTR.

To understand the roles of cytoplasmic chaperones in the temperature-dependent export of ΔF508 CFTR, we prepared a series of chaperone-deficient HEK cell lines by stably expressing chaperone-specific shRNAs. We generated a HEK Hsp105 knockdown stable cell line where Hsp105 level is reduced by ~60% without affecting the levels of Hsp70, Hsc70, Hsp90α or Hsp90β (Fig. 1 C). When ΔF508 CFTR was transiently expressed in this cell line, the simple reduction in Hsp105 increased the steady state level of the ER-localized, core-glycosylated CFTR (band B) at 37ºC (Fig. 1 A). After incubation at 30ºC for 16 hours, a proportional increase in the Golgi-modified, complex-glycosylated CFTR (band C) was observed with the C/B ratio unchanged (Fig. 1 B). This indicates that moderate knockdown of Hsp105 by itself does not have a major impact on the efficiency of the temperature-dependent maturation of ΔF508 CFTR.

We also knocked down Hsp105 in a HEK cell line stably expressing ΔF508 CFTR (HEK-ΔF) (Silvis et al., 2003). In one of the clonal stable cell lines we generated (F7), over 99% reduction was observed for Hsp105, which is accompanied by dramatic decrease in Hsc70 and Hsp90β but no changes in other major cytoplasmic chaperones (Fig. 1 F). As a result, the temperature-dependent maturation of ΔF508 CFTR was reduced (Fig. 1, D and E). This is reflected in a shift of band C to the partially Golgi-processed complex glycoforms known as band I typically seen in HEK cells expressing ΔF508 CFTR at 37ºC (Wang et al., 2008). Furthermore, a quantitative reduction of processing (including band I) is apparent as the level of the complex glycoforms is
disproportionally lower relative to the increase in band B, leading to significant decrease in the (1+C)/B ratio.

The chaperone levels shown in Fig. 1, C and F were measured on cells incubated at 37ºC, and essentially the same chaperone levels were observed after the 16-hour incubation at 30ºC (Fig. S 1). Furthermore, a similar phenotype has been observed in another Hsp105-knockdown HEK-ΔF cell line (F0) where dramatic reduction (by over 98%) in Hsp105 led to reduction in Hsc70 and Hsp90β, and an increase in the levels of Hsp70 and Hsp90α (Fig. S 2, A-C). Obviously, the compensatory increase in Hsp70 and Hsp90α levels failed to restore the temperature-dependent maturation of ΔF508 CFTR, suggesting that Hsp105 is the limiting factor in the process.

Taken together, these results suggest that Hsp105 is functionally related to Hsc70 and Hsp90β, and that the three chaperones are essential in the temperature-dependent export of ΔF508 CFTR.

**Hsp70 or Hsc70 overexpression reduces the temperature-dependent export of ΔF508 CFTR.**

Previously, Hsp70 recruitment to ΔF508 CFTR was observed shortly after the temperature downshift (Wang et al., 2008). To test the role of Hsp70 or Hsc70 in the process, we transiently overexpressed the two chaperones individually in HEK-ΔF cells. At 37ºC, overexpression of Hsp70 or Hsc70 did not have a major impact on the steady state level of ΔF508 CFTR (Fig. 2 A). At reduced temperature however overexpressing Hsp70 or Hsc70 moderately reduced the temperature-dependent processing of ΔF508 CFTR, which is reflected in significant reduction in the C/B ratio (Fig. 2, B and C). In
neither case, significant changes in other cytoplasmic chaperones have been observed (Fig. 2, B and D). These data suggest a moderate inhibitory role for Hsp70 or Hsc70 in the temperature-dependent maturation of ΔF508 CFTR.

The roles of Hsc70 and Hsp70 in the temperature-dependent maturation of ΔF508 CFTR.

It is generally believed that Hsp70 and Hsc70 have similar function but different transcriptional regulation. We found that depletion of Hsp105 dramatically reduces the level of Hsc70 but not Hsp70 (Fig. 1 F). To further probe the roles of Hsc70 and Hsp70 in CFTR folding, we stably knocked down the two chaperones individually in HEK-ΔF cells. Consistent with a connection between Hsc70 and Hsp105, Hsc70 knockdown slightly reduces Hsp105 level (Fig. 3 B). The elevation of Hsp70 level is likely due to compensation. Given that the constitutive expression of Hsp70 is much greater than that of Hsc70 in HEK-ΔF cells (Fig. 3 E), the increase in Hsp70 should more than compensate for the reduction in Hsc70 (Fig. 3 B). These changes in chaperone levels stabilize ΔF508 CFTR at 37ºC and proportionally increases processing at reduced temperature as reflected in the unchanged C/B ratio (Fig. 3 A).

Hsp70 knockdown moderately reduced the level of Hsp70 and mildly reduced the levels of Hsp90β and Hsp105 (Fig. 3 D and S2 B), suggesting a functional connection among the three. This led to reduced temperature-dependent maturation of ΔF508 CFTR as reflected in the lack of increase in band B with a dramatic increase in band C (Fig. 3 C). Notably, in this case, no switch from band C to band I was observed. Therefore, in the lack of compensatory effect, Hsp70 knockdown slightly reduced Hsp90β and Hsp105.
Fig. 3 C), which in turn led to a moderate inhibition of the temperature-dependent maturation of ΔF508 CFTR (Fig. 3 C). It is worth mentioning that, due to the much higher abundance of constitutively expressed Hsp70 than Hsc70, the moderate reduction in Hsp70 is translated into a much greater decrease in the total amount of Hsp70 and Hsc70. To further probe the relationship between Hsp105 and the two isoforms of Hsp70, we conducted quantitative co-immunoprecipitation using Hsp105-FLAG fusion protein in HEK cells. We normalized the level of Hsp70 or Hsc70 recovered from co-immunoprecipitation to the level of the same in the whole cell lysate (fractional recovery) to compare the relative strength of association of the two isoforms of Hsp70 with Hsp105 (Fig. 3 G). Despite far higher abundance for Hsp70 in HEK-ΔF cells, relatively more Hsc70 associated with Hsp105-FLAG than Hsp70 (Fig. 3 F), suggesting preferential association of Hsp105 with Hsc70. These data suggest that Hsp70, like Hsc70, is functionally related to Hsp90β and Hsp105 in mediating the temperature-dependent maturation of ΔF508 CFTR.

Taken together, these data reveal an intrinsic difference between Hsc70 and Hsp70 in their ability to be incorporated into chaperone complex. While moderate changes in the level of Hsp70 or Hsc70 have minimal impact on the efficiency of the temperature-dependent maturation of ΔF508 CFTR, a cytoplasmic chaperone network containing at least Hsc70/Hsp70, Hsp90β and Hsp105 is essential for the process.

**Hsp90 promotes the temperature-dependent maturation of ΔF508 CFTR**

To probe the role of Hsp90 in the temperature-dependent maturation of ΔF508 CFTR, we treated HEK-ΔF cells with increasing doses of Hsp90 inhibitors Geldanamycin
or Novobiocin, and assessed the efficiency of the temperature-dependent export of ΔF508 CFTR by measuring the steady state level of CFTR in bands B and C after 16-hour incubation at 30°C. Within defined concentration ranges, both Geldanamycin and Novobiocin reduce the level of band C in a dose-dependent manner, and this is accompanied by slight increase in band B (Fig. 4, A and B). Geldanamycin appears to be more potent in inhibiting the temperature-dependent maturation of ΔF508 CFTR than Novobiocin. At the highest levels for both, a reduction in the level of CFTR in band B was observed, which is consistent with the identified role for Hsp90 in stabilizing CFTR from the ER-associated degradation (ERAD) (Loo et al., 1998). These results suggest that Hsp90 is a positive regulator of the temperature-dependent maturation of ΔF508 CFTR. The slight increase in the level of CFTR band B is attributable to the reduced export.

Given the potential role for Hsp90β in the temperature-dependent export of ΔF508 (Figs. 1, D-F, and 3, C and D), we knocked down its expression in HEK-ΔF cells, and observed the most dramatic inhibition of the temperature-dependent maturation of ΔF508 CFTR (Fig. 4 C). The almost complete depletion of Hsp90β down-regulates all major cytoplasmic chaperones except Hsp70 (Fig. 4 D), suggesting that Hsp90β is an integral part of the cytoplasmic chaperone complex mediating the temperature-dependent export of ΔF508 CFTR. Among the folding components whose levels are dramatically reduced is Hop, the cochaperone that physically and functionally links Hsp70 and Hsp90, suggesting that Hop is part of the complex as well.
Hop knockdown dramatically inhibited the temperature-dependent export of ΔF508.

To confirm Hop’s role in the temperature-dependent export of ΔF508 CFTR, we knocked down Hop. A 97% decrease in Hop led to mild reduction in Hsc70 and moderate reduction in the in Hsp90β and Hsp105 without affecting the levels of Hsp70 and Hsp90α (Fig. 5B). However, the temperature-dependent maturation of ΔF508 CFTR was reduced by ~70% based on C/B ratio (Fig. 5A). These data indicate a critical role for Hop in the temperature-dependent export of ΔF508 CFTR, and further suggest that the functional cooperation between Hsp70 and Hsp90 is essential in the maturation of ΔF508 CFTR at reduced temperature.

Altered chaperone recruitment underlies the inhibition of temperature-dependent maturation of ΔF508 CFTR.

The temperature-dependent export of ΔF508 CFTR occurs post-translationally and has a slow kinetics which peaks at 6 hours of chase (Wang et al., 2008). At the steady state, conversion from band B to band I or C is hardly detectible 3 hours after the temperature downshift. It has been shown that the temperature-dependent maturation of ΔF508 CFTR involves dynamic chaperone recruitment (Wang et al., 2008). To probe the potential mechanism, we conducted quantitative co-immunoprecipitation on HEK-ΔF cells deficient in Hsp105 (F7) and compared with the control cell line. In the control cells, Hsp105 started to be recruited to ΔF508 CFTR 30 minutes after the temperature downshift. The recruitment peaks at 1 hour and then gradually declines. The almost
complete depletion of Hsp105 in the F7 cell line abolishes the Hsp105 association at reduced temperature (Fig. 6, A and B).

Hsc70 recruitment starts immediately after the temperature downshift (Fig. 6 C). Due to the reduced level of Hsc70 in F7 cells (Fig. 1 F), the magnitude of Hsc70 recruitment is also reduced but its time-dependent pattern remains unchanged (Fig. 6 C). In contrast, the recruitment of Hsp70 is increased without altering the time-dependent pattern (Fig. 6 D), suggesting that Hsp70 can functionally compensate for the deficiency of Hsc70 and this occurs in the absence of significant increase in Hsp70 level (Fig. 1 F).

Hsp105 is a nucleotide exchange factor for Hsp70s (Dragovic et al., 2006; Raviol et al., 2006b) whereas Hsp40s promote the ATP hydrolysis of Hsp70s. We probed the time-dependent recruitment of Hdj-2 which is known to be involved in CFTR biogenesis (Meacham et al., 1999; Zhang et al., 2006). In control cells, Hdj-2 association peaks at 30 minutes and then declines (Fig. 6 E). This pattern is similar to those of Hsc70 (Fig. 6 C) and Hsp70 (Fig. 6 D), except the decline is more drastic. Depletion of Hsp105 dramatically reduced initial association of Hdj-2 with CFTR (Fig. 6 E) and this occurs in the absence of reduction in Hdj-2 level in the cell (Fig. 1 F). Over time, however, Hdj-2 increases its association with ΔF508 CFTR to a level higher than that in control cells. These data suggest Hdj-2 association depends on the presence of Hsp105 even though the total amount of Hsp70 and Hsc70 association is not reduced (Fig. 6 C and D).

Previously, we have observed differential recruitment of Hsp70 and Hsp90 during the temperature-dependent maturation of ΔF508 CFTR, suggesting different roles for the two major chaperones in the process (Wang et al., 2008). In control cells, the recruitment of both Hsp90α and Hsp90β increases over time, peaks at the same time as Hsp105 (Fig.
6 B, at 1 hour) but 30 minutes later than Hsp70 (Fig. 6 D) and Hsc70 (Fig. 6 C), and then starts to decline (Fig. 6, F and G). However, upon Hsp105 depletion which leads to a dramatic decrease in Hsp90\(_{\beta}\) (Fig. 1 F), Hsp90\(_{\beta}\) fails to increase its association with ΔF508 CFTR after the temperature downshift (Fig. 6 F), and Hsp90\(_{\alpha}\) recruitment peaks 30 minutes earlier (Fig. 6 G). Despite the reduced level of Hsp90\(_{\beta}\) in F7 cells, its association with ΔF508 CFTR at time 0 was even slightly increased and so does Hsp90\(_{\alpha}\) (Fig. 6, F and G). These data suggest that depletion of Hsp105 disrupts the time-dependent recruitment of Hsp90s to ΔF508 CFTR.

ER lumenal chaperone calnexin is known to be involved in the biogenesis of CFTR (Pind et al., 1994). We found that the time-dependent pattern of calnexin recruitment is similar to those of Hsp90s and Hsp105, which peaks at 1 hour and then decline (Fig. 6, B and F-H). Such recruitment was completely abolished in the F7 cell line, suggesting that depletion of Hsp105 prevented the dynamic association of calnexin with ΔF508 CFTR.

Taken together, our data support an essential role for dynamic chaperone recruitment in ΔF508 maturation at reduced temperature. While Hsp70, Hsc70 and Hdj-2 show similar recruitment pattern, the patterns of Hsp105, Hsp90s and calnexin association are alike and are distinct from the former. The recruitment of the latter group of chaperones peaks 30 minutes later than the former group. Depletion of Hsp105 has minimal impact on the pattern of recruitment of Hsc70 or Hsp70 but dramatically affects the recruitment of Hdj-2, Hsp90s and calnexin. It is likely that Hsp105 functions earlier than Hsp90s and calnexin in the temperature-dependent maturation of ΔF508 CFTR.
DISCUSSION

Temperature-dependent maturation of ΔF508 as a model system to understand CFTR post-translational conformational maturation.

The folding of multiple domains of CFTR is largely co-translational (Kleizen et al., 2005). However, nascent CFTR needs to achieve post-translational conformational maturation before it can exit the ER and this is where the ΔF508 defect occurs (Lukacs et al., 1994). Low temperature in combination with chaperone action allows the correction of such defect leading to export.

Studying the post-translational conformational maturation of wild-type CFTR is challenging because it is hard to separate the post-translational events from the co-translational ones given the efficient processing. We use the temperature-dependent rescue of ΔF508 CFTR as a model system to understand how the chaperone machinery mediates the rescue of ΔF508 CFTR at reduced temperature. The process is much slower and can be quickly initiated or stopped by the switching of the temperature.

Previously, we identified a baby hamster kidney (BHK) cell line stably expressing ΔF508 CFTR (BHK-ΔF) that does not support the temperature-dependent export (Wang et al., 2008). In this cell line, the dynamic chaperone recruitment to ΔF508 CFTR after the temperature downshift is impaired. Due to the species difference between hamster and human, it is difficult to pinpoint which chaperone(s) are responsible for such phenotypic differences. We therefore individually knocked down major cytoplasmic chaperones Hsc70, Hsp70, Hsp90β, Hsp105 and Hop in HEK-ΔF cells and observed their effects on the temperature-dependent maturation of ΔF508 CFTR. Strikingly, we found that Hsp90β knockdown almost completely blocked the maturation of ΔF508 CFTR at

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reduced temperature, which also dramatically reduced the levels of Hsc70, Hsp105 and Hop (Fig. 4), indicating a critical role for an Hsp70-Hsp90 chaperone network containing at least Hsp70, Hsp90, Hop and Hsp105 in the temperature-dependent maturation of ΔF508 CFTR. Consistent with this, depletion of Hsp105 (Fig. 1, C-F), knockdown of Hsp70 (Fig. 3, C and D), and depletion of Hop (Fig. 5) also reduced the temperature-dependent maturation of ΔF508 CFTR, and these are accompanied by reduction in the levels of other components of the chaperone network. In further support of the importance of the integrity of the chaperone network, we found that, in the Hsp90β knockdown HEK-ΔF cells, overexpressing Hsc70, Hsp70, Hop or Hsp105 failed to restore the temperature-dependent maturation of ΔF508 CFTR (Fig. S3), and neither did compensatory upregulation of Hsp70 and Hsp90α as seen in one of the Hsp105 knockdown cell lines (Fig. S2)

**Distinct roles for Hsp70 and Hsp90 in CFTR conformational maturation.**

Hsp70 play versatile roles in protein folding within the cell. It is involved in co-translational folding and post-translation degradation of CFTR in the ER. However, whether it has a role in the post-translational conformational maturation is not known. We found that transiently overexpressing Hsp70 or Hsc70 slightly reduced the temperature-dependent maturation of ΔF508 CFTR (Fig. 2), suggesting that Hsp70s have a mild negative impact on the post-translational maturation of ΔF508 CFTR. However, Hsp70s appear to be necessary for ΔF508 maturation as well. Hsp70 knockdown reduced the ΔF508 maturation, and this is associated with the reduction in the levels of Hsp105 and Hsp90β (Fig. 3, C and D).
On the other hand, we found that Hsp90 is essential for ΔF508 maturation. Both its chaperone activity and the integrity of the Hsp70-Hsp90 chaperone network are absolutely necessary for the temperature-dependent maturation of ΔF508 CFTR (Fig. 4). This is consistent with the fact that while Hsp90 is not essential for de novo folding of most proteins but is critical in the formation of native conformation of “hard-to-fold” proteins (Nathan et al., 1997). Hsp90 is also necessary for the post-translation maturation of wild-type CFTR (Loo et al., 1998), suggesting that the cell can use the same chaperone system to mediate the maturation of even wild-type CFTR at physiological temperature.

Therefore, Hsp70 and Hsp90 have distinct roles in the temperature-dependent maturation of ΔF508 CFTR. The former appears to regulate CFTR refolding and ERAD, and pass it on to Hsp90 for conformational maturation. The latter directly mediates the conformational change in CFTR necessary for passing the ER quality control.

**Functional relationship between Hsc70 and Hsp70.**

We observed very different phenotype for the knockdown of Hsc70 and Hsp70. In HEK cells, Hsp70 is constitutively expressed in the absence of stress, and the level of Hsp70 is over ten fold higher than Hsc70 (Fig. 3 E). Hsc70 knockdown was over-compensated by induction of Hsp70 (Fig. 3 B), which did not disrupt the Hsp70-Hsp90 chaperone network and hence did not affect ΔF508 maturation (Fig. 3 A). On the other hand, Hsp70 knockdown can not be compensated, and led to disruption of the chaperone network and reduction of ΔF508 maturation (Fig. 3, C and D). We found that the difference between Hsp70 and Hsc70 is not restricted to transcriptional regulation as the two isoforms appear to have different ability to be incorporated into the Hsp70-Hsp90
chaperone network (Fig. 3, F and G). However, the reduction in the level of Hsc70 in F7 cell line was able to be complemented by Hsp70 in terms of recruitment to ΔF508 CFTR (Fig. 6, C and D). Therefore, under physiological condition, Hsc70 plays a major role in the Hsp70-Hsp90 chaperone network but Hsp70 can compensate for the loss of former at reduced efficiency.

**Functional connection between Hsp70 and Hsp90.**

Hsp70 and Hsp90 form a chaperone relay team in mediating the conformational activation of SHRs, a process coordinated by multiple cochaperones (Pratt, 1997; Pratt and Toft, 2003). Given that both Hsp70 and Hsp90 are involved in CFTR biogenesis (Yang et al., 1993; Loo et al., 1998) and that similar cochaperones were identified to associate with the ER-localized CFTR (Wang et al., 2006), it is likely that a similar mechanism operates in the conformational maturation of CFTR in the ER. In the current study, we systematically analyzed the functional roles for Hsp70, Hsp90 and some of the key cochaperones in the temperature-dependent maturation of ΔF508. Our data strongly support the existence of an Hsp70-Hsp90 chaperone network in facilitating the maturation of ΔF508 CFTR at reduced temperature: 1) Hsp70 and Hsp90 have distinct roles in the process (Figs. 2 and 4); 2) Hsp70 knockdown reduces Hsp90β level and impair ΔF508 maturation (Fig. 3, C and D); 3) Hsp70 appears to be recruited earlier than Hsp90 (Fig. 6, C, D, F and G); 4) Depletion of Hsp70 cochaperone Hsp105 impairs recruitment of Hsp90s but not Hsp70s (Fig. 6, C, D, F and G).

Based on the above findings, we propose a model for the maturation of ΔF508 CFTR at reduced temperature which is mediated by the cooperative actions of the
components of the Hsp70-Hsp90 chaperone network on the cytoplasmic side of the ER membrane (Fig. 7). Upon the temperature downshift, Hsp70 and Hdj-2 are first recruited to the ΔF508 CFTR on the ER membrane, and then Hsp105, Hop and Hsp90s are recruited, which is accompanied by the dissociation of Hsp70s and Hdj-2. In the process, Hsp90 promotes the conformational maturation of ΔF508 CFTR at the expense of ATP hydrolysis. Once conformational maturation is achieved, Hsp90 and related cochaperones such as Hsp105 then dissociate from ΔF508 CFTR and allow it to be coupled to COPII for ER export.

We have demonstrated a prominent role for Hsp105 in functionally linking Hsp70 and Hsp90. Although a largely Hsp70 cochaperone, Hsp105 knockdown downregulated Hsp90β (Fig. 1, D-F). Furthermore, the pattern of Hsp105 association with ΔF508 CFTR after the temperature downshift is more similar to Hsp90s than Hsp70s (Fig. 6, B, C, D, F and G). Even more critically, Hsp105 depletion disrupts the recruitment of Hsp90s but not Hsp70s and leads to inhibition of ΔF508 maturation at reduced temperature (Figs. 1, D-F and 6, F and G). Our finding is consistent with an early study demonstrating a functional relationship between Hsp105 and Hsp90 in yeast (Liu et al., 1999). Recently, Hsp105 was also shown to play an important role in the Hsp70-Hsp90 relay system mediating the activation of the glucocorticoid receptor (Mandal et al., 2010).

We have not been able to identify Hop association using immunoblotting. This may be due to its transient nature of interaction. However, when Hop is knocked down, we definitely observed a dramatic inhibition of ΔF508 maturation (Fig. 5). These data suggest that Hop definitely play a role in the process.
Previously, using quantitative co-immunoprecipitation, we observed differential association of Hsp70 and Hsp90 during early stage of the temperature-dependent maturation of ΔF508 CFTR (Wang et al., 2008). However, in that study, Hsp90 association appears to have high basal association and start to decrease 30 minutes after the temperature downshift (Wang et al., 2008). This result was generated using low stringency washing condition to preserve highest possible Hsp90 association. When regular washing condition was used, we did observe an increase in Hsp90 association which peaked after Hsp70. We recently optimized and standardized our quantitative co-immunoprecipitation procedure and consistently seen sequential recruitment of Hsp70 and Hsp90 (Fig. 6). The significance of high basal association of Hsp90 under low stringency wash is currently not clear. The apparently low affinity basal association of Hsp90 might be produced by the chaperone’s natural tendency to bind misfolded ΔF508 CFTR after cell lysis. Alternatively, the low affinity binding of Hsp90 with ΔF508 CFTR may reflect another dimension of complexity in chaperone-mediated folding of ΔF508 CFTR.

**Functional crosstalk between cytoplasmic and lumenal chaperones.**

We have observed that shown that the ER luminal chaperone calnexin is also recruited to ΔF508 CFTR shortly after the temperature downshift whose pattern resembles that of Hsp105 and Hsp90s (Fig. 6, B and F-H), suggesting that luminal folding is also involved in ΔF508 CFTR maturation at reduced temperature. Even more interestingly, Hsp105 depletion totally abolishes the association of calnexin with ΔF508 CFTR, suggesting an intimate crosstalk between the cytoplasmic and luminal
chaperones. It is currently unclear whether this event is the cause or consequence of the failed maturation of ΔF508 CFTR at reduced temperature in F7 cells.
EXPERIMENTAL PROCEDURES

Antibodies and Chemicals

CFTR mAbs used in this study include MM13-4, M3A7, and 13-1. Other antibodies used include anti-Hsp105 mAb (Novocastra, Newcastle upon Tyne, UK), anti-Hsc70, anti-Hop (p60), anti- Hsp90α and anti-Hsp90β mAbs (Stressgen, Ann Arbor, MI), anti-Hsp70 polyclonal antibodies (Stressgen), anti-FLAG mAb (Sigma, St. Louis, MO) and anti-actin mAb (Millipore, Temecula, CA). Anti-Hdj2 antibody was purchased from Abcam (Cambridge, MA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA). Protein G Sepharose 4 Fast Flow was purchased from GE Healthcare (Piscataway, NJ). Geldanamycin was purchased from A.G. Scientific (San Diego, CA) and novobiocin from Sigma.

Plasmids

The Hsp105α, Hsp70, Hsc70, Hop, and Hsp90β shRNA plasmids were constructed using IMG-800-1 vector (Imgenex, San Diego, CA) according to the manufacturer’s protocol. The control plasmid contains a non-targeting sequence 5’–TCAGTCACGTAATGGCGT–3’. The Hsp105α target sequence used is 5’–CAGCCATGTTGACTAAGC–3’. The Hsp70 target sequence used is 5’–GCTGCTGCAAGACTTCTTCAA–3’. The Hsc70 target sequence used is 5’–GACTGTTACCAATGCTGTT–3’. The Hop target sequence used is 5’–CCGAGAAGACTACGACAGAT–3’. The Hsp90β target sequence used is 5’–GTGGTTGATCACAAGC–3’. All target sequences were tested by Basic Local
Alignment Search Tool (BLAST) for potential off-target effects. The pCMV-SPORT6 harboring human full-length Hsp105α coding sequence was obtained from ATCC (Manassas, VA). The pBluscriptKS+ harboring human Hsp70-2 cDNA (Milner and Campbell, 1990) was kindly provided by Dr. Edwin R. Sanchez (University of Toledo College of Medicine, Toledo, OH). The Hsp70-2 coding sequence was subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) by using BamH I and Xho I sites. The pCMV-SPORT6 harboring human Hsc70 coding sequence was obtained from ATCC. The CFTR expression plasmids pcDNA3.1(+) CFTR-ΔF508 and pcDNA3.1(-)-Myc-Rab3A were provided by Dr. William E. Balch (The Scripps Research Institute, La Jolla, CA). All plasmids containing DNA fragments generated by polymerase chain reactions and all the shRNA constructs were sequence confirmed.

Cell Culture and Transfection

HEK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS) and 100 units/mL each of penicillin and streptomycin. The HEK-ΔF cells (Silvis et al., 2003) were maintained in the above medium supplemented with 150 μg/mL hygromycin B (EMD Chemicals, Gibbstown, NJ). Cell transfection was performed using Lipofectamine 2000 (Invitrogen). HEK or HEK-ΔF cells were transfected with the shRNA plasmids for Hsp105α, Hsp70, Hsc70, Hop and Hsp90β, and stable cell lines were generated by selection with 400 μg/mL G418. Once generated, the stable cell lines were maintained in appropriate media supplemented with 200 μg/mL G418.
Cell Lysis and Quantitative Immunoblotting

Cells were lysed on ice for 30 minutes in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 1% Triton X-100 (v/v) supplemented with the Complete™ protease inhibitor cocktail (Roche Diagnostics GmbH, Mannhem, Germany). The lysed material was cleared by centrifugation at 16,000 g for 20 min. Protein concentration was determined by Bradford Assay using Coomassie Protein Assay Reagent (Thermo, Rockford, IL). For quantitative immunoblotting, protein loading was adjusted based on their intracellular abundance, and antibody dilution was optimized so that the intensity of bands is within the dynamic range. Densitometry was performed using ImageJ software (National Institutes of Health).

Quantitative Co-immunoprecipitation

For Hsp105-FLAG co-immunoprecipitation, cell lysates were incubated with anti-FLAG mAb on ice for 1 hour, and then Protein G sepharose beads were added and the mixture was incubated for another hour before the precipitation, wash and elution of the beads. For CFTR co-immunoprecipitation, cell lysates were incubated with Protein G sepharose beads pre-coated with CFTR mAb at 4ºC overnight as described previously (Wang et al., 2008). HEK cells mock transfected or untransfected were used as negative control for non-specific protein association.
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FIGURE LEGENDS

**Figure 1.** (A-C) A HEK cell line stably expressing a non-targeting shRNA (Cntrl) or Hsp105α shRNA (105i, F4) were transiently transfected with DF508 CFTR. Twenty four hours post-transfection, cells were either freezeed (37°C) or were shifted to 30°C and incubated for additional 16 hours. Cell lysates were immunoblotted for CFTR, the indicated chaperones and actin. Band intensity was quantified by densitometry. For all temperature shift experiments, all values were normalized to actin and then to the values of the Cntrl to facilitate comparison. The means and the standard errors of the means (SEMs) are shown. Two tailed, unpaired t-test was performed, and * and ** indicate p values ≤ 0.05 and 0.01, respectively. n = 3. (D-F) HEK-ΔF cells stably expressing a non-targeting shRNA (Cntrl) or Hsp105α shRNA (105i, F7) were grown at 37°C, or were further shifted to 30°C and incubated for additional 16 hours. Equal amounts of cell lysates were immunoblotted as indicated.

**Figure 2.** HEK-ΔF cells were transiently transfected with plasmid expressing human Hsp70-2 (Hsp70) or Hsc70. Cells were either incubated at 37°C (A), or 28 hours post-transfection, cells were shifted to 30°C and incubated for 16 hours (B-D). Equal amounts of cell lysates were immunoblotted as indicated.

**Figure 3.** (A-D) HEK-ΔF cells stably expressing a non-targeting shRNA (Cntrl) or Hsc70 shRNA (Hsc70i) (A-B) or Hsp70 shRNA (Hsp70i) (C-D) were grown at 37°C or were shifted to 30°C and incubated for additional 16 hours. Cells were lysed and equal amounts of cell lysates were immunoblotted as indicated. Quantification and data
analysis were as described above. n = 3. (E) Whole cell lysates of HEK-ΔF and BHK-ΔF were separated with SDS-PAGE and immunoblotted with an antibody recognizing both Hsp70 and Hsc70. (F and G) HEK cells were transiently transfected with Hsp105-FLAG plasmid. Co-immunoprecipitation was conducted using anti-FLAG antibody. The precipitated proteins were immunoblotted as indicated. Mock transfected HEK cells served as control (mock). Small amount of cell lysates were also analyzed for control purpose. The level of the associated chaperones was first subtracted by the level of the mock, and then normalized to the level of the Hsp105-FLAG. To assess the relative avidity of Hsp70 and Hsc70 for Hsp105-FLAG, we further normalized the above values to the level of the respective chaperones in the lysate, and name the value as “Fractional recovery” (G).

**Figure 4.** (A and B) HEK-ΔF cells were incubated with geldanamycin (A) or novobiocin (B) at the indicated concentration at 37°C for 8 hours before they were shifted to 30°C and incubated for additional 16 hours. Equal amounts of cell lysates were immunoblotted for CFTR and actin, n=2. (C and D) HEK-ΔF cells stably expressing a non-targeting shRNA (Cntrl) or 90β shRNA (90βi) were grown at 37º or were shifted to 30ºC and incubated for additional 16 hours. Cell lysates were immunoblotted as indicated.

**Figure 5.** HEK-ΔF cells stably expressing a non-targeting shRNA (Cntrl) or Hop shRNA (Hopi) were grown at 37º, or were further shifted to 30ºC and incubated for additional 16 hours. Cell lysates were immunoblotted as indicated.
**Figure 6.** (A) HEK-ΔF cells stably expressing a non-targeting shRNA (Cntrl) or Hsp105α shRNA (105i, F7) were grown at 37º and then were shifted to 30º and incubated for the indicated periods of time. Cells were lysed, and CFTR co-immunoprecipitation was performed as described in the Materials and Methods. The precipitated proteins were immunoblotted for the indicated chaperones as well as CFTR. HEK cells not expressing CFTR served as the mock. (B-H) The level of associated chaperones were quantified by densitometry, subtracted by the level of the mock, and normalized to the total level of CFTR.

**Figure 7.** Working model for the Hsp70-Hsp90-mediated maturation of ΔF508 CFTR at reduced temperature. Calnxn, calnexin.

**Figure S1.** HEK-ΔF cells stably expressing non-targeting (Cntrl) or a specific HEK-ΔF Hsp105 knockdown cell line known as F7 were shifted from 37ºC to 30 ºC and incubated for additional 16 hours. Equal amounts of cell lysates were immunoblotted for the proteins as indicated.

**Figure S2.** (A-C) HEK-ΔF cells stably expressing non-targeting shRNA (Cntrl) or another HEK-DF Hsp105 knockdown cell line known as F0 were incubated at 37ºC (A and C) or shifted to 30ºC and further incubated for 16 hours (B). Equal amounts of cell lysates were immunoblotted for the proteins as indicated. Quantification and statistical
analysis were performed as described above. n=3. (D) The Cntrl and F0 cell lines were subjected to 30°C time course as indicated.

Figure S3. HEK-ΔF cells stably expressing shRNA for Hsp90β were transfected with increasing doses of Hsc70 (A), Hsp70 (B), Hop (C) and Hsp105 (D). Twenty-four hours post-transfection, the cells were shifted to 30ºC and incubated for additional 16 hours. Equal amounts of cell lysates were immunoblotted for the indicated proteins. Where indicated, HEK-ΔF cells expressing non-targeting shRNA and subjected to the same temperature shift served as the positive control (Cntrl).
Figure 1
Figure 2

A

37°C

Hsp70

Cntrl

Hsc70

CFTR

Hsp70

actin

Hsc70

actin

B

30°C

Hsp70

Cntrl

Hsc70

CFTR

Hsp70

Hsp90β

actin

Hsp70

Hsp105

Hsp90α

actin

C

D

CFTR level

30°C

C

B

C/B

Hsp70

Cntrl

Hsc70

Chaperone level

30°C

Hsp70

Hsc70

Hsp90β

Hsp90α

Hsp105
Figure 3
Figure 5
Figure 6
Figure 7
Figure S1
Figure S2
Figure S3
Role of Hsp105 in co-translational folding of CFTR.

Cytoplasmic chaperones play important roles in the biogenesis of integral membrane proteins. Both Hsp70 and Hsp105 have been implicated in co-translational folding of membrane proteins in the ER (Oberdorf et al., 2005; Yam et al., 2005). In the current study, we examined the roles of Hsp105 in CFTR biogenesis. Using pulse-chase analysis we show that Hsp105 affects the synthesis of CFTR in a dose dependent manner. This is likely due to the impact of Hsp105 on the co-translational folding of CFTR.

Hsp105 requires a functional ATPase domain to carry out its NEF activity and binding of ATP to Hsp105 is required for its interaction with Hsp70 (Dragovic et al., 2006; Shaner et al., 2006). The G233D mutation in yeast Sse1 blocks its ATP binding, impairing its interaction with Hsp70 and diminishing its NEF activity (Shaner et al., 2004; Shaner et al., 2005; Dragovic et al., 2006). We constructed a corresponding mutant in human Hsp105α, G232D, or G-D. We also constructed another mutant where we deleted the entire ATPase domain of Hsp105α (ΔATP). Both these mutants lack NEF activity and retain their holdase activity in vitro (Oh et al., 1999; Goeckeler et al., 2002). Utilizing these two mutants we tried to dissect the contribution of Hsp105 NEF and holdase activities to CFTR biogenesis. We show that both the G-D and ΔATP mutants reduce synthetic yield of CFTR; hence Hsp105 holdase activity regulates CFTR synthesis.
Hsp105 in maturation of CFTR.

We observed that Hsp105 has a role in the maturation of both wild-type CFTR as well as its processing mutants such as ΔF508 and DAA. However, such effects were observed within a specific concentration range, suggesting that a certain ratio between Hsp105 and Hsp70 or some other chaperones/cochaperones is essential in providing an optimal chaperone environment to promote CFTR maturation. We also observed that the rescue of ΔF508 CFTR by Hsp105 overexpression is dependent upon the presence of its NEF activity as the G-D mutant failed to rescue ΔF508 CFTR at the same dosage.

Hsp105 and ERAD of CFTR.

We have found using pulse-chase that low overexpression of Hsp105 promotes the ERAD of wild-type CFTR. This is consistent with the finding that Hsp70 has a role in CHIP-mediated ER quality control (Connell et al., 2001; Meacham et al., 2001). Such a role for Hsp105 can be attributed to its NEF activity as the overexpression of G-D and ΔATP mutants stabilized CFTR in the ER and enhanced its maturation, indicating that holdase activity of Hsp105 inhibits ERAD of CFTR and promotes its maturation. It appears that the NEF and holdase activities of Hsp105 represent two competing forces in the regulation of the ERAD of CFTR.

In the rescue of Hsp105 knockdown, we observed a greater inhibition of ERAD on ΔF508 CFTR than wild-type CFTR. This moderate difference in the effect of Hsp105 on the ERAD of two forms of CFTR might be attributed to the fact that ΔF508 CFTR associates with Hsp105 to a much greater extent than wild-type CFTR in the ER. Hence,
it is possible that the holdase activity of Hsp105 has a greater impact on the stability of ΔF508 CFTR in the ER.

**Hsp70 and Hsp90 in CFTR conformational maturation.**

Hsp70 plays versatile roles in protein folding. It is involved in the co-translational folding and post-translational degradation of CFTR in the ER. However its role in post-translational maturation of CFTR is not known yet. We found that overexpressing Hsp70/Hsc70 slightly reduced the temperature dependent maturation of ΔF508 CFTR suggesting a negative impact on the post-translational maturation of ΔF508 CFTR. However, Hsp70 knockdown was followed by reduction in the levels of Hsp105 and Hsp90β, which reduced the ΔF508 CFTR maturation, indicating that Hsp70 might be necessary for ΔF508 CFTR maturation as well.

Hsp90 is necessary for the post-translational maturation of wild-type CFTR (Loo *et al.*, 1998). Also, we show that the chaperone activity of Hsp90 and the integrity of Hsp70-Hsp90 chaperone network are absolutely necessary for the temperature dependent maturation of ΔF508 CFTR. These data suggest a direct involvement of Hsp90 in CFTR conformational maturation.

**Hsp105, a key player in the Hsp70-Hsp90 relay system in CFTR biogenesis.**

Hsp70 and Hsp90 are known to form a chaperone relay system in the activation of SHRs (Pratt and Toft, 1997, 2003). Both Hsp70 and Hsp90 are involved in the CFTR biogenesis (Yang *et al.*, 1993; Loo *et al.*, 1998). Also, Hsp70 and Hsp90 together with other cochaperones were identified to associate with the ER localized CFTR (Wang *et
Our analysis of the functional roles of Hsp70, Hsp90 and some of the key cochaperones in the temperature-dependent rescue of ΔF508 CFTR suggests the existence of an Hsp70-Hsp90 chaperone network in facilitating the maturation of ΔF508 CFTR at reduced temperature. It appears that Hsp70 maintains CFTR in a foldable state and passes it on to Hsp90 for conformational maturation.

Hsp105, an Hsp70 cochaperone, functionally relates to Hsp90 in yeast (Liu et al., 1999). It is shown to play an important role in the Hsp70-Hsp90 relay system in activating the glucocorticoid receptor (Mandal et al.). We show that Hsp105 knockdown downregulates Hsp90β. Also, after the temperature downshift, it associates with the ΔF508 CFTR with a similar pattern as Hsp90. Furthermore, Hsp105 depletion disrupts the recruitment of Hsp90 and leads to inhibition of temperature dependent rescue of ΔF508 CFTR. Our data indicate that Hsp105, together with Hop, is an integral part of the Hsp70-Hsp90 relay system in CFTR biogenesis, and is required for the conformational maturation of ΔF508 CFTR at reduced temperature.

We also observed that the ER lumenal chaperone calnexin is recruited to the ΔF508 CFTR with a pattern resembling that of Hsp105 and Hsp90. Hsp105 depletion abolishes the association of calnexin with ΔF508 CFTR. This suggests a possible functional connection between cytoplasmic and lumenal chaperones during the temperature dependent maturation of ΔF508 CFTR.

**Hsp105 escorts misfolded ΔF508 CFTR in post-ER compartments.**

We observed that Hsp105 increases the post-ER stability of ΔF508 CFTR. This can be attributed to high level of association of Hsp105 with the post-ER form of ΔF508 CFTR.
On the other hand, association of Hsp105 with the post-ER form of wild-type CFTR is much reduced. This suggests a novel role for Hsp105 in escorting ΔF508 CFTR in post-ER compartments. The extensive association of cytoplasmic chaperones with ΔF508 CFTR in post-ER compartments was observed, suggesting a potential role for the cytoplasmic chaperone machinery in regulating the post-ER stability of misfolded integral membrane proteins. However, the mechanism for such regulation remains to be elucidated.

**Hsp105 in the treatment of CF and other protein misfolding diseases.**

The current study revealed that Hsp105 is a versatile player in CFTR biogenesis. Due to conformational defect, the maturation of nascent ΔF508 CFTR in the ER is arrested in a chaperone protected, early folding intermediate. Enhancing Hsp105 activity reduced CFTR synthesis to prevent excessive ER accumulation of the mutant protein. In the meantime, its holdase activity reduces its ERAD, and promotes its refolding and maturation. Through its NEF activity, terminally misfolded ΔF508 CFTR is cleared away through ERAD. In post-ER compartments, Hsp105 escorts and stabilizes ΔF508 CFTR and enhances its functional expression at the cell surface. The interplay of the above activities makes Hsp105 an ideal target for promoting the rescue of ΔF508 CFTR. A more in-depth understanding of the functional regulation of Hsp105 in relation to its chaperone partners will reveal the molecular mechanism underlying the chaperone-mediated CFTR maturation on the cytoplasmic side of the ER membrane. Hsp105, in combination with pharmacological correctors can greatly enhance the conformational maturation of ΔF508 CFTR, which in turn can rescue CFTR misprocessing occurring in...
most CF patients. Given the wide spectrum of Hsp105 substrates (Goeckeler et al., 2002; Wang et al., 2006; Hrizo et al., 2007), we believe that Hsp105 is an ideal target for treating other protein misfolding diseases as well.


interacts with heat shock proteins and negatively regulates chaperone functions. Mol Cell Biol 19, 4535-4545.


transmembrane conductance regulator expression and maturation in epithelial cells. Mol Pharmacol 70, 1435-1442.


