THE ROLE OF MOLECULAR CHAPERONES IN THE ER ASSOCIATED DEGRADATION OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR IN THE BUDDING YEAST *S. cerevisiae*

by

Robert Thomas Youker

B.S., Manhattan College, 1998

M.H.S., Johns Hopkins University, 2001

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This dissertation was presented

by

Robert Thomas Youker

It was defended on

September 30, 2005

and approved by

Karen M. Arndt

John D. Hempel

Jeffrey D. Hildebrand

Raymond A. Frizzell

Jeffrey L. Brodsky
Dissertation Director

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Robert T. Youker, Ph.D.

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ABSTRACT

The requirements of Hsp90 and Hsp70 cytoplasmic chaperone in the proper folding/degradation of an integral membrane protein remain poorly characterized, however it was previously demonstrated that the yeast Hsp70, Ssa1p, chaperone catalyzes the degradation of the misfolded human chloride channel, CFTR. To better define the roles of these chaperones and partner co-chaperones, I characterized the involvement of two Hsp70 co-chaperones, Ydj1p and Hlj1p, in the degradation of CFTR in the budding yeast *S. cerevisiae*. Mutations in the genes encoding Ydj1p or Hlj1p alone did not affect CFTR degradation, but disruption of both co-chaperones stabilized CFTR. In contrast, the degradation of a soluble misfolded protein (CPY*) was unaffected in an *hlj1Δ ydj1-151* double mutant. Hlj1p stimulated the ATPase activity of Ssa1p and partially rescued the growth defect in a *ydj1-151* strain, suggesting that Hlj1p and Ydj1p function redundantly during CFTR degradation.

The contribution of Hsp90 to CFTR folding and degradation in mammalian cells has been examined, but disparate results have been obtained. I therefore analyzed CFTR degradation in yeast using a temperature sensitive Hsp90 mutant ($Hsp90^{G313N}$) and found that CFTR was

degraded faster in the mutant compared to the wildtype. Consistent with this result, highly enriched yeast Hsp90 prevented the aggregation of CFTR's NBD1 domain. In contrast, the degradation of CPY* was unaffected in the Hsp90 mutant. Furthermore, I found no effect on CFTR degradation upon inactivation of the yeast Hsp90 co-chaperones Sba1p, Sti1p, or Sse1p. These results suggest that Hsp90, in the absence of co-chaperones, facilitates CFTR folding, possibly through its interaction with NBD1.

Finally, I analyzed the effects of overexpressing two mammalian co-chaperones on CFTR biogenesis in yeast. I observed reduced CFTR degradation upon overexpression of FKBP8 or Bag-3 but did not observe enhanced trafficking of CFTR to the plasma membrane. This result suggests that stabilization *per se* is not sufficient to promote CFTR exit from the ER.

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LIST OF ABBREVIATIONS

AP = Adaptor Protein
Bag = Bcl-2 Anthanogene
CCP = Clathrin Coated Pits
CF = Cystic Fibrosis
CFTR = Cystic Fibrosis Transmembrane conductance Regulator
CHIP = COOH-terminal Hsc70 Interacting Protein
CME = Clathrin Mediated Endocytosis
COP = Coatomer Protein
COPII = Coat Protein complex II
CPY* = mutant form of CarboxyPeptidase Y
CRT = Calreticulin
CXN = Calnexin
EDEM = ER Degrading Enhancing Mannosidase-like protein
EGFR = Epidermal Growth Factor Receptor
ER = Endoplasmic Reticulum
ERAD = ER Associated protein Degradation
ERGIC = ER-Golgi-Intermediate Compartment
FKBP = FK506 Binding Protein
GGA = Golgi-localized G-ear containing Adenosine diphosphate ribosylation factor-binding protein
HIP = Hsc70 Interacting Protein
HOP = Hsp70-Hsp90 Organizing Protein
HSP = Heat Shock Protein
LDL = Low Density Lipoprotein
NSF = NEM Sensitive Factor
PACS = PhosphoAcidic Cluster Sorting
RNC = Ribosome Nascent chain Complex
SNARE = Soluble NSF Associated Receptor
SR = SRP Receptor
SRP = Signal Recognition Particle
TGN = Trans-Golgi Network
UGGT = UDP-Glucose Glucosyltransferase

PREFACE

I had been told by a professor once, that I was not cut-out to be a Ph.D. scientist and that I should set my professional goals lower. I came to Pittsburgh unsure about my future in scientific research and I questioned my own abilities. Five years after coming to Pittsburgh, I am a confident, skillfull scientist and educator, but I could not have completed this journey alone.

I would like to thank Dr. Jeffrey Brodsky for being a patient, enthusiastic and caring mentor. For providing me with a home to conduct my research and giving me an exciting project to work on. You helped me to see the big picture in science and life and I am truly indebted. I hope we can have many scientific discussions in the future. I would also like to thank my thesis committee Dr. Karen Arndt, Dr. Jeffrey Hildebrand, Dr. John Hempel and Dr. Raymond Frizzell for taking time out of their busy schedules to guide my research and for providing comments on my thesis.

Thank you Jen, Annette, Rob, Mick, Sheara, Craig, Christine, Nancy, Stacy, Shruthi, and Kunio for friendship, advice on experiments, and general discussions about life, the universe and everything. Thank you to all the undergraduates who have made reagents and media over the years, without their help my experiments would have taken a lot longer to complete.

I would also like to thank Dr. Barry Zirkin at Johns Hopkins University for providing me with the opportunity to perform research in his lab and for believing in my abilities, when others did not.

I would like to thank my beautiful wife Xiaoli for giving moral support and always providing plenty of dumplings to eat during those long nights in the lab. Finally, I thank my parents (John and Sandra) and brothers (Steven, David, John) for all their love and encouragement.

1. Introduction

There are 25,000 - 30,000 protein-encoding genes in the human genome (McPherson *et al.*, 2001; Venter *et al.*, 2001; Southan, 2004), but if alternative splicing and post-translational modifications are taken into consideration then the total complement of proteins in a "typical" human cell is much larger. The numbers of proteins that reside in or are transported through the secretory pathway, a major intracellular transport pathway, are estimated to range from 10-20% of the total proteins expressed in a human cell (Emanuelsson *et al.*, 2000; Lander *et al.*, 2001). To ensure that properly folded proteins are transported to their correct final intracellular or extracellular destinations, the cell possesses complex quality control mechanisms. Endoplasmic reticulum associated protein degradation (ERAD) is one quality control mechanism that functions early in the secretory pathway (see section, 1.2 ER Quality Control). An important group of proteins that modulate ERAD are cytoplasmic molecular chaperones (see section 1.3, Cytoplasmic Molecular Chaperones). Only recently has the importance of ERAD become evident with the identification of a large number of secretory proteins that are substrates for ERAD and when mutated cause human disease (Plemper and Wolf, 1999). One of these is the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Mutations in CFTR cause Cystic Fibrosis, one of the most common lethal diseases in Caucasians (Quinton, 1990)(see section 1.4, Cystic Fibrosis and CFTR).

1.1. Secretory Pathway

1.1.1. Co-Translational and Post-Translational Translocation

Before soluble or integral membrane proteins can be transported through the secretory pathway, they must first be targeted to the endoplasmic reticulum (ER). Protein import into the ER can occur either during synthesis of a nascent polypeptide (co-translational translocation) or after its synthesis is complete (post-translational translocation). The majority of ER translocation in mammalian cells occurs co-translationally, while post-translational translocation is more common in yeast and bacteria (Walter and Johnson, 1994). However, even in organisms where post-translational translocation is common, almost all membrane spanning proteins are inserted co-translationally (Valent *et al.*, 1998). What signals direct translating ribosomes to the surface of the ER during co-translational translocation? Approximately 30 years ago Blobel and Doberstein observed that dog pancreatic microsomes stripped of polysomes could sequester newly synthesized immunglobin κ light chain, and the immature light chain was proteolytically processed only if microsomes were present during translation (Blobel and Dobberstein, 1975). Milstein and colleaguaes observed in a cell-free system that immunoglobin light chain synthesized from myeloma mRNA was larger then when the protein was synthesized in the presence of microsomes (Milstein *et al.*, 1972). These investigators hypothesized that the extra "piece" of the protein could be at the NH_2-terminus and that it served as a "signal" to coordinate secretion. Shortly thereafter signal sequences were identified in the amino-terminus of immature

secretory proteins (Schechter, 1973; Kemper *et al.*, 1974). Further work by the Blobel and Doberstein groups led to the identification of two protein complexes known as the signal recognition particle (SRP) and the docking protein (DP), that bind to and coordinate synthesis of pre-secretory nascent proteins (Walter and Blobel, 1981a; Meyer *et al.*, 1982). In mammalian cells, The SRP particle is a multi-protein complex composed of 6 proteins (SRP19, SRP54, SRP68/72, SRP9/14) and a 7S RNA that acts as a scaffold to organize the complex (Siegel and Walter, 1988a, 1988b). The DP, now called the SRP receptor (SR), is composed of two GTPase proteins (SRα, SRβ). Today, all the major players in the co-translational translocation cycle have been identified (see Figure 1)(reviewed in Egea *et al.*, 2005).

The first step in co-translational translocation is the binding of SRP to the signal sequence as it emerges from the polypeptide exit tunnel of the ribosome. The signal sequence is a short hydrophobic stretch of amino acids (7-15 amino acids) that is near the N-terminus for soluble secreted proteins but in membrane proteins can be internal (Goder and Spiess, 2001). The SRP54 subunit binds to the emerging signal sequence, translation is arrested and the ribosome is targeted to the ER membrane (Walter and Blobel, 1981b). The Ribosome-SRP-nascent chain complex (RNC) binds to the SR via an interaction with SRα at the ER membrane. SRP54 and SRα bind GTP and act as reciporal GTPase activating proteins, causing hydrolysis of each other's GTP and transfer of the RNC to the translocation channel (Song *et al.*, 2000). The RNC engages the translocation channel (Plath *et al.*, 1998) that initiates a tight association between the two complexes (Jungnickel and Rapoport, 1995; Belin *et al.*, 1996; Rapiejko and Gilmore, 1997) and opens the luminal gate of the channel (Hamman *et al.*, 1998), creating an aqueous pore from the ribosome exit site to the ER lumen (Crowley *et al.*, 1993; Crowley *et al.*, 1994). Translation

resumes and the N-terminus of the growing polypeptide enters the lumen of the ER where signal peptidase cleaves the signal sequence (Landry and Gierasch, 1991; von Heijne, 1996). The driving force behind translocation of the peptide is controversial and could be provided by the translating ribosome "pushing" the nascent chain through the pore (Gorlich and Rapoport, 1993). However, co-translational translocation into microsomes can be blocked by depletion or mutation of luminal proteins, suggesting that the luminal proteins also drive translocation (Nicchitta and Blobel, 1993; Brodsky *et al.*, 1995).

Yeast and bacteria perform both co- and post-translational translocation. Post-translational translocation proceeds through an SRP-independent mechanism. The SRP-independent pathway was discovered because yeast cells deleted for either SRP or SR are viable (Felici *et al.*, 1989; Amaya and Nakano, 1991; Hann and Walter, 1991)(Stirling and Hewitt, 1992; Brown *et al.*, 1994). In mammalian cells post-translational translocation is strictly used by small proteins (< 70 amino acids) and proteins that are anchored to membranes at their extreme COOH-terminus (Anderson *et al.*, 1983; Kutay *et al.*, 1993; Pfeffer, 1996; Adams and Cory, 1998). The post-translational translocation mechanism is not as well characterized as the co-translational translocation pathway, but cytoplasmic and luminal molecular chaperones are known to be required to keep the newly synthesized protein unfolded because the Sec61p translocon (diameter 20-60 Å) cannot accommodate a fully folded protein (Hamman *et al.*, 1997). Furthermore, unlike co-translational translocation, there is no ribosome present and hence no energy or driving force exists to "push" the nascent polypeptide through the translocation pore. Instead, luminal molecular chaperones have been implicated in providing the energy necessary for translocation (Brodsky, 1998). One current model (see Figure 2) for post-translational

translocation is that the newly synthesized polypeptide is bound by cytoplasmic molecular chaperones to prevent folding/aggregation. Next, it is targeted to the translocation channel by a mechanism that is not fully characterized. At the channel, molecular chaperones on the cytosolic (Ssa1p, Ydj1p) and luminal (BiP, Sec63p) side of the channel aid in the translocation process (Brodsky *et al.*, 1995; McClellan *et al.*, 1998; McClellan and Brodsky, 2000). Both translocation pathways use the same translocation channel (Sec61p) at the ER membrane.

How is the permeability barrier of the ER maintained during translocation? The ER is a major storage compartment for calcium, a potent second messager in cells, and calcium leakage from the ER during translocation would cause havoc in the cell. In mammalian and yeast cells the permeability barrier of the ER is maintained during co- and post-translational translocation by the luminal molecular chaperone BiP (Hamman *et al.*, 1998).

The translocation of membrane proteins is more complex then soluble proteins and occurs co-translationally. The final topology of a membrane protein depends on the orientation, length and composition of the signal sequence and stop-transfer signal, or anchor sequences (Goder and Spiess, 2001). The signal sequence targets the ribosome-nascent chain complex to the translocon and translation resumes, but when the stop-transfer signal (~20 hydrophobic residues) enters the channel translation stops and the signal is inserted into the ER membrane, hence acting as an anchor sequence. The alternation of start and stop sequences allows for the insertion of both bi-topic and polytopic membrane proteins. What determines the orientation of the membrane protein? The best method to predict the orientation of the NH_2 and COOH-termini of the transmembrane helix is by the "positive-inside" rule (von Heijne, 1986). The rule was discovered

first for bacterial proteins where it was observed that positive amino acids were found to be four times more abundant at cytoplasmic loops then periplasmic loops. In eukaryotes, it is not the positive charge, but the charge difference between the flanking sequences of the hydrophobic core of the signal sequence or the transmembrane segments that correlates with the orientation for insertion at the ER membrane (Hartmann *et al.*, 1989). Usually the more positively charged flanking sequence is cytoplasmic.

Figure 1: Model of Mammalian Co-Translational Translocation.

For Simplicity only the SRP54 subunit of the SRP complex is shown and proteins involved in channel gating are not depicted: 1) SRP54 scans for signal sequences emerging from the ribosome; 2) SRP54 recognizes and binds the signal sequence, arresting translation; 3) The Ribosome-SRP nascent chain complex (RNC) is targeted to the Sec61 translocon; 4) The RNC binds to the SRP receptor; 5) SRP54 and SRα bind GTP; 6) SRP54 and SRα stimulate each other's GTPase activity, causing transfer of the RNC to the translocation channel and SRP54 is released; and 7) GDP is released from SRP54 and the cycle can repeat.

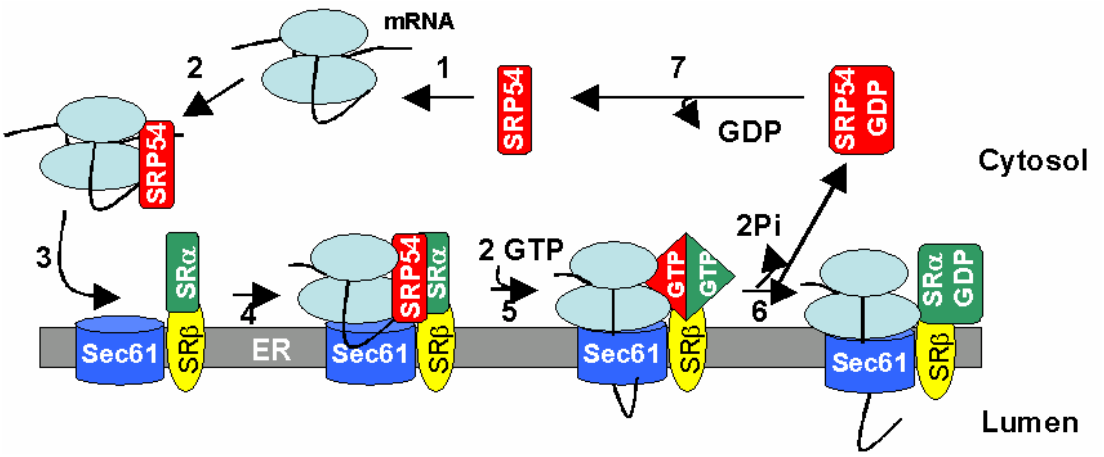


Figure 1: Model of Mammalian Co-Translational Translocation

Figure 2: Model of Yeast Post-Translational Translocation.

1) The newly synthesized secretory precursor protein is released into the cytosol and is bound by molecular chaperones; 2) The secretory precursor-chaperone complex is targeted to the Sec61p complex, and other ER resident proteins (Sec62p, Sec71p, Sec63p, Sec72p) are also required at this step; 3) The polypeptide is translocated through the Sec61p complex and the BiP molecular chaperone may pull the polypeptide into the ER. BiP may also gate the Sec61p complex and is required to maintain the permeability barrier of the ER.

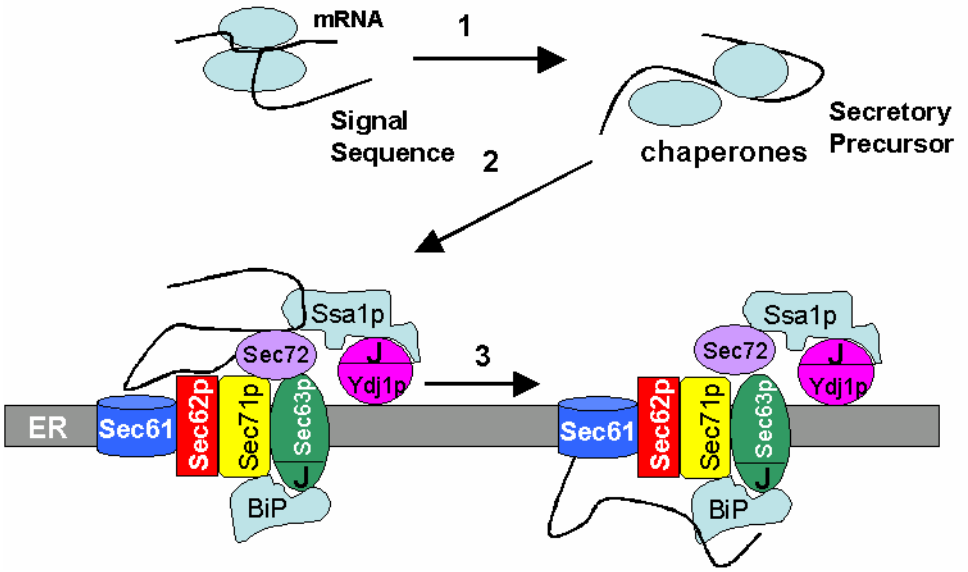


Figure 2: Model of Yeast Post-Translational Translocation

1.1.2. Protein Transport from the Endoplasmic Reticulum to the Golgi

After translocation, properly folded and matured proteins that are not ER residents are transported from the ER to the Golgi. If the protein is not properly folded then it is targeted to the ERAD pathway (see section 1.2) for degradation. Secreted proteins are moved through the secretory pathway by vesicle-mediated transport. Distinct regions of the ER where vesicles form and bud off are termed ER exit sites (Orci *et al.*, 1991; Kuge *et al.*, 1994) (Hobman *et al.*, 1998; Pagano *et al.*, 1999). At these exit sites, secreted proteins or "cargo" are concentrated into vesicles (Malkus *et al.*, 2002). The vesicles are formed by the ordered assembly of coat proteins and it is these coat proteins that cause deformation and budding off of the ER membrane (Gorelick and Shugrue, 2001)(see Figure 3). Specifically, vesicles that bud from the ER membrane are covered with coat protein complex II (COPII). The identities of these coat proteins and their roles in vesicle budding were determined by genetic screens (Novick and Schekman, 1983; Kaiser and Schekman, 1990)and by *in vitro* budding assays using purified coat proteins and lipososmes of a known composition (Barlowe *et al.*, 1994).

The process of vesicle formation at the ER membrane is conserved from yeast to mammals (Dunphy *et al.*, 1986). I will describe the process of vesicle formation at the yeast ER membrane, keeping in mind that mammalian homologues exist for most of the proteins mentioned, (reviewed in Lee *et al.*, 2004a). First, the small ras-related protein Sar1p in its GDP bound form (Sar1p-GDP) is recruited to the ER membrane by the ER resident membrane protein Sec12p. Sar1p-GDP exchanges GDP for GTP through action of Sec12p, which is a guanine nucleotide

exchange factor (GEF). The exchange of GDP for GTP leads to a conformational change in Sar1p, whereby the NH_2-terminus of Sar1p is inserted into the membrane. Sar1p-GTP then recruits the heterodimer coat protein Sec23p/Sec24p. The Sec13p/Sec31p heterodimer then binds to Sec23p/Sec24p, and this process is repeated until a vesicle buds from the ER. Sec13p/Sec31p stimulates the GTPase activating protein (GAP) function of Sec23p; causing Sar1p to hydrolyze GTP. This hydrolysis event causes the release of the coat proteins and uncoating of the newly formed vesicle. The uncoated vesicle then trafficks to its destination compartment, in this case the Golgi apparatus either directly, or by way of the ER-Golgi-intermediate-compartment (ERGIC) (see Figure 4). Only mammalian cells have an ERGIC and in yeast the vesicles fuse directly with the cis-Golgi. Since proteins included in these COPII vesicles can be either soluble or membrane bound it is important to separate these cargo proteins from ER resident proteins. There are two competing theories for how cargo proteins are incorporated into vesicles: the bulk flow model and the regulated cargo selection model. In the bulk flow model, secreted proteins are trafficked to the plasma membrane by default but ER resident proteins contain a signal that allows for their retention in the ER. This view of transport was reinforced by early studies performed in Chinese hamster ovary (CHO) cells using a glycoslated tri-peptide. The tri-peptide was secreted in a reasonable amount of time and possessed no "transport signal" (Wieland *et al.*, 1987). This model for ER-to-Golgi transport was the prevailing model until the mid 1990s when new data by Balch and colleagues gave evidence for a regulated model of transport (Balch *et al.*, 1994). These researchers examined the relative concentration of vesicular stomatitis virus glycoprotein (VSV-G) protein at exit sites of the ER by immuno-electron microscopy and found that VSV-G was sorted from ER resident proteins and enriched 5-10 fold in vesicles.

Figure 3: Assembly of Coat Proteins to form COP II Budded Vesicle.

1) Sar1-GDP is recruited by Sec12p to the ER membrane and GDP is replaced by GTP, causing a conformational change in Sar1p and insertion of Sar1p's NH_2-terminus into the membrane; 2) Sar1p-GTP recruits coat proteins Sec23p/Sec24p; 3) Membrane protein cargo may bind to Sec23p/Sec24p; and Sec13p/Sec31p is recruited and deformation of the ER membrane begins.

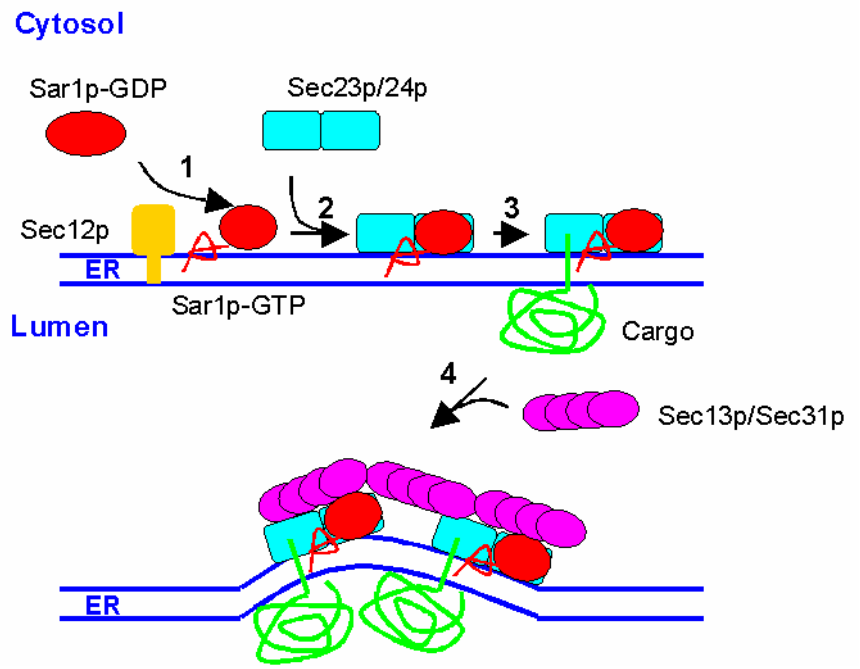


Figure 3: Assembly of Coat Proteins to form COP II Budded Vesicles

This was the first direct evidence that regulated trafficking existed. Since these intial studies other groups have documented the enrichment of cargo protein and the absence of ER resident proteins at ER exit sites in mammalian cells (Aridor *et al.*, 1998). Furthermore, Muniz and colleagues immunoisolated COPII vesicles and determined that the majority of cargo protein inside was of one type (Muniz *et al.*, 2001). Studies in yeast by Barlowe and colleagues led to the identification of Erv29p, a conserved transmembrane protein that is required for the packaging of a soluble protein, pro-α-factor, into COPII vesicles; lending more credence to the importance of regulated export from the ER (Belden and Barlowe, 2001b). There are also packaging receptors for membrane proteins: The ER membrane protein Shr3p facilitates the incorporation of the general amino acid permease (Gap1p) into vesicles through interactions with COPII components (Kuehn *et al.*, 1996; Gilstring *et al.*, 1999). Since transport in the secretory pathway can be anterograde (ER-to-Golgi) or retrograde (Golgi-to-ER), it is the composition of the coat proteins and adaptor proteins that determine vesicle targeting. While COPII-coated vesicles carry cargo proteins from the ER to the Golgi, it is COPI (coatomer complex) coated vesicles that are responsbile for retrograde transport (see Figure 4). Any ER resident proteins that are accidentally packaged for transport to the Golgi are retrieved by COPI coated vesicles in a receptor-dependent manner. The major function for COPI is in the retrograde transport of cargo from the cis-Golgi back to the ER (Ostermann *et al.*, 1993; Spang and Schekman, 1998; Lee *et al.*, 2004a) but there is some evidence for COPI function in the transport of cargo between the Golgi stacks (Waters *et al.*, 1991). The assembly mechanism for COPI is similar as for COPII vesicles, the only differences are the identity of the small GTPase (ARF1) and the coat protein is a 7 subunit complex. How is fidelity and directionality controlled between the ER and Golgi?

SNAREs and Rabs are two classes of proteins that aid in maintaining fidelity and directionality in the secretory pathway (see section 1.1.3).

Most soluble ER resident proteins contain a COOH-terminal KDEL sequence (HDEL in yeast) which allows binding to the KDEL receptor in the Cis-Golgi and subsequent incorporation into COPI vesicles for retrieval (Pelham, 1991). Membrane proteins that contain di-lysine (KKXX) or di-arginine sequences (RxR) can be retained in the ER or retrieved from the Golgi (Cosson and Letourneur, 1994; Teasdale and Jackson, 1996). For some membrane substrates, such as K_{ATP} channels, these motifs are masked upon proper folding/assembly to ensure that only properly folded/assembled proteins are released (Hill and Stevens, 1994; Chang *et al.*, 1999; Zerangue *et al.*, 1999)(see ER quality control 1.2).

As mentioned above, soluble and membrane proteins can be selected for incorporation into COPII vesicles. What recognition or targeting sequence enhances incorporation into COPII vesicles? Analysis of the COOH-terminus of membrane proteins led to the identification of transport or export signals that target their inclusion into COPII vesicles. The VSV-G protein contains a di-acidic motif at its C-terminus (DxE) that interacts with Sar1p and Sec23p/Sec24p (Nishimura *et al.*, 1999; Sevier *et al.*, 2000). A growing number of membrane proteins contain DxE or ExE motifs that are required for efficient packaging into COPII vesicles (Wang *et al.*, 2004) (Ma *et al.*, 2001; Scott *et al.*, 2001; Votsmeier and Gallwitz, 2001; Ma and Jan, 2002). A second class of export motifs contain hydrophobic and aromatic amino acids at their COOH-terminus, such as two phenylalanines (FF) which are present in the p23/24 family of proteins (Belden and Barlowe, 2001a; Nufer *et al.*, 2002). The p23/24 proteins are ER transmembrane

proteins that bind to soluble secreted proteins and facilitate incorporation into COPII vesicles. The ER export signal may be on the secreted protein itself or in a receptor that binds to the protein. Cargo receptors are not restricted to soluble proteins and specific receptors have been identified for transmembrane proteins (Powers and Barlowe, 1998, 2002).

The current model for ER-to-Golgi transport is a combination of regulated export and bulk flow mechanisms (Pelham and Rothman, 2000). The vast majority of membrane proteins (e.g. VSV-G, Gap1p) are concentrated into COPII coated vesicles by means of export sequences. In contrast, many but not all soluble proteins are concentrated into vesicles through interaction with coat proteins, while a handful have been identified that use a cargo receptor (Belden and Barlowe, 2001b; Otte and Barlowe, 2004). The bulk flow mechanism or passive sampling of the ER luminal contents is a very inefficient mode of transport (Malkus *et al.*, 2002), however it is used by specialized secretory cells to transport some soluble enzymes, such as amylase and chymotrypsinogen (Martinez-Menarguez *et al.*, 1999).

Figure 4: ER to Golgi Vesicle Transport of Proteins in Mammalian Cells.

COPII-coated vesicles bud from the ER and either directly fuse with the cis-Golgi or the vesicles fuse together to form the ER-Golgi-intermediate-compartment (ERGIC), before the ERGIC fuses with the cis-Golgi. ER resident proteins (retrograde cargo) that are mistargeted to the Golgi are retrieved by COPI vesicles and returned to the ER by retrograde transport. In addition to distinct coat proteins, there are also distinct sets of SNAREs (see section 1.1.3) that play a role in anterograde or retrograde transport between the two compartments. Note: plants and yeast do not contain an ERGIC, and COPII vesicles fuse directly with the cis-Golgi.

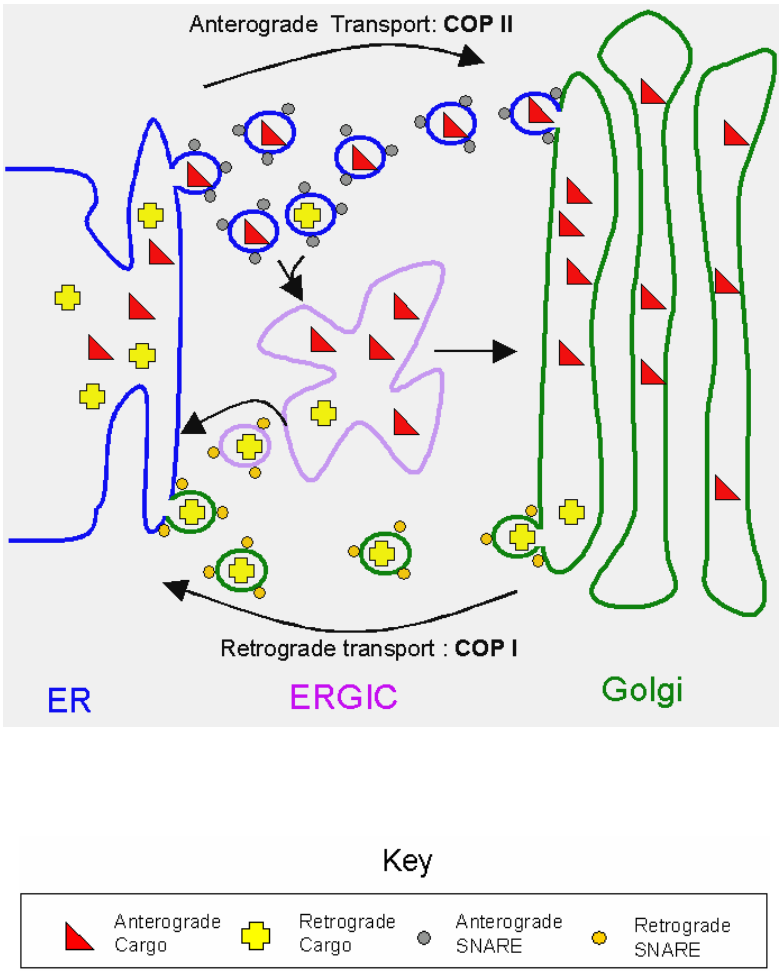


Figure 4: ER to Golgi Vesicle Transport of Proteins in Mammalian Cells

1.1.3. Transport of Proteins from the Golgi to the Plasma Membrane

1.1.3.1. Fusion of ER Vesicles with the Cis-Golgi

After a newly created vesicle is released from the ER, it must find and fuse with the correct target membrane, in this case the cis-Golgi. What machinary is involved in membrane fusion? Soluble NSF attachment protein receptor or "SNARE" proteins are implicated in membrane fusion reactions in all compartments of the eukaryotic cell. Syntaxin was the first SNARE identified by researchers studying syntaptic vesicle docking in neuronal cells (Bennett *et al.*, 1992). Since then over 100 SNARES have been identified in a diverse set of organisms (Jahn and Sudhof, 1999). Rothman and Sollner proposed the SNARE hypothesis in 1993 (Sollner *et al.*, 1993) to explain the role of SNAREs in membrane fusion. The hypothseis stated that two types of SNAREs existed, one type on vesicles (v-SNAREs) and another type on target membranes (t-SNAREs). In their model, v-SNAREs and t-SNAREs paired-up, causing membrane fusion and subsequent release of vesicle contents into the target membrane. The hypothesis stated that specificity was determined by the unique pairing of v- and t-SNAREs and that only SNAREs are needed for membrane fusion, a conclusion that was based on *in vitro* experiments (Sollner *et al.*, 1993). However, this hypothesis was postulated when very little was known concerning the mechanism of action of SNAREs. Today it is known that other proteins are required in addition to SNAREs for efficient membrane fusion *in vivo*. The accepted model for SNARE mediated membrane fusion is depicted in Figure 5 (for review see (Chen and

Scheller, 2001)) and the mechanism can be divided into several steps which include targeting of vesicles, vesicle tethering and docking, and finally fusion.

The most well characterized SNARE complex (SNAP-25-Syntaxin1-VAMP) complex has been crystallized (Sutton *et al.*, 1998), and this has helped to decipher their mechanism of action. All three proteins interact to form a four helix bundle during the docking phase. It is the formation or "zippering" of this four helix bundle (the "SNAREpin") that proceeds in an NH_2 to COOH-terminal fashion that brings the vesicle and target membranes into close proximity, thus allowing the membranes to fuse. To break apart this highly stable SNAREpin complex requires energy from the ATPase NEM-sensitive factor (NSF). NSF is a soluble ATPase protein that was identified before SNAREs as being important in membrane fusion (Malhotra *et al.*, 1988). Once this complex is dissociated, another round of membrane fusion can occur. SNARE proteins exist on all membraneous compartments and their role in either anterograde or retrograde transport depends on the Rabs and effector proteins recruited to the membrane surface (Zerial and McBride, 2001).

As mentioned earlier, Rabs regulate membrane fusion events and vesicle trafficking at the TGN and other compartments in the secretory pathway. Rabs are monomeric GTPase proteins of the ras protein superfamily. Rabs are found in all eukaryotes and there are more than 60 rabs in the human genome (Seabra *et al.*, 2002). Rabs are thought to function by recruiting effector proteins to the membrane surface during budding, transport and docking of vesicles and act as anchors to ensure a proper fit between SNAREs. Rabs cycle between the cytosol and membrane surface by a GTP switch mechanism similar to Sar1p and ARF1 (Seabra *et al.*, 2002). Together, the

SNARE proteins in concert with Rabs and coat proteins are responsible for controlling the directionality and fidelity of vesicle transport between compartments in the secretory pathway. As noted above, vesicles can fuse directly with the cis-Golgi or they may fuse to each other forming the ER intermediate compartment (ERGIC) or vesicular tubular cluster (VTC). It is this intermediate compartment that can fuse with the Golgi in mammalian cells. However, yeast appear to lack an ERGIC and therefore ER-derived vesicles are thought to fuse directly with the Golgi. In any event, a secretory protein that is delivered to the cis-Golgi by SNARE-mediated membrane fusion undergoes sidechain modification of its N-linked glycans (attached in the ER) and in some cases proteolytic processing as it progresses through the *cis*-, medial, and *trans*-compartments of the Golgi. The transport of proteins between stacks is believed to be either by vesicular transport, or cisternal progression/maturation. Cisternal maturation is necessary for a subset of proteins that traverse the secretory pathway, such as pro-collagen, which forms 300 nm rigid rods in the ER and is thus to large to "fit" into classical 40-50 nm secretory vesicles (Bonfanti *et al.*, 1998).

Figure 5: Model for SNARE-mediated Vesicle Fusion.

1) An ER-derived vesicle attaches initially to its target membrane (in this case the Golgi) through interactions with a tethering complex, which could contain Rabs and effectors; 2) the v-SNARE makes contact with the Golgi SNARE (t-SNARE); 3) The loose association between v-SNAREs and t-SNAREs tightens to form a SNAREpin complex; 4) The SNAREpin "zippers-up"; bringing the two membranes in close contact for fusion to proceed.

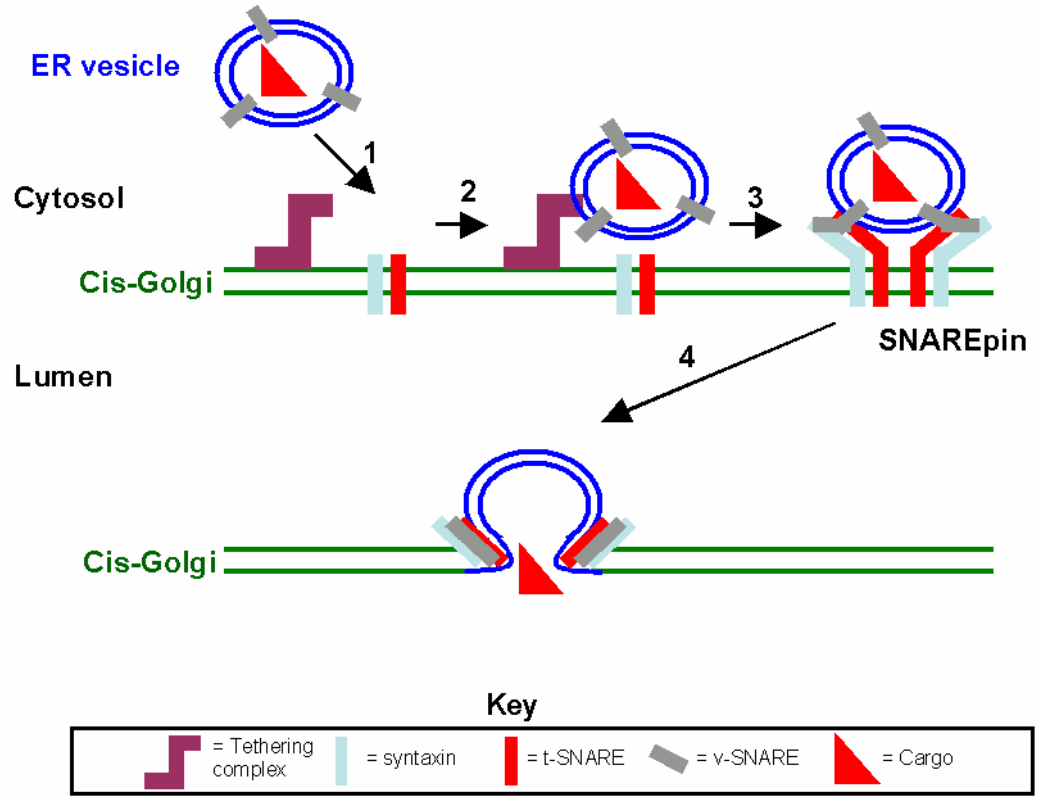


Figure 5: Model for SNARE-mediated Vesicle Fusion

1.1.3.2. Protein Transport from the Trans-Golgi-Network to the Plasma Membrane

After passage through the Golgi compartment secreted proteins enter the *trans*-Golgi network (TGN). The TGN is a sorting-station that directs cargo to the plasma membrane, lysosome (vacuole in yeast), or endosomal compartments of the cell. The vesicles that bud from the TGN are not coated with COPII or COPI coat proteins but are usually coated with a clathrin-coat that facilitates membrane deformation.

Clathrin is a multiprotein complex composed of 3 heavy chains (180,000 Da) and 3 light chains (35,000 Da) that form a three-legged structure named the triskelion. Multiple clathrin complexes assemble to form a basket-like structure composed of pentagons and hexagons. In 1976 Barbara Pearse identified clathrin as the most abundant protein in plasma membrane coated pits (Pearse, 1976). Purified clathrin was able to form cages *in vitro* of a similar size to coated pits, but this occurred only under non-physiological conditions. The search for clathrin co-factors that could facilitate the formation of clathrin cages under physiologic conditions led to the discovery of two adaptor complexes (AP1 & AP2) that colocalized with clathrin (Pfeffer *et al.*, 1983; Virshup and Bennett, 1988). Recently, two additional adaptor complexes (AP3, AP4) were identified bringing the total number of basic adaptor complexes to four (Dell'Angelica *et al.*, 1997; Simpson *et al.*, 1997) (Dell'Angelica *et al.*, 1999; Hirst *et al.*, 1999).

Adaptor complexes are heterotetramic complexes composed of two large adaptins (AP1 = γ,β1, AP-2 = α,β2, AP-3 = δ,β3, AP-4 = ε,β4), a medium subunit (μ1-4) and a small subunit (σ1-4). The AP-1 adaptor is predominatly located on TGN and endosomal membranes, while AP-2 is

located at the plasma membrane (Robinson and Pearse, 1986; Robinson, 1987; Pearse, 1989). The two recently discovered adaptors AP-3 and AP-4 appear to be localized pre-dominantly to the endosome and TGN respectively (Simpson *et al.*, 1996; Dell'Angelica *et al.*, 1997; Dell'Angelica *et al.*, 1998; Dell'Angelica *et al.*, 1999). Adaptors are localized to their respective compartments by various mechanisms and require interactions with different subunits. For example, the α subunit of AP-2 binds to poly-phosphoinositols located in the plasma membrane (Beck and Keen, 1991; Gaidarov *et al.*, 1996; Gaidarov and Keen, 1999). The γ subunit of AP-1 binds to ARF at the TGN (Traub *et al.*, 1993) (Seaman *et al.*, 1996). Adaptors assist in recruiting clathrin to the surface of membranes and can bind cargo for incorporation into clathrin coated vesicles, thus providing a link between clathrin vesicle formation and cargo inclusion.

Adaptors recognize and bind to cargo proteins based on a recurrent theme in vesicular transport: the presence of short amino acid sequences in the tails of the cargo proteins. In ER-to-Golgi transport the sequences are called export sequences, while in trafficking to and from the TGN they are called sorting signals. The first sorting signal identified that could bind to an adaptor was YXXΦ (X = any amino acid, Φ = bulky hydrophobic amino acid), which binds to the μ2 subunit of AP2 (Ohno *et al.*, 1995; Ohno *et al.*, 1996; Ohno *et al.*, 1998). Tyrosine-based sorting signals are the most well characterized of the sorting signals and are involved in the sorting of cargo at the TGN, endosome, lysosome and even in internalization from the plasma membrane.

Proteins destined for the lysosome/endosome compartments are sorted away from proteins destined for the plasma membrane. Proteins trafficking through the TGN can be divided into three categories: 1) Proteins that are to be secreted or inserted into the plasma membrane

immediately (constitutive secretion); 2) Proteins that are incorporated into secretory vesicles for later release (regulated secretion); 3) Proteins targeted to the endosome/lysosome compartments (see Figure 6). Immediate secretion or insertion into the plasma membrane is considered a default pathway (Traub and Kornfeld, 1997). The complexities of regulated secretion in neuroendocrine cells go beyond the range of this introduction (reviewed in Jena, 2005). Many plasma membrane proteins, including CFTR (see below), are inserted by constitutive secretion. The best studied example of protein trafficking in the TGN is the sorting of lysosomal hydrolases from the TGN to the lyosome by the mannose-6-phosphate receptor (MPR). Newly synthesized hydrolases are co-translationlly translocated into the ER, core-glycosylated and transported to the Golgi. In the Golgi, the core gylcans are modifed with the addition of 6-phosphomannosyl sugars. The MPR binds to this modification on the hydrolase protein and incorporates it into clathrin coated vesicles. The MPR contains an adaptor binding domain and an acidic di-leucine motif (binds to β subunit) that connects it to AP-1 (Mauxion *et al.*, 1996).

In yeast there are two pathways for sorting proteins to the vacuole, which is a degradative compartment analogous to the lysosome. The two proteins that define these pathways are carboxypeptidase Y (CPY) and alkaline phosphatase (ALP) (see Figure 7). CPY is delivered to the vacuole along the classical TGN-endosome-vacuole, or vacuolar protein sorting pathway (VPS) (Banta *et al.*, 1988; Robinson *et al.*, 1988). The CPY receptor Vps10p mediates transport of CPY to the pre-vacuolar compartment (precursor to vacuole). This pathway is similar to the delivery of hydrolyases by MPR to the lysosome in mammalian cells. In contrast, ALP is delivered directly from the TGN to the vacuole, and deletion of the yeast homologue of AP-3 blocks transport of ALP but not CPY (Cowles *et al.*, 1997a; Cowles *et al.*, 1997b; Stepp *et al.*,

1997). In general, the correct sorting of proteins in the TGN to their other compartments is accomplished by the recognition of different sorting signals using coat protein/adaptor complexes. For example, clathrin/AP-1 coats direct sorting of proteins from the TGN to the lysosome/endosome compartments, while clathrin/AP-2 regulates endocytosis from the plasma membrane.

Adaptors are recruited to membranes by small GTP-binding proteins, such as ARF in the case of AP-1. In mouse cells deficient for the μ1A subunit of AP-1, the MPR is localized under steady-state conditions to the endosome instead of the TGN, but delivery of lysosomal hydrolases is normal (Meyer *et al.*, 2000). This experiment implicated AP-1's involvement in retrograde transport of proteins from endosomes/lysosomes to the TGN. How could AP-1 be involved in anterograde and retrograde transport? One answer is that there might be unique effectors of ARF and AP-1 to help explain the role of AP-1 in TGN sorting. Indeed, γ-synergin was identified by yeast two hybrid analysis as binding to γ-adaptin, a subunit of AP-1 (Page *et al.*, 1999). Recently, the GGA (Golgi-localized, G-ear-containing, Adenosine diphosphate ribosylation factor-binding protein) adaptor proteins were identified by several groups based on homology to domains of ARF and AP-1 (Poussu *et al.*, 2000) (Takatsu *et al.*, 2000) (Boman *et al.*, 2000; Dell'Angelica *et al.*, 2000) (Hirst *et al.*, 2000). Yeast contain two homologues of GGAs and deletion of both genes leads to the missorting of carboxypeptidase Y (CPY) (Hirst *et al.*, 2000). Overexpression of GGAs causes mislocalization of MPR and the Golgi protein TGN38, implicating GGAs in TGN to endosome sorting (Boman *et al.*, 2000). There is very little information known about the effectors for retrograde transport from the endosome to the TGN in mammalian cells. Recently, TIP47 and PACS-1 were identified as adaptors that play a role in retrograde transport in

mammalian cells. TIP47 appears to only be involved in the retrieval of MPRs from the lysosome back to the TGN (Diaz and Pfeffer, 1998).

In contrast, PACS-1 can bind to mutliple substrates, including furin, for retrieval from the endosome/lysosome back to the TGN (Wan *et al.*, 1998) (Crump *et al.*, 2001; Crump *et al.*, 2003) (Blagoveshchenskaya *et al.*, 2002; Kottgen *et al.*, 2005). PACS-1 acts as a connector between furin and AP-1. In fact, PACS-1 (Phospho-Acidic Cluster Sorting protein) was identified in a yeast two hybrid screen using a mutant of the COOH-terminus of furin. PACS-1 specifically binds to the phosphorylated form of furin (Wan *et al.*, 1998). The demonstration that not only transport signals, but also the phosphorylation state of the COOH-terminal tail of the cargo protein, are important in trafficking adds a new layer of complexity to recognition by adaptor proteins.

Figure 6: Cargo Sorting Pathways from the Trans-Golgi Network (TGN) to the Plasma Membrane in mammalian cells.

Clathrin/AP-1 and GGAs regulate protein trafficking from the TGN to EE. AP-2/Clathrin regulate endocytosis and exocyotsis at the PM. Direct transport of proteins from the TGN to the Lysosome is regulated by AP-3. AP-1/PACS-1 regulate retrograde transport from LE back to the TGN. Dotted lines represent pathways that are not fully elucidated (see text for complete details).

TGN =Trans Golgi Network, EE = Early Endosomes, LE = Late Endosome, Lys = Lysosome

PM = Plasma Membrane

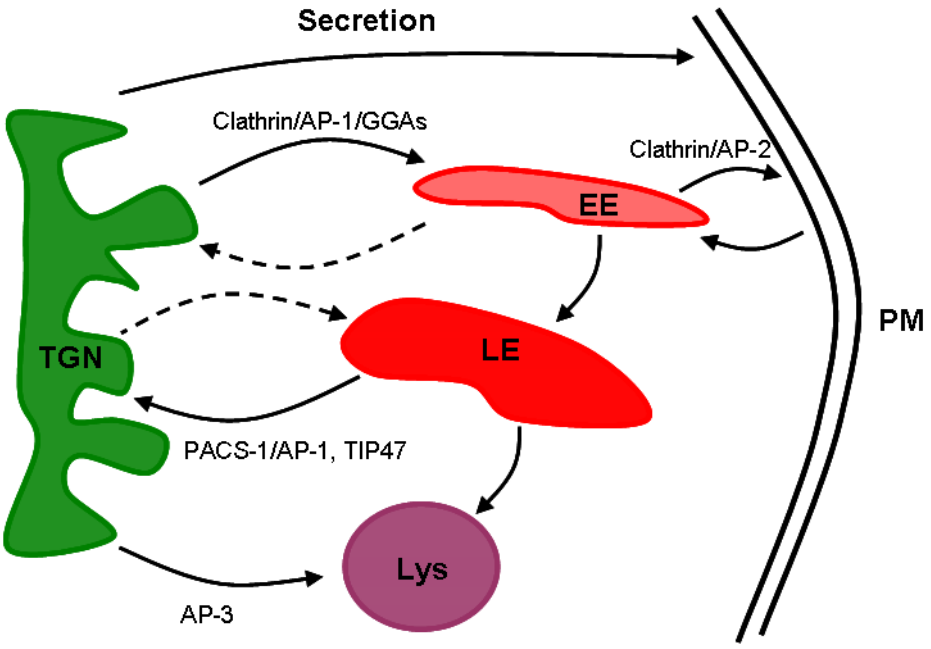


Figure 6: Cargo Sorting Pathways from the Trans-Golgi Network (TGN) to the Plasma Membrane

Figure 7: Golgi to Plasma Membrane Sorting Routes in Yeast.

Homologues of mammalian adaptor complexes exist in yeast but are not depicted for clarity. The CPY and ALP pathways are highlighted because these are the two most well defined in yeast (see text for details). Vps52/vps53/vps54 is a heterotrimeric protein complex that along with the retromer complex are required for protein sorting at the TGN (Conibear MBC 2000). Endosomes are not depicted but do exist and endocytosis and exocyotsis at the plasma membrane are dependent on clathrin in yeast.

TGN = Trans Golgi Network PVC = Prevacuolar Compartment Vac = Vacuole PM = Plasma Membrane

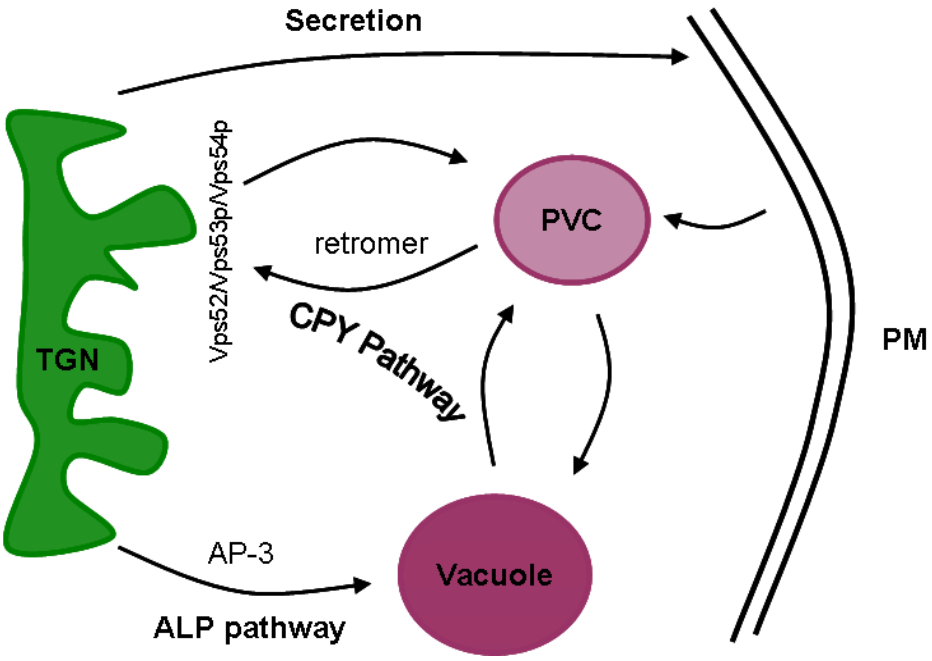


Figure 7: Golgi to Plasma Membrane Sorting Routes in Yeast

1.1.4. Endocytosis from the Plasma Membrane

The secretory pathway or biosynthetic pathway is the cellular highway for transport of proteins and lipids to the preiphery or outside of the cell. However, a cell needs to bring proteins, lipids and nutrients from the outside to the inside of the cell. The endocytic pathway in the cell is the return pathway for proteins and lipids to gain entry to the cellular interior (reviewed in Conner and Schmid, 2003). The two types of endocytosis are constitutive and receptor-mediated. Constitutive endocytosis includes pinocytosis or "cellular drinking" and phagocytosis or "cellular eating". The vesicles for pinocytosis are smaller (150 nm or less) compared to phagocytosis (250 nm or greater). Both processes occur constantly as the cell samples its extracellular space "looking" for nutrients and proteins for energy. Clathrin-mediated endocytosis (CME), originally called receptor mediated endocytosis, involves the concentration of macromolecules through binding to receptors at the plasma membrane and subsequent internalization through clathrin coated pits.

The CME process is similar to the packaging of lysosomal hydrolases in the TGN, although many plasma membrane receptors and channels are down-regulated from the plasma membrane by CME. The four steps to CME are clustering, internalization, uncoating and fusion (Brodin *et al.*, 2000). The process begins with the recruitment of clathrin to the cytosolic side of the plasma membrane by AP-2. The accumulation of clathrin on the membrane surface causes a high curvature deformation in the membrane called a clathrin coated pit (CCP). Receptors that are activated, such as the epidermal growth factor receptor (EGFR), by ligand binding are recruited to the CCPs by AP-2 through their cytoplasmic tails. Calthrin and AP-2 bind to adaptor proteins

unique for endocytosis (amphiphysin, epsin, Eps15). The initial steps and machinery to form a CCP are similar to the budding of clathrin vesicles at the surface of the TGN. The CCP is pinched off by the GTPase dynamin, but the exact mechanism of dynamin's action in the scission of the vesicle is controversial (Hinshaw and Schmid, 1995; Sweitzer and Hinshaw, 1998) (Stowell *et al.*, 1999). Once the vesicle is pinched off, uncoating and fusion with an endosomal compartment can occur. In the endosome a ligand may dissociate from its receptor and the receptor may recycle back to the plasma membrane; go to the lysosome for degradation; or traffic to another plasma membrane domain (transcytosis). However, most receptors and plasma membrane proteins, such as transporters, are recycled back to the plasma membrane. If a receptor-ligand complex is endocytosed, the receptor and ligand dissociate at a given pH; this determines into which compartment the ligand is sorted. As the receptor progresses from early (sorting and recycling) to late endosomes the pH drops from ~6.5 to ~5 (Yamashiro *et al.*, 1984; Mellman *et al.*, 1986). The well studied transferrin receptor, which carries iron into all dividing and hematopoeitic cells, and the Low Density Lipoportien (LDL) receptor are examples of receptors that are constantly recycled back to the plasma membrane (Maxfield and McGraw, 2004). In contrast, the Epidermal Growth factor Receptor (EGFR) bound to EGF is sent to the lyosome for degradation. This leads to a reduction in EGFR at the plasma membrane, a process termed receptor down-regulation (Smythe and Warren, 1991; Sorkin and Waters, 1993).

Endocytic receptors that lack their COOH-terminal tails are poorly endocytosed (Adams and Cory, 1998) (Verrey *et al.*, 1990; Huang *et al.*, 1995). This observation led to the discovery of an endocytic sorting motif (Davis *et al.*, 1987). The most extensively studied endocytic sorting motif is the tyrosine-based sorting motif Yxψϕ (x = any amino acid, ψ = bulky hydrophilic, and

a ϕ = hydrophobic residue) of the transferrin receptor (Canfield *et al.*, 1991), but there are other variations of this motif (Chen *et al.*, 1990). The motif in the transferin receptor is recognized by the μ2 subunit of AP-2, but it appears that receptors utilize different endocytic motifs and adaptor machinery (Warren *et al.*, 1998). For example, the adaptor protein disabled-2 (Dab2) recognizes the FxNPxY (x = any amino acid) motif in the LDL receptor-family and binds to AP-2, thus connecting the receptor to the endocytic coat proteins (Morris and Cooper, 2001; Mishra *et al.*, 2002). Overall, different receptors do not appear to compete for sorting into the same CCPs, but use distinct motifs and adaptors for incorporation into these vesicles.

1.2. ER Quality Control

The first of many sorting events in the secretory pathway occurs in the ER, where secretory proteins are sorted away from ER resident proteins into COPII vesicles. Properly folded secretory proteins must also be sorted away from misfolded or incompletely assembled proteins, which are dangerous byproducts of protein folding in the ER that could disrupt normal cellular function. The process of conformation-dependent sorting of newly synthesized proteins in the ER was given the name ER quality control (Hurtley and Helenius, 1989; Ellgaard *et al.*, 1999). The mechanisms by which aberrant proteins are identified and retained in the ER are complex and overlapping. Moreover, three main chaperone systems operate in the ER lumen to aid in the folding of proteins or retention of misfolded/unassembled proteins until they can be refolded or degraded and these systems are discussed in the following section.

1.2.1. Folding of a Secretory Protein

During translocation most proteins undergo N-linked glycosylation on asparagine residues within the consensus sequence Asn-X-Ser/Thr. The $Glc_3Man_9GlcNAc_2$ (Glc = glucose, Man = mannose, GlcNac = N-acetyl glucosamine) glycan is transferred from dolichol to the protein by oligosaccharyl transferase on the luminal side of the ER. Multiple chaperone systems exist to correctly fold proteins in the ER lumen. A chaperone is a protein that prevents protein aggregation and catalyzes the folding of that protein (for a detailed explanation see section 1.3). The best described chaperone-mediated retention/folding pathway is for glycoproteins in mammalian cells.

First, as a glycoprotein folds, the two outermost glucoses of the Glc_3Man_9-glycan ($Glc_3Man_9GlcNAc_2$) are trimmed off by glucosidase I and II. The calcium-binding lectins calnexin/calreticulin bind to monoglucosylated proteins (Hammond *et al.*, 1994; Peterson *et al.*, 1995) and the oxidoreductase ERp57 forms transient mixed disulfide bonds with any cysteine residues in the glycoprotein (Molinari and Helenius, 1999) to catalyze proper disulfide bond formation. On cleavage of the final glucose, the glycoprotein is released from the lectin/oxidoreductase complex and if the protein has reached its native conformation it exits the ER. If the protein is still misfolded then the "folding sensor" UDP-glucose glucosyltransferase (UGGT) binds to the glycoprotein and reglucosylates the protein to generate the Glc_1Man_9 glycan, thus causing calnexin/calrecticulin to re-bind the protein. The cycle of binding and

release can be repeated multiple times in an attempt to fold the glycoprotein (see Figure 8), (reviewed in Ellgaard *et al.*, 1999). If the protein cannot be folded, then an enzyme called mannosidase I cleaves mannose to generate either a Glc_1Man_8 or Man_8 containing glycan. Another lectin, called EDEM (ER Degrading Enhancing Mannosidase-like protein), has been proposed to target the glycoprotein for degradation (Molinari *et al.*, 2003; Oda *et al.*, 2003). Yeast do not contain a calnexin/calrecticulin cycle, but do contain an EDEM homologue, called Mnl1p or Htm1p, that facilitates the proteolysis of misfolded glycoproteins (Jakob *et al.*, 2001; Nakatsukasa *et al.*, 2001).

Second, proper disulfide bond formation is critical for the folding and activity of secretory proteins (Rietsch and Beckwith, 1998) and a chaperone system exists to catalyze the correct formation of disulfide bonds in the ER lumen (reviewed in Fewell *et al.*, 2001). Proteins unable to form the correct disulfide bonds may aggregate in the ER, which can lead to disease (Plemper and Wolf, 1999).

And finally, the luminal chaperone BiP, an Hsp70 chaperone, which is essential for protein translocation can bind to exposed hydrophobic patches on newly synthesized proteins in the ER lumen and help catalyze protein folding through a binding/release cycle that requires ATP hydrolysis (see section 1.3). It is the cooperation of these three main chaperone systems that ultimately catalyze the folding of newly synthesized proteins in the ER lumen. If the proteins are misfolded/unassembled then these systems retain the protein in the ER lumen until they can attain their native state. Prolonged retention of the misfolded/unassembled protein can lead to

aggregation or targeting of the aberrant protein to the cytosol where it is degraded by the 26S proteasome.

Figure 8: Timing Mechanism for Glycoprotein Quality Control.

Misfolded polypeptide (brown line) enters the lumen of the ER and the terminal two glucoses of its NH_2-linked glycans are trimmed by Glucosidase I and II. The mono-glucosylated polypeptide can bind to calnexin (CXN) or calreticulin (CRT). When the substrate polypeptide is released from CXN/CRT after a round of folding then the remaining glucose is trimmed off by Glucosidase II and the native protein can exit the ER. But, if the polypeptide is still misfolded then it becomes a substrate for UDP-glucose:glycoprotein glucosyltransferase (UGGT), which attaches a single glucose back onto to the NH_2-glycan to force re-association with CXN/CRT. After several rounds of association with CXN/CRT, if the protein remains misfolded then it becomes a substrate of α1,2-mannosidase-I, which cleaves off the Mann9 moiety to yield a Mann8 glycan. This allows the ER degradation enhancing α1,2-mannosidase-like (EDEM) protein to bind and target the misfolded protein for degradation.

Man = mannose, Glc = glucose, GlcNac = N-acetylglucosamine

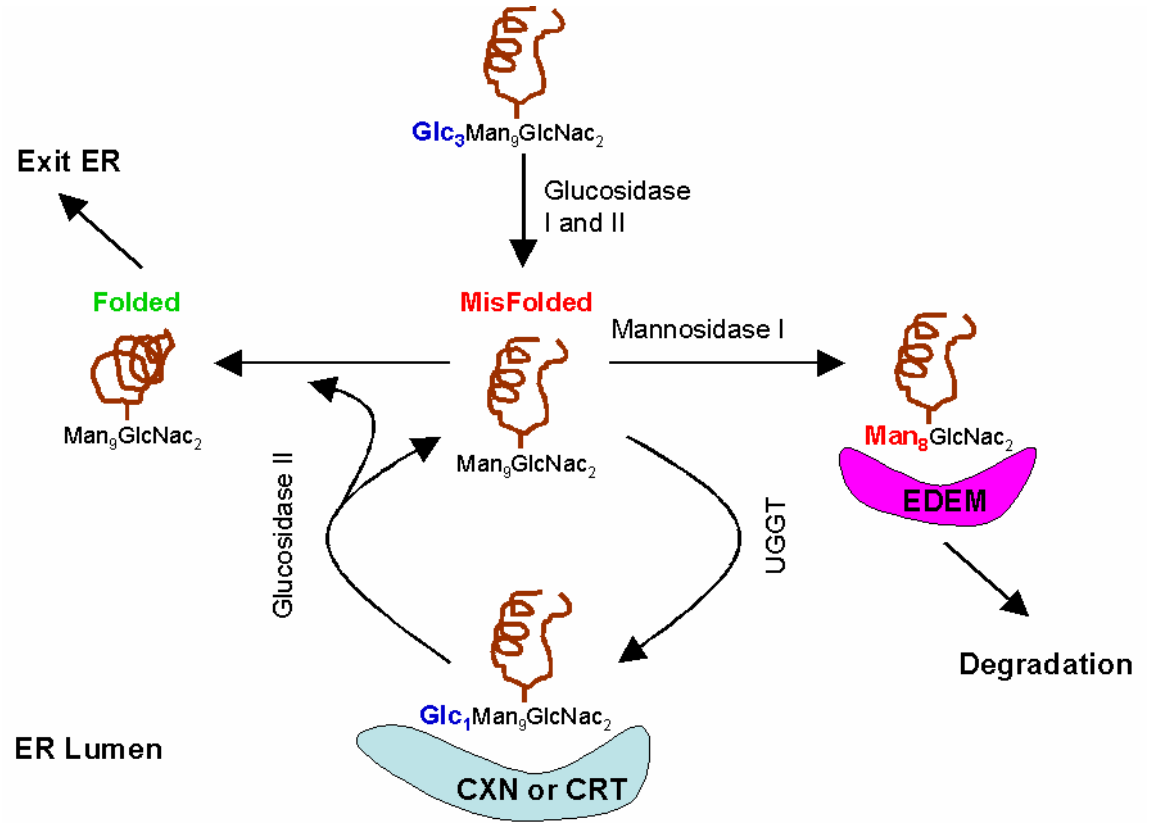


Figure 8: Timing Mechanism for Glycoprotein Quality Control in the ER

1.2.2. Ubiquitin-Proteasome Degradation Pathway

For much of the early 20th century it was believed that only dietary proteins were degraded to provide energy for the body, while proteins in the body were viewed as relatively stable. This notion was challenged by the protein labeling studies of Scheonheimer (Ratner *et al.*, 1987). The identification of a compartment in the cell, called the lysosome, which is dedicated to protein degradation gave birth to the idea that proteins are continually being synthesized and degraded. Mounting experimental evidence from the 1950s through the 1970s suggested that some cellular proteins were not degraded by the lysosome and that a second unidentified proteolytic system must exist in the cell. Rabinovitz and Fisher demonstrated that aberrant hemoglobin that contained amino acid analogues was efficiently degraded in rabbit reticulocytes, which do not contain lysosomes, and this was the first evidence for a non-lysosomal proteolytic pathway (Rabinovitz and Fisher, 1964). Biochemical fractionation of rabbit reticulocyte lysate by Ciechanover and Hershko led to the identification of a ~8.5 kDa heat stable protein, later identified as ubiquitin, which modifies proteins targeted to the non-lysosomal pathway (Hershko *et al.*, 1979; Ciechanover *et al.*, 1980). The rabbit recticulocyte lysate assay proved to be a powerful cell-free system to study proteolysis and was used by several groups to identify the enzymes and protease involved in non-lysosomal protein degradation (Ciechanover *et al.*, 1982; Hershko *et al.*, 1983; Tanaka *et al.*, 1983; Waxman *et al.*, 1987).

The core components of the ubiquitin-proteasome pathway include three enzymes (E1, E2, E3), and the cytosolic 26S proteasome (see Figure 9). Ubiquitin is conjugated to a primary amine usually lysine on a target proteins through its COOH-terminal glycine residue (Gly76) in a

process that requires E1, E2 and E3 enzymes. First, ubiquitin is actviated through thioester bond formation with E1. Next, the E1 enzyme transfers the ubiquitin to the E2 enzyme by a transthiolation reaction. The ubiquitin is then transferred to the E3 ubiquitin ligase enzyme and finally the E3 conjugates the ubiquitin to the ε-amino group of lysine of a target protein. Some E3s do not directly receive ubiquitin but instead facilitate its transfer from E2 to the target protein. This process can be repeated with each subsequent ubiquitin being attached to K48 of the previously added ubiquitin moiety to generate a ubiquitin chain on the substrate protein (Glickman and Ciechanover, 2002). A minimum of four ubiquitins or a tetra-ubiquitin tag is required to target proteins to the proteasome for degradation (Thrower *et al.*, 2000). Most but not all proteins targeted to the proteasome for degradation contain poly-ubiquitin chains (Verma and Deshaies, 2000). In most organisms there is one E1, several E2s and a large number of E3s (Wong *et al.*, 2003). It is believed that the combination of E2/E3 pairs provide substrate specificity for the tagging of proteins for destruction. Once tagged, the protein is sent to the proteasome for degradation.

The proteasome is an ~1.5 Mda multicatalytic protease composed of a 19S regulatory cap (Glickman *et al.*, 1998a; Glickman *et al.*, 1998b)and a 20S core (Pickart and Cohen, 2004). The 19S cap contains subunits that bind to poly-ubiquitin tags (Lam *et al.*, 2002; Walters *et al.*, 2002) (Elsasser *et al.*, 2002) and function to gate or control which polypeptides enter the catalytic core. The cap also contains six AAA-ATPase subunits that are thought to unfold substrates and feed them into the 20S core. The catalytic core is composed of four stacked rings and each ring is composed of seven distinct subunits to give the general structural arrangement of $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$. The core possesses three distinct protease activities. The 20S core may have one or both ends

bound by a 19S cap complex. The proteasome is known to reside in the cytosol and in the nucleus and is attached to the ER/nuclear membrane (Enenkel *et al.*, 1998).

1.2.3. ER Associated protein Degradation (ERAD)

ER Associated protein Degradation (ERAD) is the process by which misfolded proteins are retro-translocated/dislocated from the ER compartment and targeted to the cytosolic proteasome for degradation. For a long time it was believed that ERAD was mediated by ER resident proteases. This belief was upheld by the observations that in a permeabilized cell system the degradation of ER, misfolded proteins was independent of cytosol (Stafford and Bonifacino, 1991). Second, the isolation of an apparent protease (ER-60) that preferentially associated and degraded misfolded lysozyme versus wildtype enzyme reinforced the belief of an ER resident protease (Otsu *et al.*, 1995). However, there was a high probability that the ER membranes used in the semi-permeabilized system were contaminated with cytosolic factors (McGee *et al.*, 1996), and it was later discovered that ER-60 is a molecular chaperone required for ERAD (Oliver *et al.*, 1997). In contrast, studies by several groups demonstrated that drugs that inhibited the proteasome, or *in vivo* studies using proteasome mutants led to the stabilization of ERAD substrates (Jensen *et al.*, 1995; Ward *et al.*, 1995; Hiller *et al.*, 1996; Oda *et al.*, 1996). It is now known that cytosolic and ER luminal proteins are degraded by the cytosolic 26S proteasome.

Figure 9: Ubiquitin-Proteasome Pathway.

Ubiquitin is linked to and activated by the E1 enzyme in a step requiring ATP. The activated ubiquitin is transefered to an E2 enzyme and the E2 enzyme associates with an E3 enzyme. The E2/E3 pair function to poly-ubiquitinate the target protein. The ubiquitinated protein is targeted to the proteasome, de-ubiquitinated and degraded in a process requiring ATP.

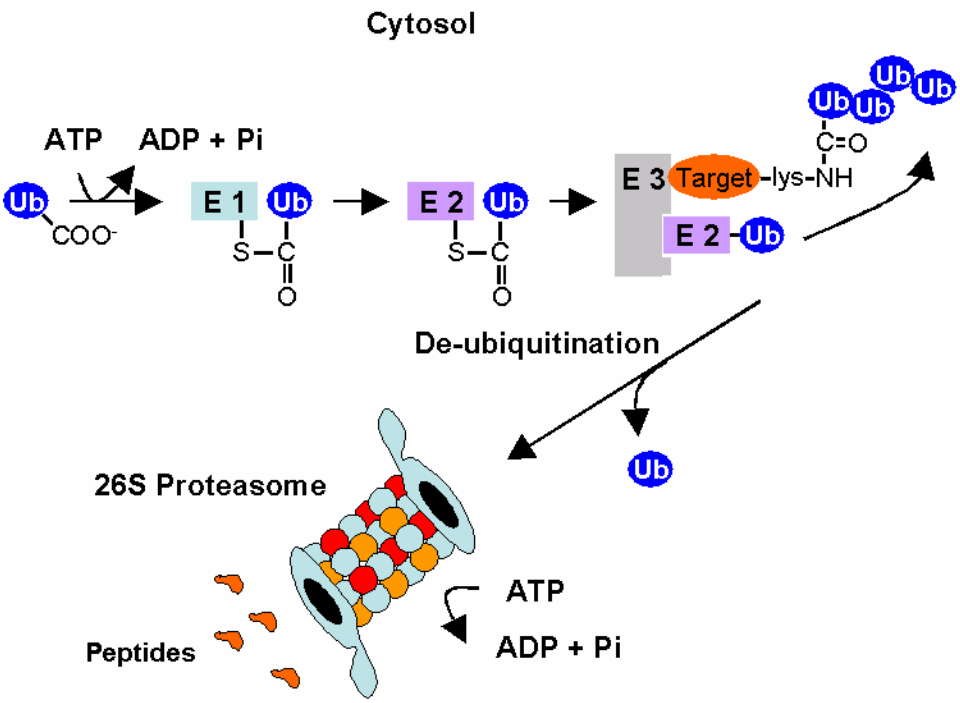


Figure 9: Ubiquitin-Proteasome Pathway

The process of ERAD can be broken down into several discrete steps: 1) identification of misfolded/unassembled proteins by molecular chaperones; 2) retro-translocation/dislocation of the protein from the ER membrane; 3) deglucosylation and poly-ubiquitination of the protein (if necessary); 4) degradation by the proteasome. The main function of ERAD is to dispose of misfolded or aberrant proteins in the ER, but some wildtype enzymes in the cell are degraded by ERAD as a mechanism to control their activity (Hampton and Rine, 1994; Hampton *et al.*, 1996).

Molecular chaperones function at all stages of ERAD. Many molecular chaperones bind to hydrophobic-rich patches in unfolded proteins to prevent aggregation and catalyze the folding of the protein substrate. Molecular chaperones were originally identified as proteins that were over-expressed when cells were subjected to heat stress, hence molecular chaperones were originally called heat shock proteins (Ellgaard *et al.*, 1976), (McKenzie *et al.*, 1975). Today it is known that molecular chaperone expression is induced by a wide variety of cellular stresses and not just heat stress. There are multiple classes of molecular chaperones and they are named based on their molecular weights (e.g. Hsp70, Heat Shock Protein of MW ~70,000). Molecular chaperones are highly conserved from single cell bacteria to metazoan eukaryotes and can be found in almost every compartment of the cell.

The three main classes of molecular chaperones that are involved in folding or degrading soluble/membrane proteins in the ER are Hsp40, Hsp70, and Hsp90 (See Table 1). Co-factors called co-chaperones can shift the chaperone activity from folding to degradation if the substrate protein cannot be correctly folded (see section 1.3 for more details). Over the past ten years,

genetic and biochemical studies in yeast and mammalian cells have begun to elucidate the roles of chaperones and accessory factors in the ERAD of aberrant proteins.

1.2.3.1. Chaperone Requirements for ERAD of a Soluble Protein

Studies conducted in yeast have demonstrated that calnexin (McCracken and Brodsky, 1996), BiP (Plemper *et al.*, 1997; Brodsky *et al.*, 1999) and peptidyl-proyl isomerase (PDI) (Gillece *et al.*, 1999; Norgaard *et al.*, 2001) are required for the efficient degradation of some soluble ERAD substrates. Interestingly, the substrate used in the PDI studies lacked cysteines, suggesting that PDI possesses a chaperone activity separate from its function in catalyzing disulfide bond formation. Furthermore, a mutant form of pro-α-factor that is not glycosylated requires calnexin for degradation suggesting that calnexin has a lectin independent chaperone function. Export of the misfolded soluble protein from the ER requires the Sec61p translocon (Pilon *et al.*, 1997) (Plemper *et al.*, 1997; Zhou and Schekman, 1999) even though the retro-translocation or dislocation of a misfolded soluble protein is mechanistically different from translocation (Zhou and Schekman, 1999) (Brodsky *et al.*, 1999). In both cases the substrate protein must be kept in a soluble state to allow transport, but the co-chaperone requirements for BiP, an Hsp70, are different depending upon its action during translocation, or retro-translocation. For example, the BiP co-chaperone Sec63p (see Figure 2) is required for the translocation of proteins into the ER but is dispensible for ERAD (Pilon *et al.*, 1997; Plemper *et al.*, 1997; Nishikawa *et al.*, 2001). In contrast, the BiP co-chaperones Scj1p and Jem1p are required for the ERAD of pro-α–factor and a mutant form of a vacuolar enzyme (CPY*) but not for translocation (Nishikawa *et al.*, 2001). It is not entirely clear how misfolded soluble proteins are targeted to the translocon for retro-

translocation, since no signal sequence has been identified to direct proteins to the pore as is the case for translocation. Results from Romisch and colleagues suggest that PDI can target at least one soluble substrate to BiP positioned at the translocon (Gillece *et al*., 1999). BiP may then deliver substrates to the translocon, but it is not clear how it performs this function (Schmitz *et al.*, 1995; Brodsky *et al.*, 1999; Skowronek *et al.*, 1998).

Table 1: Molecular Chaperones & Co-factors

Yeast	Mammals	Class	Location	Function
Cne1p	Calnexin	lectin	ER	Glycoprotein Folding
-----	Calreticulin	lectin	ER	Glycoprotein Folding
BiP	BiP	Hsp70	ER	Translocatio/retro-translocation Protein folding
Ssa1p	Hsc/Hsp70	Hsp70	Cytosol	Protein folding/degradation
Ydj1p	Hdj2	Hsp40/ J-protein	ER/Cytosol	Protein folding/degradation
Hlj1p	-----	Hsp40/ J-protein	ER	Protein folding/degradation
-----	CSP1	Hsp40/ J-protein	Cytosol	Protein folding/ Exocytosis
Fes1p	HspBP1	Nucleotide Exchange Factor (NEF)	Cytosol	Stimulate ADP release from Hsp70
-----	Bag-3	Nucleotide Exchange Factor (NEF)	ER/Cytosol	Stimulate ADP Release from Hsp70
-----	CHIP	E3 Ligase	Cytosol	Protein degradation
Hsc/Hsp82	Hsp90	Hsp90	Cytosol	Protein folding/degradation
Sba1p	p23	Hsp90 co-chaperone	Cytosol	Protein folding
Sti1p	HOP	Hsp90 co-chaperone	Cytosol	Protein folding
Sse1p	Hsp110	Hsp90 co-chaperone	Cytosol	Protein folding
Aha1p	Aha1	Hsp90 co-chaperone	Cytosol	Protein folding

Since BiP (luminal Hsp70) plays an important role in the ERAD of misfolded soluble proteins, are cytosolic Hsp70 chaperones are involved in the ERAD of soluble proteins? Most likely not, since the major cyotosolic Hsp70 in yeast, Ssa1p, is dispensible for the degradation of CPY*, pro-α-factor and the Z-variant of the human protein α1-anitrypsin inhibitor (A1PiZ) (Brodsky *et al.*, 1999; Huyer *et al.*, 2004). Though it is not entirely clear why, some misfolded soluble proteins are stabilized in yeast mutants that block ER to Golgi transport, suggesting that trafficking to the Golgi is a pre-requisite for efficient ERAD of soluble proteins (Caldwell *et al.*, 2001; Vashist *et al.*, 2001). In fact, a pool of UGGT enzyme may be located in the Golgi (Roth *et al.*, 1994) (see section 1.2.1). Together, these results suggest that ER luminal chaperones are required, but cytosolic chaperones are dispensable for the degradation of soluble misfolded proteins in the ER and degradation is dependent on a functional Sec61p translocon.

1.2.3.2. Chaperone Requirements for ERAD of a Membrane Protein

The chaperone requirements for the ERAD of a membrane protein are distinct compared to the requirements for soluble proteins. BiP is not required for the efficient degradation of a mutant form of the yeast ABC transporter Ste6p (Ste6p*), a mutant form of the yeast multidrug transporter Pdr5p (Pdr5p*), the human chloride channel CFTR, or a subunit of the vacuolar ATPase Vph1p, even though Ssa1p (the yeast cytosolic Hsp70) is required for the efficient degradation of these transmembrane proteins (Plemper *et al.*, 1998; Hill and Cooper, 2000; Zhang *et al.*, 2001; Huyer *et al.*, 2004). Furthermore, the Sec61p translocon is not required for the ERAD of Ste6p* (Huyer *et al.*, 2004). However, yeast contain two other putative translocons that could be involved in retro-translocation, so it can not be completely ruled out that retro-

translocation is dispensible for Ste6p* degradation (Finke *et al.*, 1996; Walter *et al.*, 2001; Hitt and Wolf, 2004). Some membrane proteins are stabilized in *sec61* mutant yeast, providing support that retro-translocation through Sec61p could be required for the ERAD of certain membrane proteins (Plemper *et al.*, 1998; Zhou and Schekman, 1999; Huyer *et al.*, 2004). Additionally, some membrane-bound ERAD substrates can be co-immunoprecipitated with Sec61p (Bebok *et al.*, 1998) (de Virgilio *et al.*, 1998) . Overall it appears that cytosolic chaperones are required for ERAD of most membrane proteins, while luminal chaperones are required for the ERAD of luminal substrates.

1.2.3.3. Additional Factors Required for ERAD

What is the driving force that dislocates soluble or membrane protein from the ER compartment? There is evidence that some membrane proteins can be extracted from the ER membrane directly by the proteasome (Mayer *et al.*, 1998; Walter *et al.*, 2001). The Sec61p translocon is required for retro-translocation of soluble proteins; therefore it is possible that the extraction machinery associates with Sec61p. In fact, Romisch and colleagues recently published data suggesting that the Sec61p translocon may be a receptor for the proteasome at the ER (Kalies *et al.*, 2005). In addition, the proteasome is sufficient for the dislocation of a non-ubiquitinated soluble protein from microsomes *in vitro* (Lee *et al.*, 2004c). Alternatively, the multiprotein complex Cdc48p-Ufd1p-Npl4p (Bays and Hampton, 2002) has been shown to catalyze the retro-translocation of soluble and membrane proteins from the ER. Mutations in subunits of these complex result in the accumulation of poly-ubiquitinated subtrates at the ER membrane suggesting that this

complex acts after ubquitination to target substrates to the proteasome for degradation (Ye *et al.*, 2001).

Several genetic screens in yeast have been performed that have led to the identification of additional factors that target substrates to the proteasome for degradation. Wolf and colleagues identified three *DER* genes (Degradation in the ER) in a screen looking for mutants that stabilized CPY* (Knop *et al.*, 1996). Der2p encodes an E2 ubiquitin conjugating (Ubc) enzyme called Ubc7p, and Der3p encodes an E3 ubiquitin ligase. Hampton and colleagues (Hampton *et al.*, 1996)used a genetic screen to look for mutants that stabilized the yeast HMG-CoA-Reductase, an enzyme whose activity is regulated by ERAD, and identified the *HRD* genes (HmgCoA Reductase Degradation), three of which are involved in ERAD (Knop *et al.*, 1996; Gardner *et al.*, 2000; Bays *et al.*, 2001). Hrd1p is the same E3 ligase as identified in Wolf's screen (Der3p) and it associates with Hrd3p to form an ER membrane spanning complex that preferentially ubiquitinates misfolded proteins (Bays *et al.*, 2001). Hrd2p is a component of the 19s cap of the 26s proteasome. Finally, a genetic screen performed in yeast uncovered mutants that accumulate A1PiZ and identified seven complementation groups that could represent novel ERAD components (McCracken *et al.*, 1996).

Evidence from early studies suggested that the *HRD/DER* genes and the ER-associated ubiquitin conjugating enzymes Ubc6p/Ubc7p form a central ERAD machine that mediates the degradation of a diverse set of soluble and membrane proteins (Hampton *et al.*, 1996; Bordallo *et al.*, 1998; Plemper *et al.*, 1998; Plemper *et al.*, 1999), but subsequent studies have demonstrated a diverse requirement for the *HRD/DER* genes in the degradation of membrane proteins (Wilhovsky *et al.*,

2000; Huyer *et al.*, 2004). Some membrane proteins require the E3 ligase Hrd1p (Bays *et al.*, 2001) while others require the action of the E3 ligase Doa10p (Swanson *et al.*, 2001). The current thinking is that ubiquitin E3 ligases may interact with different ERAD substrates, consistent with distinctions within the ERAD pathway.

Recent studies using chimeric soluble and membrane proteins have begun to identify several novel ERAD checkpoints. Ng and colleagues created a series of chimeric proteins that had misfolded domains either on the luminal or cytosolic side of the ER in order to determine which ERAD machinery was required for their degradation. Their results suggested that it is not whether the substrate is soluble or membrane-bound that controls the ERAD requirements. Instead, the site of the lesion in the protein determines the requirements and dependence on ER-to-Golgi trafficking. They therefore proposed the existence of the ERAD-L (Luminal) and ERAD-C (cytosolic) pathways (Vashist and Ng, 2004). The proposed ERAD-L pathway detects misfolded luminal domains, while ERAD-C detects misfolded cytosolic domains. Finally, they made a chimeric protein with lesions on both sides of the ER membrane and discovered that the protein behaved similarly to an ERAD-C substrate. This result supports the idea that the ERAD-C checkpoint precedes the ERAD-L checkpoint. Similarly, Wolf and colleagues studied the ERAD requirements for several chimeric proteins that were anchored to the ER membrane and contained misfolded luminal domains. In opposition to Ng and colleagues, however they found that their substrates behaved like ERAD-C substrates and not ERAD-L as would have been predicted (Taxis *et al.*, 2003). Thus, the exact signals that target the specific ERAD machinery to ERAD substrates is still not clear, but what is clear is that substrates cannot be merely defined as soluble luminal or membrane anchored substrates as previously thought.

1.3. Cytoplasmic Molecular Chaperones

Anfinsen discovered over 30 years ago that the amino acid sequence contained all the information necessary to dictate the correct tertiary structure of a protein *in vitro* (Anfinsen, 1973). However, Afinsen's experiments were performed *in vitro* with dilute solutions of a small globular protein (ribonuclease A) and these conditions are completely opposite to the highly crowded environment inside a cell where protein concentrations are at least 300 mg/ml (Zimmerman and Trach, 1991). Most newly synthesized proteins inside a cell would fail to fold efficiently in this crowded environment without the assistance of other cellular machinary. Specifically, molecular chaperones aid in protein folding and prevent protein misfolding inside the cell. Three major classes of molecular chaperones in the cytosol include Hsp70, Hsp40 and Hsp90 (See Table 1). The Hsp70 and Hsp40 classes of molecular chaperones can bind to hydrophobic patches on unfolded or misfolded proteins to prevent aggregation and catalyze refolding. In contrast, the Hsp90 class of molecular chaperones can bind to polypeptides but shows no preference for hydrophobic patches (Joachimiak, 1997). Furthermore, if the native state of the protein cannot be reached, then the molecular chaperones can target the protein for degradation.

1.3.1. Hsp70

The Hsp70 class of molecular chaperones participate in a wide variety of processes that include folding newly synthesized proteins, prevention of protein aggregation, refolding of misfolded

proteins, translocation of proteins across organellar membranes， and dissociation of protein complexes. （See Table 1）

Hsp70 is composed of a ~45 kDa N-terminal ATPase domain, a ~15 kDa peptide binding domain and a ~10 kDa COOH-terminal lid domain. Like other chaperone classes, there is a stress inducible form of Hsp70 and a constitutively expressed form of Hsc70. Their functions are genetically and biochemically interchangeable and the only difference seems to be in their level and mechanism of expression. Hsp70 is expressed at low levels under normal physiological conditions and its expression is induced to high levels during times of stress. Conversely, Hsc70 expression is not stress inducible, but its levels are constant. Hsp70 binds to short hydrophobic stretches of amino acids normally buried in the native conformation of the protein, and assists in protein folding through a cycle of substrate binding and release that is regulated by ATP hydrolysis (Mayer and Bukau, 2005). In the ATP-bound state, Hsp70 binds with low affinity to its susbtrates due to a fast peptide off-rate. In the ADP-bound state, Hsp70 binds with high affinity to its substrates due to a slow peptide off-rate. The ATPase activity of Hsp70 is inherently weak ($3x10^{-4}$ to $1.6x10^{-2}$ s^{-1}) (Zylicz *et al.*, 1983; McCarty *et al.*, 1995; Bukau, 1999), and this rate is too slow to promote substrate binding and drive productive folding. Therefore, Hsp40 co-chaperones are required to stimulate the ATPase of Hsp70 and drive substrate binding (see section 1.3.2) (see Figure 10). In addition, while all Hsp70 family members require ATP hydrolysis for chaperone activity, there are clearly differences in the rates of ATP hydrolysis and ADP dissociation rates. These differences in kinetic rate constants between Hsp70s can be partly explained by the subtle differences in an exposed loop and the

absence or presence of salt bridges in the cleft of their ATPase domains (Mayer and Bukau, 2005).

The x-ray crystal structure for several Hsp70 ATPase domains have been solved, including bovine Hsp70 (Flaherty *et al.*, 1990), and reveals that the ATPase domain is composed of two globular subdomains with a deep cleft that is formed at the interface of the subdomains. The nucleotide is bound at the bottom of the deep cleft, in complex with one Mg^{2+} and two K^{+} ions, by four binding loops (two each for β and γ phosphate) and a hydrophobic pocket for the adenosine ring. A crystal structure of the central peptide binding domain of a bacterial Hsp70 (DnaK) complexed with peptide was also solved (Zhu *et al.*, 1996). This structure revealed that the peptide-binding domain is composed of β-sheets that form a cleft that can accommodate an ~7 residue peptide. The COOH-terminal domain is α-helical and forms a lid over the peptide-binding domain.

Due to the high concentrations of cytoplasmic ATP, ADP release becomes rate-limiting for substrate release for the bacterial Hsp70 (DnaK). The cleft in the ATPase domain of DnaK must be opened in order to release the ADP and allow binding of a new ATP molecule, and for this Hsp70 co-chaperones called nucleotide exchange factors (see section 1.3.3) promote the release of ADP. The first nucleotide exchange factor identified was the bacterial protein GrpE, which enhances the release of ADP from DnaK (bacterial Hsp70). GrpE and DnaJ working together can stimulate the ATPase activity of DnaK by up to 5000-fold (Karzai and McMacken, 1996). In fact, the binding of the nucleotide exchange factor to Hsp70 causes a conformational change that opens the nucleotide cleft and facilitates ADP release (Harrison *et al.*, 1997). The absence

of a GrpE-like homologue and significant spontaneous release of ADP from mammalian Hsp70 led to the assumption that nucleotide exchange factors were not present in the eukaryotic cytosol. However, several groups identified Bcl-2 athanogene 1 (BAG-1) as a nucleotide exchange factor for mammalian Hsc70 that accelerates the ATPase activity by enhancing ADP release (Hohfeld and Jentsch, 1997; Packham *et al.*, 1997; Takayama *et al.*, 1997; Zeiner *et al.*, 1997; Takayama *et al.*, 1998). Interestingly, a negative regulator of Hsc70 was discovered by Hartl and colleagues called Hsc70 interacting protein (HIP). HIP stabilizes the ADP bound form of Hsc70 and is important for Hsc70 cooperation with other chaperone systems, such as Hsp90 (Ziegelhoffer *et al.*, 1996; Frydman and Hohfeld, 1997). Together, the concerted action of Hsp40, nucleotide exchange factors, and these other co-chaperones modulate the ATPase cycle of Hsp70 and facilitate peptide capture, or release.

Figure 10: ATPase Cycle of Hsp70.

In the ATP bound state Hsp70 binds peptide weakly and the COOH-terminal lid domain is open. Upon stimulation by Hsp40, ATP is hydrolyzed and the COOH-terminal lid closes, locking the peptide onto Hsp70 to favor tight binding. A nucleotide exchange factor (NEF) releases ADP to allow ATP to bind to Hsp70. Upon ATP binding the COOH-terminal lid on Hsp70 opens and the peptide is released.

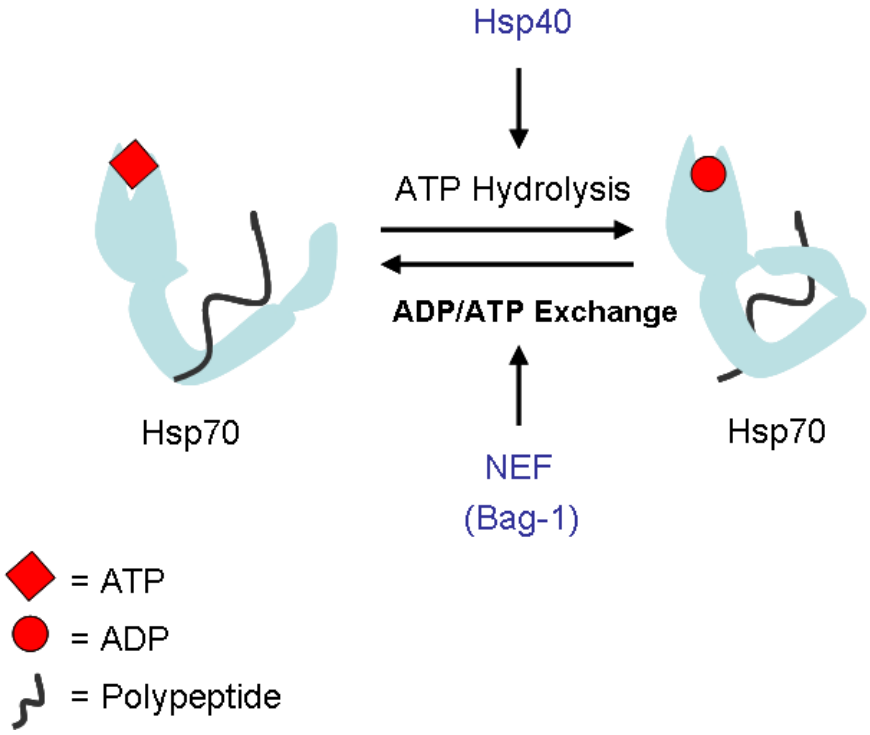


Figure 10: ATPase Cycle of Hsp70

1.3.1.1. Regulators of Hsp70 Function

HSP40 CHAPERONES

The founding member of this class of chaperones is the *E. coli* DnaJ protein, which stimulates DnaK in the *E. coli* cytosol. This canonical Hsp40 contains the ~70 residue NH_2-terminal J-domain, an adjacent glycine/phenylalanine rich domain (G/F), a cysteine rich zinc finger domain and a COOH-terminal domain. It is the J-domain that contacts and stimulates the ATPase domain of Hsp70. The J-domain is composed of four α-helices (I,II,III,IV) that form a finger-like projection (Szyperski *et al.*, 1994; Hill *et al.*, 1995; Qian *et al.*, 1996). Helices II and III are anti-parallel amphipatic helices that are tightly packed (Szyperski *et al.*, 1994; Qian *et al.*, 1996). A loop connecting helices II and III contains the invariant tripeptide HPD which is required for stimulation of Hsp70 ATPase activity (Greene *et al.*, 1998). The G/F domain is a flexible linker that connects the J-domain to the rest of the protein. A second function of some Hsp40 chaperones is to present substrates to Hsp70. The zinc finger domain, which is part of the larger central peptide-binding domain, can bind to polypeptides and is required for some Hsp40 chaperones to present or transfer substrates to Hsp70 (Banecki *et al.*, 1996; Szabo *et al.*, 1996). In fact, the zinc finger domain of DnaJ can prevent the *in vitro* aggregation of denatured rhodanese, but the entire full-length DnaJ protein is required for refolding (Banecki *et al.*, 1996). The function of the COOH-terminal domain is less well characterized and is thought to function in substrate binding (Szabo *et al.*, 1994; Banecki *et al.*, 1996; Fan *et al.*, 2005).

A large number of J-domain containing proteins have been identified in multiple organisms. Cheetham and Caplan developed a nomenclature to organize the many J-domain containing proteins (J-proteins) based on their similarity to DnaJ (see Table 1). Class I proteins contain an NH_2-terminal J-domain, G/F domain and a zinc finger domain. Class II Hsp40, contain an NH_2-terminal J-domain, an enlarged G/F domain, but lack a zinc finger domain. Class III proteins only contain a J-domain and the J-domain can be located anywhere in the protein. It is known that the G/F domain is required for the formation of a Hsp70-peptide-DnaJ ternary complex (Wall *et al.*, 1995) but the J-domain alone can stimulate a preformed Hsp70-peptide complex, suggesting that the G/F domain interacts with DnaK and that peptide binding mimics this interaction. Since class III J-proteins do not contain the G/F domain, they may not be able to present substrates to Hsp70. However, this may not be true for all class III J-proteins because cysteine-string protein (CSP) can stimulate Hsp70 in a substrate independent manner similar to type I J-proteins (Braun *et al.*, 1996; Chamberlain and Burgoyne, 1997). More detailed anaylsis of class III proteins will be required to clearly define their substrate specificity and ability to bind to unfolded polypeptides.

Not all J-proteins stimulate Hsp70s and there appears to be partner specificity that seems to dictate Hsp70-Hsp40 interaction. For example, in yeast the cytosolic Hsp40 Ydj1p stimulates the ATPase activity of the cytosolic yeast Hsp70 (Ssa1p) up to 10-fold but weakly stimulates ($\leqslant$2-fold) the ER luminal Hsp70 (Kar2p) (McClellan *et al.*, 1998). The converse is also true: The ER luminal Hsp40 (Sec63p) weakly stimulates (1.6-fold) Ssa1p (McClellan *et al.*, 1998). There is also some selectivity between Hsp70 and potential Hsp40 partners in the cytosol. For example, the yeast Hsp70 chaperone Ssz1p interacts exclusively with Zuo1p (Hsp40) on

translating ribosomes (Walsh *et al.*, 2004b). In contrast, the yeast Hsp70 chaperone Ssa1p interacts with Sis1p on translating ribosomes, Swa2p to assist in clathrin coat disassembly, and Ydj1p/Hlj1p to facilitate the ERAD of membrane proteins (Horton *et al.*, 2001; Lemmon, 2001; Youker *et al.*, 2004). It is not completely understood how the specifity between Hsp70/Hsp40 pairs are mediated within compartments (e.g., the cytosol and ER), especially since Ssa1p can interact with all three classes of J-proteins. The specificty of interaction between different Hsp70-Hsp40 proteins has been proposed to be partly conferred by residues on the surface of the J-domain (Schlenstedt *et al.*, 1995; Pellecchia *et al.*, 1996). In fact, Genevaux and colleagues identified a set of new mutations in DnaJ that abolish J-domain function and all the mutations mapped to a small solvent exposed region in helix II and III (Genevaux *et al.*, 2002). Furthermore, mutations engineered into the J-domain of Hdj1 in an Hdj1/DnaJ chimeric protein also abolished function suggesting an evolutionarily conserved surface that interacts with Hsp70 proteins (Genevaux *et al.*, 2002).

Hsp40 stimulation is abolished if the conserved last four COOH-terminal amino acids (EEVD) of Hsp70 are deleted (Freeman *et al.*, 1995). This result indicates that Hsp40 interacts with at least two domains in Hsp70 in order to stimulate its activity. The protein surfaces that dictate specificity between Hsp40 and Hsp70 remain to be fully elucidated, but whichever surfaces mediate Hsp70/Hsp40 specificity it is clearly a complex multivalent interaction.

BAG PROTEINS AND NUCLEOTIDE EXCHANGE FACTORS

The BAG (Bcl-2 –associated athanogene) family of proteins are conserved from yeast to humans and affect diverse cellular processes that include cell differentiation, migration, division and even apoptosis. The founding family member BAG-1, also known as RAP46/HAP46, was identified in a screen for Bcl-2 binding proteins (Takayama *et al.*, 1995). The human BAG-1 gene generates four isoforms by alternative translation initiation (BAG-1S, BAG-1, BAG-1M, BAG-1L) (Packham *et al.*, 1997; Takayama *et al.*, 1998; Yang *et al.*, 1998),(Zeiner and Gehring, 1995). The BAG-1 proteins differ in the length of their NH_2-terminal regions and all contain a ubiquitin-like domain (UBL). Additional human BAG family members (BAG-2, BAG-3, BAG-4, BAG-5) were disocvered by Reed and colleagues (Takayama *et al.*, 1999), and there are now a total of seven Bag proteins in humans (not counting isoforms), one in *S. cerevisiae*, two in *S. pombe*, two in *C. elegans*, and one each in *Drosophila*, *Xenopus*, and *A. thaliana* (Takayama and Reed, 2001). All family members possess a BAG domain at the COOH-terminus that binds to the ATPase domain of Hsc70 and stimulates nucleotide release. Family members possess a wide variety of domains, in addition to the BAG domain, that include UBL (ubiquitin-like) domains (BAG-1, BAG-6), a nuclear localization domain (BAG-1L), a WW protein interaction domain (BAG-3). These additional domains facilitate interaction with target proteins and/or target BAG proteins to different locations in the cell. For example, BAG-1 can target Hsc70 chaperone to the proteasome through its UBL domain (Luders *et al.*, 2000). It is thought that BAG-1, through its nucleotide exchange activity, then facilitates substrate unloading and transfer from Hsp70 to the proteasome for degradation. Because the binding sites are non-overlapping, Hsp70 is able to interact with both Hsp40 and BAG-1 (Demand *et al.*, 1998). Hence, BAG-1 can turn the Hsp70

chaperone from a protein folding to a protein degrading machine (Luders *et al.*, 2000; Demand *et al.*, 2001; Hohfeld *et al.*, 2001).

Consistent with its binding to Bcl-2, cells overexpressing BAG-1 are more resistant to apoptotic inducing stimuli (Takayama *et al.*, 1995). It has been suggested that BAG-1 in conjunction with Hsp70 can cause conformational changes in Bcl-2 to regulate apoptotic pathways (Takayama *et al.*, 1997). In addition, the serine/threonine kinase Raf-1 competes with Hsp70 for binding to BAG-1. During times of cellular stress Hsp70 levels increase and there is a shift from BAG-1/Raf-1 to BAG-1/Hsp70 complex formation. This reduction in BAG-1/Raf-1 complex levels leads to depressed Raf-1 signalling and inhibition of cell growth. Morimoto and colleagues hypothesize that this mode of competition represents a molecular switch to control cell growth (Song *et al.*, 2001).

The mechanism by which BAG-1 stimulates ADP release from Hsp70 is quite different from GrpE stimulation of DnaK. In the presence of inorganic phosphate BAG-1 can stimulate release of ADP from Hsc/Hsp70 by up to 100-fold, and in the absence of inorganic phosphate by 600-fold (Gassler *et al.*, 2001). In contrast, GrpE activity is not inhibited by inorganic phosphate. Unlike GrpE, BAG-1 cannot stimulate the release of ATP from Hsc/Hsp70 and the mechanism of BAG-1 binding to Hsc/Hsp70 is different compared to the nucleotide exchange factor HspBP1 (see below) (Shomura *et al.*, 2005). These observations suggest that BAG-1 and GrpE stimulate release of nucleotides by different mechanisms.

Recently, additional nucleotide exchange factors have been identified in mammalian cells and yeast. The yeast proteins Sls1p and Lhs1p are nucleotide exchange factors for the ER luminal Hsp70, BiP. Deletion of both exchange factors is lethal in yeast, highlighting the importance of nucleotide exchange for the BiP ATPase cycle (Kabani *et al.*, 2000; Tyson and Stirling, 2000). Hendershot and colleagues idenitfied BiP-associated-protein (BAP) in a yeast two hybrid screen and demonstrated that it too is a nucleotide exchange factor for mammalian BiP and exhibits some homology to Sls1p (Chung *et al.*, 2002). The yeast cytosolic exchange factor Fes1p and its mammalian homologue Hsp70-binding-protein 1 (HspBP1) are also homologous to Sls1p/BAP and can stimulate the nucleotide release from cytosolic Hsp70 but not luminal Hsp70 (Kabani *et al.*, 2002a). Interestingly, both HspBP1 and Fes1p inhibit Hsp40 dependent stimulation of Hsp70, suggesting they may be negative regulators of Hsp70s (Kabani *et al.*, 2002b). In fact, BAG proteins can act as both positive and negative regulators of Hsp70 dependent refolding *in vitro*, depending on protein and phosphate concentration and the Hsp40 co-chaperone used in the assay (Zeiner *et al.*, 1997; Takayama *et al.*, 1999; Thress *et al.*, 2001; Gassler *et al.*, 2001). The importance of the nucelotide exchange activity of BAG proteins *in vivo* is still controversal.

CHIP

CHIP (C-terminal Hsc70 interacting Protein) was identified in a yeast two-hybrid screen for human TPR containing proteins (Ballinger *et al.*, 1999). Tetratricopeptide repeat (TPR) domain is a protein-protein interaction motif and is composed of incompletely-conserved 34 amino acid repeats. CHIP contains three TPR domains at its NH_2-terminus and also contains a COOH-terminus U-box domain, which binds ubiquitin (Hatakeyama *et al.*, 2001). Many co-factors bind to Hsp70 and/or Hsp90 through the use of TPR domains.

The combination of TPR and U-box domains in one protein strongly suggest that CHIP couples chaperone complexes to the ubiquitin-proteasome machinary. CHIP can bind to Hsp70 through its TPR domain and bind to E2 enzymes of the Ubc4/5 family, thus targeting Hsp70 substrates for degradation by the proteasome (Demand *et al.*, 2001; Jiang *et al.*, 2001; Murata *et al.*, 2001; Pringa *et al.*, 2001). CHIP also prevents premature release of Hsp70 substrates before ubiquitination by blocking the ATPase cycle of the chaperone (Ballinger *et al.*, 1999; Connell *et al.*, 2001). In mammalian cells, overexpression of CHIP leads to the enhanced degradation of a growing number of chaperone substrates that include the glucocorticoid receptor, CFTR and p53 (Meacham *et al.*, 2001; Galigniana *et al.*, 2004; Esser *et al.*, 2005). CHIP-induced degradation requires both a functional U-box domain and a TPR domain. In fact, mutation of the U-box domain leds to a dominant-negative effect *in vivo* (Connell *et al.*, 2001). *In vitro* ubiquitination of heat denatured firefly luciferase by CHIP and Ubc5p requires either Hsp70 or Hsp90 (Murata *et al.*, 2001) and the ligase activity exhibited by CHIP *in vitro* is specific because native luciferase is not ubiquitinated. CHIP also has the ability to ubiquitinate some substrates *in vitro* in the absence of Hsp70/Hsp90 (Demand *et al.*, 2001) and perhaps by direct interaction with the substrate (He *et al.*, 2004). Since both CHIP and BAG-1 are involved in targeting chaperone-substrate complexes to the proteasome, it is plausible to hypothesize that both proteins may interact with each other. In, support of this hypothesis, heteromeric complexes of BAG-1/Hsp70/CHIP have been isolated from mammalian cells (Demand *et al.*, 2001). Furthermore, BAG-1 and CHIP can interact *in vitro* (Demand *et al.*, 2001). This result is not surprising considering that BAG-1 binds to the NH_2-terminus and CHIP binds to the COOH-terminus of Hsp70. The CHIP-induced degradation of the glucocorticoid receptor can be stimulated by

BAG-1, providing further evidence for the cooperation of these two proteins in the sorting of chaperone substrates to the proteasome (Demand *et al.*, 2001). However, BAG-1 is dispensable for CHIP-induced degradation of some proteins *in vitro* (Xu *et al.*, 2002). It remains to be seen whether BAG-1 is an essential component for the CHIP ubiquitin ligase *in vivo* and it should be noted that yeast lack a CHIP homologue.

Interestingly, HspBP1 binds to the ATPase domain of Hsp70 and enhances the association of CHIP with the chaperone. The E3 ligase activity of CHIP is inhibited when it is bound in a ternary complex with Hsp70 and HspBP1. Consequently, overexpression of HspBP1 prevents the CHIP-induced degradation of CFTR and promotes maturation in mammalian cells (Alberti *et al.*, 2004). HspBP1 also inhibited CHIP-mediated ubiquitination of CFTR *in vtiro*. These results suggest that CHIP may be able to modulate the activity of Hsp70 without targeting the chaperone to the proteasome. Recently, several groups have reported that overexpression of CHIP aids in the maturation or activation of the androgen receptor (Cardozo *et al.*, 2003), endothelial nitric oxide synthase (Jiang *et al.*, 2003) and the heat shock transcription factor (Dai *et al.*, 2003). These results suggest that HspBP1 may regulate a novel function of CHIP that is independent of its role in proteasome degradation.

CHIP not only ubiquitinates substrates bound by chaperones but can directly ubiquitinate chaperones and chaperone co-factors. CHIP ubiquitinates both Hsc/Hsp70 and BAG-1 (Jiang *et al.*, 2001; Alberti *et al.*, 2002). Curiously, ubiquitination of BAG-1 by CHIP leads to association with the proteasome but not its degradation. Furthermore, Hsc/Hsp70 and BAG-1 are not degraded upon ubiquitination by CHIP. One explanation for this apparent paradox is that the

linkages between ubiquitin moeities made by CHIP are at the non-canoncial Lys27 instead of the typical Lys48 that target substrates for degradation. Hohfeld and colleagues proposed that these ubiquitin signals on BAG-1 and Hsc/Hsp70 allow for multiple and possibly stronger association with the proteasome (Esser *et al.*, 2004), perhaps via contact with any one of a number of potential ubiquitin receptors in the 19S cap of the proteasome (Deveraux *et al.*, 1994; Young *et al.*, 1998; Hiyama *et al.*, 1999; Lam *et al.*, 2002).

The emerging picture now is that Hsp70 and other chaperone complexes function as either protein folding or protein degradation machines depending on the co-factors with which they associate. The co-factors or co-chaperones seem to function as either pro-folding or pro-degrading factors. For example, Hsp70 co-factors HiP (Hsc70 Interacting Protein) and Hsp70-Hsp90 organizing protein (HOP) promote protein folding (Pratt and Welsh, 1994; Smith *et al.*, 1995; Pratt and Toft, 2003). In contrast, BAG-1 and CHIP promote degradation, converting Hsp70 from a protein folding to protein degrading machine (Esser *et al.*, 2004). Interestingly, the pro-folding and pro-degrading co-factors appear to compete for the same binding sites on Hsp70. Both CHIP and HOP use TPR domains to bind to the COOH-terminal EEVD motif on HSP70 (Connell *et al.*, 2001), and similar competition occurs at the NH_2-terminus ATPase domain of Hsp70 where BAG-1 and HIP compete for binding (Hohfeld and Jentsch, 1997; Takayama *et al.*, 1999). Thus, the cellular levels of the co-factor may set the balance between protein folding or degradation. The cellular levels of BAG-1 and CHIP are low compared to HIP or HOP, and thus under normal conditions the cell appears to shift the balance in favor of protein folding. Elucidating the mechanisms that regulate the expression levels of these co-factors will provide insight into how the cell decides to fold or degrade a protein.

1.3.2. Hsp90

Hsp70 chaperones are considered to function broadly in protein folding and up to 20% of all bacterial proteins require the Hsp70-Hsp40 chaperone system for efficient maturation (Bukau *et al.*, 2000; Hartl and Hayer-Hartl, 2002). In contrast, the folding activity of the Hsp90 class of molecular chaperones was considered to be limited to a much smaller subset of signaling proteins (e.g. steroid hormone receptors and tyrosine kinases). However, the identification of ansamycin antibiotics as specific inhibitors of Hsp90 has led to the identification of a growing list (> 100) of proteins that require Hsp90 for their maturation (reviewed in Pratt and Toft, 2003). Indeed, Hsp90 is now known to play a role in the maturation of a plethora of proteins, including ion channels such as CFTR (Loo *et al.*, 1998; Ficker *et al.*, 2003).

Hsp90 is a higly conserved molecular chaperone that is found in all organisms from prokaryotes to metazoan eukaryotes. Hsp90 is also an abundant cytosolic protein, making up 1-2% of total protein in non-stressed cells (Buchner, 1996; Caplan, 1999; Young *et al.*, 2001). The Hsp90s of different species are named based on their molecular weights. In humans Hsp90 is called Hsp90α and Hsp90β (major and minor form), in *S. cerevisiae* Hsc82 and Hsp82, in Drosophila Hsp83, in mice Hsp84 and Hsp86, and in *E. coli* HtpG (see Table 1). Humans also have an ER Hsp90 called Grp94 which is important for the loading of peptides onto MHC I proteins (Melnick *et al.*, 1994). Hsp90s from all species possess the same basic structural elements with a highly conserved 25 kDa NH_2-terminal ATPase domain, a 35 kDa middle domain, and a 12 kDa COOH-terminal domain. Hsp90 exists as a homodimer in the cytosol and dimerization is

mediated by the last 190 amino acids of the COOH-terminal domain (Nemoto *et al.*, 1995). Hsp90 has a weak but measureble ATPase activity of 0.02 min^{-1} which is ~15 fold lower than unstimulated BiP and ~250 fold lower than GroEL monomer (Todd *et al.*, 1993; Scheibel *et al.*, 1998). Binding and hydrolysis of ATP by Hsp90 is absolutely required for Hsp90's biological activity (Panaretou *et al.*, 1998; Obermann *et al.*, 1998).

The structure of the ATPase domain of Hsp90 is radically different from that in Hsp70. Hsp90 is a member of the dimeric GHKL family of ATPase proteins that is characterized as having a split ATPase domain in which the NH_2-terminal halves of the homodimer must associate for ATPase hydrolysis to occur (Dutta and Inouye, 2000; Meyer *et al.*, 2003). A crystal structure of the NH_2-terminal domain of Hsp90 with ADP or the anasamycin antibiotic geldanamycin (GA) has been solved. Nucleotide binds to the pocket in an unusual kinked conformation which is mimicked by GA (Roe *et al.*, 1999). Furthermore, the ribose ring of the nucleotide is buried in the binding pocket with the phosphates facing out and the γ-phosphate completely exposed to solvent. A mechanism for the hydrolysis of ATP has been proposed based on the crystal structure of the NH_2-terminal and middle domains of Hsp90 in conjunction with biochemical experiments with Hsp90 mutants (Prodromou *et al.*, 2000; Weikl *et al.*, 2000; Meyer *et al.*, 2003). In the absence of ATP the NH_2-terminal domains of the dimer are relaxed and do not associate. Upon ATP binding a lid in the NH_2-terminal domain closes around the nucleotide, exposing a hydrophobic patch and the two NH_2-terminal domains associate through their newly exposed patches. Hydrophobic patches on the closed lid then associate with the middle domain, which provides catalytic residues to the active site. This closing of the NH_2-terminal and middle domains traps the substrate between the two Hsp90 monomers.

Is the ATPase activity of Hsp90 regulated? Indeed, there are a group of co-chaperones that assist in modulating the ATPase activity of Hsp90 like Hsp70. Hch1p (high copy Hsp90 suppressor 1) and Aha1p were identified as activators of Hsp90 that could stimulate the ATPase activity up to 12-fold (Nathan *et al.*, 1999; Panaretou *et al.*, 2002). In an *aha1* deletion strain v-Src activity is reduced, suggesting that Aha1p is required for the activation of an Hsp90 client protein (Panaretou *et al.*, 2002). Interestingly, Aha1p binds to the middle domain of Hsp90 and can bind to Hsp90-HOP or Hsp90-p23 hetero-complexes (Panaretou *et al.*, 2002; Lotz *et al.*, 2003), suggesting a mode of regulation distinct from other Hsp90 co-chaperones (see Hsp90 cycle below). CHIP was also determined to be able to bind to the MEEVD of Hsp90 and shift Hsp90 from a protein folding to a protein degrading machine (Ballinger *et al.*, 1999).

The first protein identified as a substrate of Hsp90 was the tyrosine kinase $pp60^{v\text{-}src}$ (Brugge *et al.*, 1981; Oppermann *et al.*, 1981), but the mechanism of Hsp90 action has been best studied in the maturation of steroid hormone receptors (SHRs). Early results from sedimentation experiments confirmed that SHRs are part of a large heteromeric complex (Wilson *et al.*, 1977; Pratt and Toft, 1997), and Hsp90 was isolated in complex with SHRs by co-immunoprecipitation (Ziemiecki *et al.*, 1986; Sanchez *et al.*, 1987). In addition, the binding of Hsp90 was required for receptor binding to the hormone (Denis and Gustafsson, 1989b, 1989a) (Ohara-Nemoto *et al.*, 1990). Purified Hsp90 and SHR alone could not form the 9S complex observed by sedimentation analysis, nor could it bind hormone (Scherrer *et al.*, 1990), unless the proteins were incubated with rabbit reticulolysate. These results suggested that additional factors were required for SHR maturation (Denis and Gustafsson, 1989b). The additional proteins were identified as Hsp70,

HOP and p23 (Kost *et al.*, 1989; Smith *et al.*, 1990). Hsp70, HOP, and Hsp90 were sufficient in an *in vitro* assembly reaction to promote steroid binding to the SHR, (Dittmar and Pratt, 1997) but p23 was required for efficient binding (Dittmar *et al.*, 1997).

It is now known that five proteins are required to re-capitulate the maturation of a SHR and the order of action has been determined from *in vitro* assembly reactions (Dittmar *et al.*, 1998) (Kosano *et al.*, 1998). The five proteins are Hsp90, Hsp70, Hsp40, p23, and an immunophilin. The proposed Hsp90 cycle for SHR maturation is as follows (see Figure 11)(reviewed in Pratt and Toft, 2003). In Step 1, Hsp70 stimulated by Hsp40 hydrolyzes ATP and binds to the SHR. In Step 2, HOP binds to the Hsp70/Hsp40/SHR complex and delivers it to Hsp90. The first TPR domain of HOP binds to the last eight amino acids of Hsp70 (GPTIEEVD) and the middle TPR domain binds to the MEEVD of Hsp90, thus linking the two chaperone machines (Smith *et al.*, 1993; Johnson *et al.*, 1998). The specificity of binding is maintained by hydrophobic interactions with the divergent NH_2-terminal of the EEVD sequences (Scheufler *et al.*, 2000). In Step 3, HOP binds to the ATPase domain of Hsp90 and inhibits its ATPase activity, thus allowing substrate transfer from Hsp70 to Hsp90. In Step 4, p23 and an immunophilin bind to the COOH-terminus of Hsp90, causing dissociation of Hsp70/Hsp40/HOP and binding of ATP to Hsp90. The binding of ATP causes a conformation change in the NH2-terminal and middle domains (as described above) and results in tight binding of the substrate. Finally in Step 5, ATP hydrolysis causes a conformational change in Hsp90 that opens the steroid hormone binding cleft. Upon steroid binding, Hsp90 and complex members shuttle the SHR to the nucleus where the transcriptional activation of hormone-sensitive genes is induced.

Figure 11: Proposed Chaperoning Cycle for Hsp90 Substrates (simplified).

(see text for in depth explanation)

The immature client protein (orange hexagon) is transferred from the Hsp70 machinery to the Hsp90 dimer (blue) by way of the bridging co-chaperone, HOP; 2) the co-chaperones p23 (yellow trapezoid), and ATP bind to stabilize the client-Hsp90 interaction, then an immunophilin (purple polygon) binds; 3) ATP binding causes dimerization of the NH_2-terminal and middle domains of Hsp90; 4) ATP hydrolysis causes conformational changes in the client protein and opening of the Hsp90 NH_2-terminal for release of the mature client (orange circle)

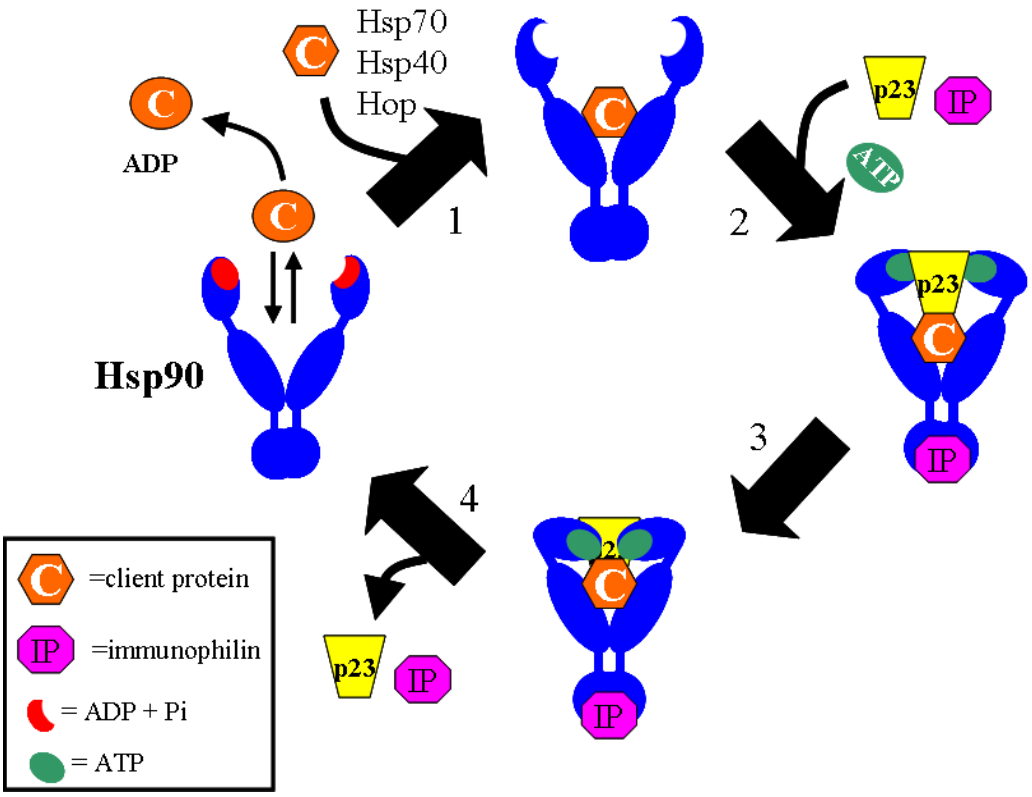


Figure 11: Proposed Chaperoning Cycle for Hsp90 Substrates (simplified)

1.4. Cytsic Fibrosis and the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

1.4.1. Cystic Fibrosis

Cystic Fibrosis (CF) is the most common lethal autosomal recessive disease in Caucasians of Northern European descent. CF is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) (Riordan *et al.*, 1989). Over 1000 disease-causing mutations have been identified in the *CFTR* gene but ~70% contain a deletion of phenylalanine at position 508 (ΔF508), and > 90% of patients have at least one ΔF508 allele (www.genet.sickkids.on.ca/cftr)(Riordan *et al.*, 1989; Davis *et al.*, 1996; Mickle and Cutting, 1998). Mutations that result in CFTR loss-of-function can be divided into four classes: I) defects in protein translation II) defects in protein folding/processing (as exemplified by ΔF508); III) defects in ion conduction and IV) defects in channel gating (Cheng *et al.*, 1990; Welsh and Smith, 1993; Rosenstein and Zeitlin, 1998; Kopito, 1999). The disease affects 1 in 3,500 live births in the USA and approximately 1 in 25 Caucasians of Northern European descent are carriers for the disease. Some, research suggests that carriers have a higher protection from cholera then non-carriers (Gabriel *et al.*, 1994; Wiuf, 2001) due to the presence of half as many CFTR channels in the intestine, which results in less water loss (see below), providing one explanation for the prevelance of carriers.

CFTR is a cAMP-dependent chloride channel (Anderson *et al.*, 1991; Drumm *et al.*, 1991) (Tabcharani *et al.*, 1991; Bear *et al.*, 1992) that is expressed in the epithelial cells of many tissues, including the lungs, pancreas, intestines and kidneys (Kartner *et al.*, 1992; Sarkadi *et al.*, 1992; Zeitlin *et al.*, 1992; Kalin *et al.*, 1999; Farinha *et al.*, 2004). CFTR function is required for proper ion and water homeostasis across epithelial tissues (reviewed in Field and Semrad, 1993). CF affects a multitude of organ systems including the pancreas, sweat glands, respiratory airways, reproductive tract and salivary glands. Due to the numerous exocrine systems affected, CF has been classified as a polyexocrinopathy disease (Davis and di Sant'Agnese, 1980). Many CF patients experience pancreatic insufficiency and chronic obstruction of their airways due to thickened mucous. Most morbidity and mortality in CF is the result of a progressive decline in pulmonary function due to a cruel cycle of airway obstruction, infection and inflammation.

1.4.2. CFTR Protein Structure

CFTR is a 1,480 residue polytopic membrane protein of the ATP-binding cassette (ABC) transporter superfamily. CFTR is composed of two nucleotide binding domains (NBD1, NBD2), two 6 helix membrane spanning domains (TMD1, TMD2), and a regulatory domain (R-domain). These domains are structurally arranged as NH_2-TMD1-NBD1-R-domain-TMD2-NBD2-COOH (see Figure 12). There are two N-linked glycosylation sites on the loop connecting transmembrane spanning 7 and 8. It is important to note that 77% of the protein is cytoplasmically disposed, 19% is composed of transmembrane domains and 4% is composed of extracellular loops, especially when folding of CFTR is considered (see section 1.4.3). Residues

in transmembrane 1, 3, and 6 that line the aqueous pore (Cheung and Akabas, 1996, 1997) (Akabas, 2000) were identified by Akabas and colleagues who introduced cysteines into transmembrane segments of wildtype CFTR and performed cross-linking studies with methanethiosulfonate. Other studies indicate residues in transmembrane 6 are particularly important for the conductance and anion selectivity of the pore (Tabcharani *et al.*, 1993; Cheung and Akabas, 1996; Smith *et al.*, 2001). Studies by Riordan and colleagues using photo-affinity labeling with 8-azidio-ATP indicated that NBD1 is a site for stable binding of ATP, while ATP is hydrolzyed rapidly by NBD2 (Aleksandrov *et al.*, 2002).

Figure 12: Domain Structure of CFTR.

MSD1 = Membrane Spanning Domain 1 (composed of six helices)

NBD1 = Nucleotide Binding Domain 1

R = Regulatory Domain

MSD2 = Membrane Spanning Domain 2 (composed of six helices)

NBD2 = Nucleotide Binding Domain 2

Blue callouts on loop between helix seven and eight represent sites of glycosylation

Pink arrow points to NBD1 domain where the phenylalanine at position 508 (F508) resides

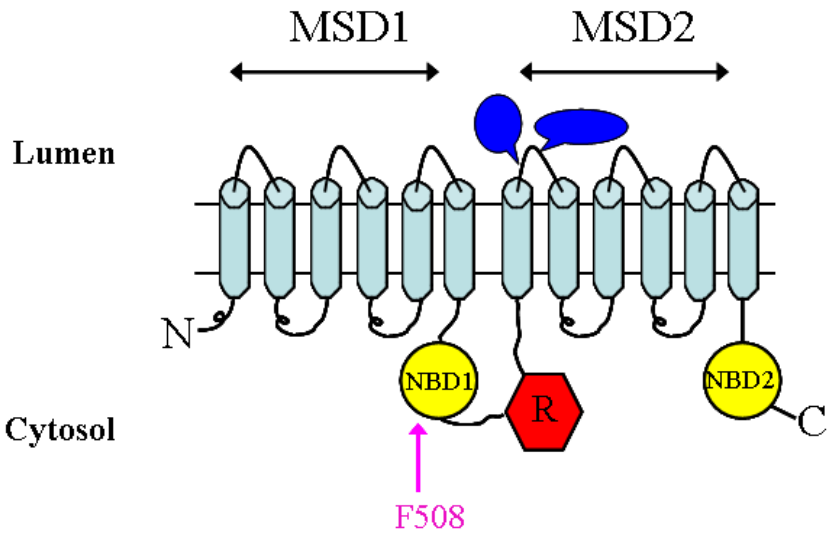


Figure 12: Domain Structure of CFTR

It is generally believed that ATP hydrolysis is coupled to the transport of solutes for many ABC transporters. However, opening of the CFTR channel can occur upon binding of non-hydrolyzable ATP analogues or ATP without divalent cations being present (Aleksandrov *et al.*, 2000). These results suggest that ATP hydrolysis is not required for channel opening. In opposition to these results, work by several groups suggest that opening and closing of the pore is linked to ATP hydrolysis (Carson *et al.*, 1995; Gunderson and Kopito, 1995). Crystal structures of many NBDs indicate that ABC transporters have a core subdomain or "head" that binds ATP and a α-helical "tail" that contains the signature sequence LSGGQ. Furthermore, structures of dimeric NBDs from several transporters reveal a homodimer head-to-tail arrangement containing two ATP molecules (Hopfner *et al.*, 2000),(Smith *et al.*, 2002) (Locher *et al.*, 2002; Chen *et al.*, 2003). The composite ATP binding sites are composed of the head of one monomer and the tail of the other. Experiments using prokaryotic NBD domains demonstrate that ATP binding causes dimerization, while hydrolysis causes dissociation (Hopfner *et al.*, 2000; Moody *et al.*, 2002; Smith *et al.*, 2002). Based on these biochemical and structural observations, CFTR gating (opening and closing) can be thought of in terms of cycles of dimerization. Recent work by Gadsby and colleagues provided direct measurements of the ATP-driven tight dimerization and opening of the CFTR channel (Vergani *et al.*, 2005). Their results strongly suggest that ATP hydrolysis at the NBD2-based site (Walker A of NBD2, signature sequence of NBD1) and channel opening are tightly coupled. Before ATP hydrolysis can open the channel, the CFTR R-domain must be phosphorylated by PKA and PKC (Riordan *et al.*, 1989; Gadsby and Nairn, 1999) (Lohmann *et al.*, 1997). Multiple consensus sites for

phosphorylation are present and the greater the number of phosphorylated sites; the increase in the probability that the channel will remain open (Hwang *et al.*, 1993; Gadsby and Nairn, 1999).

Recently, the crystal structure of residues 389-673 of the murine NBD1 (mNBD1) was solved using a pan-genomic approach (Lewis *et al.*, 2004). The domain organization consists of an F1-like core ATP binding domain that is similar to the structures of several bacterial ABC transporters but contain additional regulatory sections, such as an extension in the β-subdomain` that contains a flexible loop (see Figure 13). Interestingly, there is no structural change upon binding to nucleotide or nucleotide derivatives; most likely the conformational changes are not evident because the changes are "transmitted" to other domains that are only evident in the context of the whole protein. Surprisingly, the F508 residue is on the surface of the domain and is thought to interact with the cytosolic tail of a transmembrane helix. Furthermore, the main chain contacts for F508 are not critical for structural stability and deletion of the residue can be accommodated. From the crystal structure, it has been suggested that F508 does not disrupt packing of NBD1 but is important in domain/domain interactions during the folding of CFTR.

There is still debate over whether the active form of CFTR is a monomer or dimer *in vivo*. Initial co-immunoprecipitation studies suggested that CFTR was a monomer (Marshall *et al.*, 1994). However, measurements of CFTR particles from freeze-facture micrographs suggested that CFTR is a dimer (Eskandari *et al.*, 1998). Ramjeesingh and colleagues purified CFTR from SF9 insect cells and found that two species of CFTR migrated on electrophoretic gels, one corresponding to a monomer and the other to a dimer (Ramjeesingh *et al.*, 2001). Furthermore, both the monomer and dimer possessed ATPase activity and exhibited channel properties

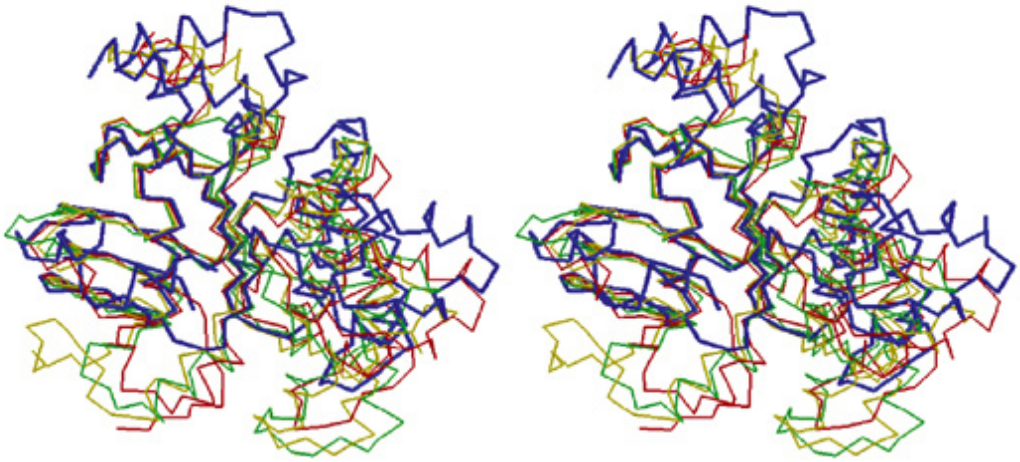
(Ramjeesingh *et al.*, 2001). Naren and colleagues used chemical cross-linking and velocity gradient centrifugation, and observed dimers as the predominant species (Li *et al.*, 2004). Recently, it has been shown that co-expression of N-terminal pieces of CFTR with full length ΔF508 mutant rescues the maturation defect, providing support that CFTR forms dimers *in vivo* (Owsianik *et al.*, 2003; Clarke *et al.*, 2004; Cormet-Boyaka *et al.*, 2004). It is clear that both monomers and dimers can form active channels, and one recent study demonstrated that CFTR can exist as a dimer at the plasma membrane (Ramjeesingh *et al.*, 2003). Whether the active form of CFTR at the plasma membrane is a monomer or dimer is still an unresolved issue.

Figure 13: Structural fold of murine NBD1 (adapted from figure 3 Lewis *et al.*, 2004).

Top: Stereo diagram of Cα backbone of ABC NBD1 structures superimposed onto murine NBD1. TAP1 (red line), HisP (yellow line), MJ0796 (green line), murine NBD1 (blue line). Note the overall structural similarity. The superposition is based on least-squares alignment of F1-type core and antiparallel β-subdomains.

Bottom: Worm diagram of murine NBD1 structure illustrating sites of phosphorylation (purple = Ser422, Ser659, Ser660, Ser670) and CF-causing muations (sidechains in yellow = Ala455, Gly480, Ile506, Ile507, Ser549, Gly551, Ala559, Arg560, Tyr569, Asp648, Phe508 (green), di-acidic code residues Asp565 & Asp567 in gold). Gold = F1-type core domain, Cyan = α-subdomain, green = β-subdomain, grey = unique to murine NBD1 (extra regulatory sections). Regulatory helix shown as red ribbon. The right structure is turned 80° toward the viewer compared to the left structure. ATP is depicted as a ball-stick molecule. Red dotted line not resolved in crystal structure.

Top



Bottom

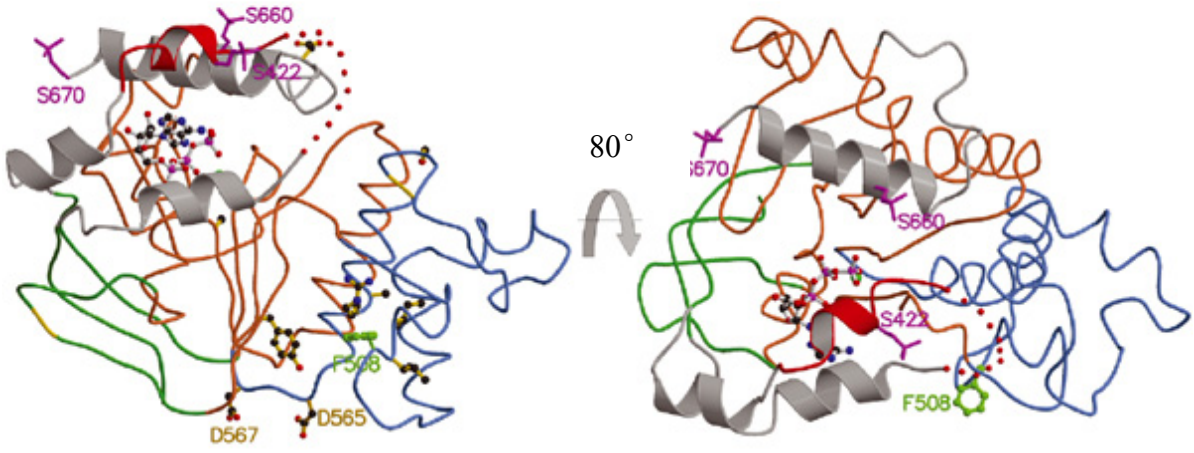


Figure 13: Structural Fold of Murine NBD1

1.4.3. The CFTR Folding Pathway

The first step in CFTR biogenesis is its co-translational insertion into the ER membrane. The first transmembrane helix (TM1) of CFTR contains two charged residues (E92, K95) which are required for chloride channel activity but cause TM1 to have weak signal sequence activity. Due to the weak signal sequence identity, TM1 inefficiently inserts into the ER membrane (Lu *et al.*, 1998). However, redundant signal sequence information in TM2 acts as a backup in case TM1 insertion fails. Thus, there are two alternative co-translational translocation pathways to ensure proper NH_2-terminal assembly in the membrane (Xiong *et al.*, 1997).

After proper assembly of the NH_2-terminal domain, the cytosolic NBD1 domain emerges from the ribosome and the chaperones Hsc70/Hdj2 bind to the domain to stabilize it and help it fold (Meacham *et al.*, 1999). These chaperones dissociate from the NBD1 domain upon synthesis of the R-domain, suggesting that the R-domain stabilizes NBD1 (See Figure 14). Interestingly, about twice the Hsc70/Hdj2 associates with ΔF508 than wildtype CFTR, suggesting that the mutant needs more help to fold. In fact, the mutant does not attain a native conformation and cannot traffic past the ER. Other chaperones that bind CFTR include the luminal chaperone calnexin (Yang *et al.*, 1993; Pind *et al.*, 1994), which likely associates with the two N-linked glycans on a loop between TM7-8; the immature core glycosylated CFTR is known as band B (~130-150 kDa). In contrast, the soluble luminal chaperones BiP and Grp94 do not bind to the newly synthesized CFTR molecule (Yang *et al.*, 1993; Pind *et al.*, 1994). However, the cysteine string protein (CSP), which contains a J-domain, is localized to the ER membrane, interacts with

Hsc70 (Zhang *et al.*, 2002a), and binds to several cytosolic domains of CFTR. Indeed, CSP also plays a role in the maturation of CFTR (Zhang *et al.*, 2002a).

Once CFTR is synthesized and folded correctly, the di-acidic motif ($_{563}$YKDAD$_{567}$) in NBD1 is recognized by the COPII machinery and CFTR is incorporated into COPII vesicles for export to the cis-Golgi (Yoo *et al.*, 2002; Wang *et al.*, 2004). Mutation of the di-acidic motif in wildtype CFTR blocks export of the protein (Wang *et al.*, 2004). In the Golgi, the N-linked glycans are modified, producing mature CFTR (band C, ~175 kDa). Although wildtype CFTR can be detected in post-ER compartments of the secretory pathway (Bradbury, 1999) it is present at ~2 fold lower levels compared to the ER (Bannykh *et al.*, 2000). In contrast to several other soluble/membrane bound proteins (Balch *et al.*, 1994; Aridor *et al.*, 1998; Martinez-Menarguez *et al.*, 1999), CFTR surprisingly does not co-localize with syntaxin-5 or p58, two markers commonly used to assess the selective concentration and export of proteins from the ER (Dascher *et al.*, 1994; Rowe *et al.*, 1998). Furthermore, dominant negative mutants in Arf1 (component of COPI), syntaxin-5 or Rab1a/Rab2 did not affect maturation of CFTR. Thus, CFTR trafficking from the ER to Golgi may occur by a non-conventional route (Yoo *et al.*, 2002) in some cell types.

Figure 14: Proposed domain interactions during CFTR maturation (adapted from Figure 2 Cyr, 2005).

The bracketed CFTR represents ER-localized CFTRDF508 that is conformationally blocked because NBD1 cannot productively interact with membrane spanning domain 1 (MSD1). Global changes in CFTR conformation are depicted by different shapes of the domains.

Yellow upright hexagon = NBD1ΔF508, yellow star = misfolded NBD2, purple circle = misfolded MSD1 and MSD2, yellow sideways hexagon = properly folded NBD1 and NBD2, purple rectangles = properly folded MSD1 and MSD2, blue oval = R-domain, green circles = (ub) ubiquitin, orange circle = ATP

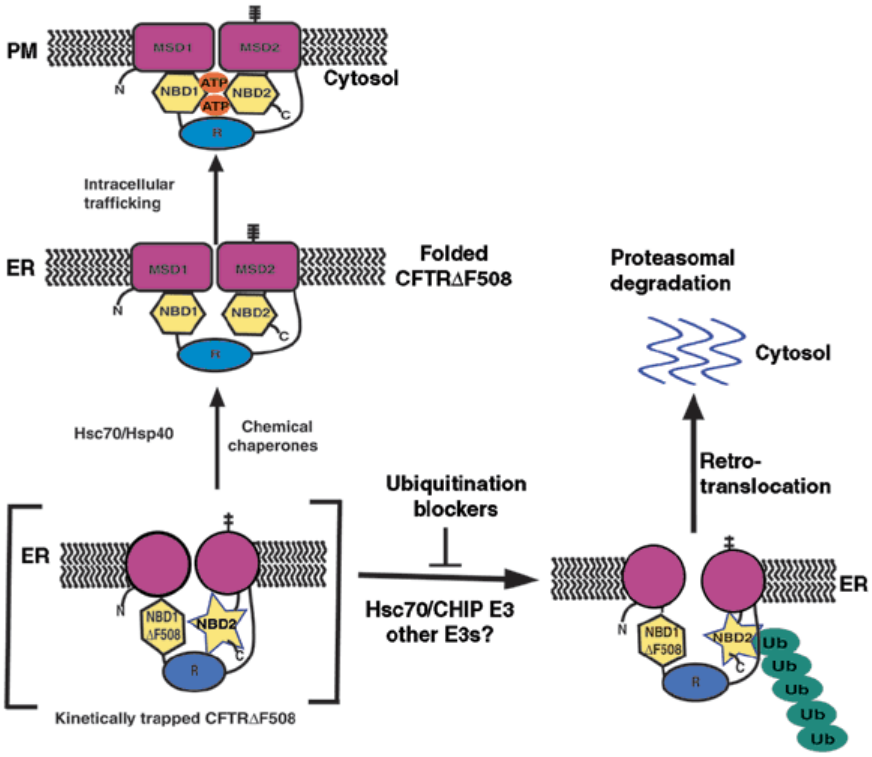


Figure 14: Proposed Domain Interactions during CFTR Maturation

Overexpression of syntaxin-13 blocked the maturation of CFTR, suggesting that recycling through a late Golgi/endosomal compartment may be important for the maturation of CFTR (Yoo *et al.*, 2002). The half-life ($t_{1/2}$) of wildtype CFTR at the cell surface is ~16 hrs and a sub-apical population of CFTR has been detected in cell lines endogenously or exogenously expressing CFTR (Lukacs *et al.*, 1992; Biwersi and Verkman, 1994; Demolombe *et al.*, 1994; Webster *et al.*, 1994). This pool of CFTR colocalizes with Rab4, which is known to reside in recycling endosomes (Webster *et al.*, 1994). Furthermore, the internalization of CFTR from the plasma membrane is constitutive and occurs through clathrin-coated vesicles (Bradbury *et al.*, 1994; Lukacs *et al.*, 1997; Bradbury *et al.*, 1999). CFTR contains multiple endocytic motifs, including a Tyr^{1424}-based motif that links CFTR to AP-2 (Weixel and Bradbury, 2000; Hu *et al.*, 2001) and thus clathrin. The level of CFTR that is present in the apical plasma membrane is not determined strictly by endocytosis but also by the rate of recycling from early endosomes back to the cell surface (Sharma *et al.*, 2004). Recently, Riordan and colleagues identified several distinct endocytic trafficking routes that CFTR can traverse and that are regulated by unique Rab proteins and are dependent on the folding status of the protein (Gentzsch *et al.*, 2004). COOH-terminal truncations of CFTR, which cause CF, do not impair the biogenesis of CFTR but divert the endocytosed mutant protein from early endosomes to the lysosome through enhanced protein mono-ubiquitination (Haardt *et al.*, 1999; Benharouga *et al.*, 2001; Sharma *et al.*, 2004). Thus, misfolded CFTR is recognized by a quality control mechanism both at proximal and distal regions of the secretory pathway.

1.4.4. The CFTR Degradation Pathway

The biogenesis of the ΔF508 mutant of CFTR (CFTRΔF508) is inefficient, with close to 100% of the protein becoming trapped in the ER and targeted for degradation by the ubiquitin-proteasome system. Suprisingly, even the wildtype CFTR protein folds slowly and 60-75% is targeted for degradation (Ward and Kopito, 1994; Jensen *et al.*, 1995). The non-native biogenic intermediates of CFTR and CFTRΔF508 that are targeted for degradation appear to attain similar conformations (Zhang *et al.*, 1998). F508 is located on the surface of NBD1 (Lewis *et al.*, 2004) & see Figure 13) and therefore it has been proposed that the CFTRΔF508 protein is a late-stage off-pathway intermediate (Qu and Thomas, 1996; Lewis *et al.*, 2004). Recent structural studies support this hypothesis, and confirm that the ΔF508 disrupts inter-domain packing between NBD1, MSD1, and NBD2 (Du *et al.*, 2005a; Thibodeau *et al.*, 2005). The NBD1 domain of CFTRΔF508 is prone to aggregation (Qu and Thomas, 1996) and a fraction of the CFTRΔF508 intermediates are present in detergent-insoluble aggregates (Ward *et al.*, 1995).

Because the ER quality control pathway and ERAD are conserved from yeast to humans (Fewell *et al.*, 2001; Ellgaard and Helenius, 2003), studies in yeast have been used to indicate that molecular chaperones and the ubiquitin-proteasome pathway are important for the degradation of CFTR. The yeast cytosolic Hsp70 (Ssa1p) is required for the efficient degradation of CFTR (Zhang *et al.*, 2001). In addition, the Hsp70 co-chaperones Ydj1p and Hlj1p function redundantly during the ERAD of CFTR (Youker *et al.*, 2004) (see section 2). Degradation of CFTR is dependent on the ubiquitin conjugating enzymes Ubc6p and Ubc7p and degradation is

blocked in a proteasome mutant (Zhang *et al.*, 2001). It is not clear if the ubiquitin ligase Der3p/Hrd1p is involved in CFTR degradation because of conflicting results (Kiser *et al.*, 2001; Zhang *et al.*, 2001). However, mammalian homologues of Ubc6p, Ubc7p and Der3p/Hrd1p have been identified and these also appear to play a role in the ERAD of mammalian membrane proteins, such as the alpha subunit of the T-cell receptor and CFTR (Fang *et al.*, 2001; Tiwari and Weissman, 2001; Lenk *et al.*, 2002). Although, there is no stabilization of CFTR in yeast individually mutated for the E3 ligases *hrd1Δ* or *doa10Δ*, there is a slight reduction in degradation in a *hrd1Δ doa10Δ* double mutant, suggesting compensatory roles for these ligases (Gnann *et al.*, 2004). The Cdc48p-Ufd1p-Npl4p complex is also required for the degradation of CFTR in yeast (Gnann *et al.*, 2004) and presumably is involved in extracting the protein from the ER membrane (Ye *et al.*, 2004) or simply in maintaining its solubility (Rape *et al.*, 2001). Wolf and colleagues showed that CFTR degradation is slightly reduced in yeast cells mutated for the luminal lectin Htm1p/Mnl1p (Gnann *et al.*, 2004). Furthermore, the mammalian EDEM protein could rescue the defect in degradation of CFTR in a *Δhtm1* yeast strain suggesting that these two lectins are functional homologues, thus providing a link between glycoprotein quality control and CFTR degradation. But these results are puzzling because there is no effect on CFTR degradation in a *cne1Δ* yeast strain and yeast do not have a calnexin quality control cycle. Fu and Sztul observed stabilization and accumulation of CFTR into distinct subdomains of the ER upon disruption of Sar1p (component of COPII coat) function, suggesting that Sar1p is required for sorting/targeting of CFTR to the proteasome (Fu and Sztul, 2003). However, Balch and colleagues demonstrated that Sar1p plays no role in the targeting of mutant or wildtype CFTR to the proteasome (Wang *et al.*, 2004). The exact role of Sar1p in the degradation of CFTR is still controversial.

Overexpression of the E3 ligase CHIP in mammalian cells accelerates degradation of immature CFTR (Meacham *et al.*, 2001). Recently, Cyr and colleagues were able to reconstitute the *in vitro* ubiquitination of a fragment of CFTRΔF508 (GST-NBD1-R) that was dependent on the activities of Hsc70, Hdj2, UbcH5a and CHIP (Younger *et al.*, 2004). Ubc6p or Ubc7p could not substitute for UbcH5a, suggesting that CHIP recognizes a specific E2. Inactivation of CHIP led to the accumulation of non-aggregated CFTRΔF508 *in vivo* that could be rescued and trafficked to the cell surface (Younger *et al.*, 2004). These results demonstrate that off-pathway non-native CFTRΔF508 is not a dead-end product.

1.4.5. Chemical and Pharmacological Rescue of CFTRΔF508

In heterologous expression systems a small amount of CFTRΔF508 can traffic to the plasma membrane and function as a cAMP-responsive chloride channel but with reduced activity (Dalemans *et al.*, 1991; Drumm *et al.*, 1991). Incubation of mammalian cells and *Xenopus* oocytes at low temperature (20-30^{o}C) increase CFTRΔF508 trafficking to the plasma membrane (Denning *et al.*, 1992) , suggesting that if the folding/degradation pathways are slowed down by reduction in temperature (kinetic effect), then the mutant CFTR can adopt a mature conformation. It has also long been known that low molecular weight polyols, e.g. glycerol, can stabilize protein conformation, enhance the assembly of oligomeric subunits and accelerate *in vitro* protein refolding (Gekko and Timasheff, 1981; Shelanski *et al.*, 1973; Sawano *et al.*, 1992); sugars and amino acids such as betaine and taurine similarly protect proteins from denaturation (Back *et al.*, 1979; Taylor *et al.*, 1995). These small organic compounds are referred to as chemical chaperones. Chemical chaperones are thought to stabilize protein conformations through unfavorable interactions with the peptide backbone, causing preferential hydration of the peptide and shifting of the equilibrium in favor of protein folding (Lee and Timasheff, 1981; Arakawa *et al.*, 1990; Bolen and Baskakov, 2001). Not surprisingly, treatment of mammalian cells expressing CFTRΔF508 with glycerol, TMAO, or deuterated water enhance trafficking and result in a 5-8 fold increase in cAMP-stimulated Cl^- currents (Brown *et al.*, 1996; Adams and Cory, 1998). Guggino and colleagues demonstrated that lower, more physiologically revelant concentrations (10 mM) of chemical chaperones (myoinositol, taurine, betaine) alone or in combination could rescue the folding defect of CFTRΔF508 (Zhang *et al.*, 2003). Treatment of

mice expressing CFTRΔF508 with TMAO partially restored chloride conductance based on rectal potential difference measurements (Fischer *et al.*, 2001). Unfortunately chemical chaperones are not specific and relatively high concentrations are required to elicit an effect which would be difficult to obtain in CF patients. A more effective and safer alternative would be the use of pharmacological chaperones. Pharmacological chaperones are small molecules that bind specifically to the protein of interest in order to stabilize the native conformation and promote release from the ER (Morello *et al.*, 2000; Bernier *et al.*, 2004). To date, pharmacological chaperones for CFTR have not been identified conclusively.

In contrast to chemical chaperones, other small molecules have been reported to rescue the CFTRΔF508 trafficking defect. For example, 4-phenylbutyrate, an ammonia scavenger used to treat patients with urea cycle disorders, enhances the trafficking of CFTRΔF508 by regulating gene expression of molecular chaperones, including Hsp70 (Choo-Kang and Zeitlin, 2001; Rubenstein *et al.*, 1997; Rubenstein and Lyons, 2001; Wright *et al.*, 2004). Recently, the ER calcium pump inhibitors thapsigargin (Egan *et al.*, 2002) and curcumin (Egan *et al.*, 2004) were reported to rescue the trafficking defect of CFTRΔF508 by disrupting chaperone interactions or possibly by acting as pharmacological chaperones, but subsequent studies by several groups have refuted these claims (Loo *et al.*, 2004; Song *et al.*, 2004; Mall and Kunzelmann, 2005). In one case, curcumin did not aid in the maturation of mutant CFTR but instead stimulated channel activity at the plasma membrane (Berger *et al.*, 2005). Rescued CFTRΔF508 displays reduced activity at the plasma membrane and the rescued mutant protein must also be stimulated. To this end, Verkman and colleagues have identified several classes of small molecules that activate or inhibit the channel activity of CFTR using cell-based high throughput screens (Becq *et al.*, 1999;

Ma *et al.*, 2002; Yang *et al.*, 2003). Therefore, it is apparent now that a cocktail of drugs will be required to rescue the trafficking defect and activate CFTRΔF508 if treatment of CF is to be successful (Dormer *et al.*, 2001).

1.5. Yeast as a Model System

It is difficult to rapidly and specifically block molecular chaperone functions in mammalian cell culture systems. Many studies performed in mammalian cells employ the use of small molecule inhibitors or RNA interference (RNAi) to lower the activity of molecular chaperones. Even "specific" molecular chaperone inhibitors such as geldanamycin (GA) can exert pleiotropic effects on cells (Lawson *et al.*, 1998). Recent experiments have revealed that target recognition is more degenerative then previously thought, casting doubt on the specificity of RNAi action (Du *et al.*, 2005b). In contrast, in yeast the function of molecular chaperones can easily be abrogated through simple one-step gene replacement (Longtine *et al.*, 1998) or through the use of readily available temperature sensitive alleles (www.yeastgenome.org). Furthermore, the trafficking and degradation pathways are conserved between yeast and humans. The well established genetic and biochemical tools available in yeast, make it an excellent model system to study the trafficking and degradation of proteins.

1.6. Thesis Overview

Mutated proteins that are unstable or fold slowly can accumulate in the ER, aggregate, and/or induce apoptosis (Thomas *et al.*, 1995; Kaufman, 1999). However, eukaryotic cells have evolved the ability to identify and degrade these aberrant proteins by a pathway termed ER associated protein degradation (ERAD). After their identification ERAD substrates are "retro-translocated" (or "dislocated") to the cytosol, ubiquitinated and targeted to the 26S proteasome for degradation (Ellgaard *et al.*, 1999; Romisch, 1999; Tsai *et al.*, 2002; McCracken and Brodsky, 2003). How ERAD substrates are selected is not completely clear, but a family of proteins, known as molecular chaperones are involved in this process. Previous work indicated unique chaperone requirements for the degradation of soluble and integral membrane proteins in yeast (reviewed in Fewell *et al.*, 2001). In this work, I describe the function of two classes of molecular chaperones (Hsp40, Hsp90) in the ERAD of CFTR in the budding yeast, *S. cerevisiae*. Furthermore, I describe the effects of overexpressing two mammalian co-chaperones (FKBP8, BAG3) on the degradation of CFTR in yeast.

2. Distinct Roles for the Hsp40 and Hsp90 Molecular Chaperones during CFTR Degradation in Yeast

2.1. Introduction

To date, it has been difficult to ascertain whether the interactions between specific chaperones and CFTR result from their attempts to fold the protein and/or to target it for degradation. This fact is highlighted by conflicting studies when the Hsp90 chaperone is disabled and CFTR biogenesis is measured. When CFTR biogenesis was examined in BHK and CHO cells treated with the Hsp90 inhibitor geldanamycin (GA), enhanced CFTR degradation was observed (Loo *et al.*, 1998). However, extended GA treatment may exert pleiotropic effects, including activation of heat shock factor (Zou *et al.*, 1998) and up-regulation of ER chaperones (Lawson *et al.*, 1998). In contrast, CFTR was stabilized when GA was added to a reaction containing dog pancreas microsomes into which CFTR was inserted after *in vitro* transcription/translation (Fuller and Cuthbert, 2000). In neither study was the effect of Hsp90 co-chaperones on CFTR degradation investigated.

To better define the roles of molecular chaperones, especially Hsp90, on CFTR biogenesis, and more generally to elucidate how membrane proteins are targeted for ERAD, I expressed CFTR in the budding yeast *S. cerevisiae*. In this chapter, I show that the two ER-associated Hsp40 chaperones, Ydj1p and Hlj1p, function redundantly to facilitate the degradation of CFTR. Hsp90 aids in the folding of CFTR, in agreement with the data of Loo *et al.*, and the Hsp90 co-chaperones Sti1p, Sba1p, Sse1p do not function in the folding/degradation of CFTR in yeast.

Finally, I show that defects in Hsp90 and the Ydj1p/Hlj1p chaperones have no impact on the degradation of the soluble ERAD substrate, CPY*. These results further delineate the unique chaperone requirements for membrane and soluble proteins.

2.2. Materials and Methods

2.2.1. Yeast Strains and Growth Conditions

S. cerevisiae strains used are: the temperature sensitive *hsp82* strain G313N (*MAT**a**, ade2-1, leu2-3,112, his3-11,15, trp1-1, ura3-1, can1-100, hsc82::LEU2, hsp82::LEU2, pTGPD-Hsp82-G313N*) and isogenic wild type p82a (*MAT**a**, ade2-1, leu2-3,112, his3-11,15, trp1-1, ura3-1, can1-100, hsc82::LEU2, hsp82::LEU2, pTGPD-HSP82*), a kind gift from Dr. Avrom Caplan (Mount Sinai Medical Center) (Nathan and Lindquist, 1995); *sti1Δ* (*MAT**a**, his3, leu2, met15, ura3, sti1::KAN*), *sba1Δ (MAT**a**, his3, leu2, met15, ura3, sba1::KAN)*, and the isogenic wild type (*MAT**a**, his3, leu2, met15, ura3*) (Invitrogen, Carlsbad, CA); ACY17b (*MATα, ade2, his3, leu2, ura3, trp1, can1-100, ydj1-2::HIS3, ydj1-151::LEU2*) (Caplan *et al.*, 1992), E0020 (*MATα, ura3, leu2, his3, trp1, sse1::HIS3*) and the W3031b wild type (*MATα, ade2, his3, leu2, ura3, trp1, can1-100*) (Shirayama *et al.*, 1993); *hlj1Δ* (*MAT**a**, ade2, his3, leu2, ura3, trp1, Δhlj1::HIS3*), *hlj1Δ ydj1-151* (*MATα, ade2, his3, leu2, ura3, trp1, can1-100, ydj1-2::HIS3, ydj1-151::LEU2, hlj1::TRP1*), and the isogenic wild type (*MAT**a**, ade2, his3, leu2, ura3, trp1*), *sti1Δ sse1Δ* (*MAT**a**, GAL2, his2-11, leu2-3, 112, lys1, lys2, trp1Δ1, ura3-5, sti1::HIS3, sse1::KAN)* (Liu *et al.*, 1999)

and the isogenic wildtype STI1 SSE1 *(MAT**a**, GAL2, his2-11, leu2-3, 112, lys1, lys2, trp1Δ1, ura3-52)* (Nicolet and Craig, 1989).

Yeast strains were grown at 26°C unless indicated otherwise and standard methods for growth, preparation of media, and transformation of yeast cultures were used (Adams, 1997). The *hlj1Δ ydj1-151* and the *hlj1Δ* mutant strain were constructed by Peter Walsh in Trevor Lithgow's Laboratory using PCR based gene disruption as described previously (Longtine *et al.*, 1998, Beilharz, 2003 #38).

2.2.2. ERAD Assays

Yeast strains expressing HA-CFTR were grown to logarithmic phase (OD_{600} = 0.4 – 0.8) at 26°C in synthetic complete medium lacking uracil, but supplemented with glucose to a final concentration of 2% (SC–ura) and protein synthesis was stopped by the addition of cycloheximide to a final concentration of 50 μg/ml. Cells were shifted to 37°C and 2.0 - 2.5 ODs of cells were removed at the indicated time-points. The cells were washed and total protein was precipitated (Zhang *et al.*, 2002b). Proteins were resolved on either 10 or 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose, probed with mouse monoclonal anti-HA antibody (12CA5, Roche Molecular Biochemicals, Indianapolis, IN) and polyclonal anti-Sec61p (Stirling *et al.*, 1992) and the signals were quantified using ^{125}I-secondary antibody and phosphorImage analysis (Fuji Medical Systems, Stamford, CT). The degradation of a mis-folded, HA-tagged form of carboxypeptidase Y (HA-CPY*) was measured by ^{35}S-metabolic labeling/pulse-chase analysis as described (Zhang *et al.*, 2001).

2.2.3. Protein Purifications

The following proteins were purified as described: Ssa1p (McClellan and Brodsky, 2000), Ydj1p (Cyr *et al.*, 1992), NBD1 (G404 – L644) of CFTR (Qu and Thomas, 1996) and Sba1p (Fang *et al.*, 1998). Yeast Hsc90 (Hsc82p) was purified using a modified protocol provided by Dr. David Toft (Mayo Clinic, Rochester, MN) from yeast strain ECUpep4 (Jakob *et al.*, 1995) with deletions in the chromosomal *HSC82* and *HSP82* genes, and that encodes *HSC82* on a 2μm plasmid. ECUpep4 cells were grown to logarithmic phase (OD_{600} = 0.8 - 1.0) in YPD (1% yeast extract, 2% peptone and 2% dextrose) at 26°C, the cells were harvested, and the cell pellets were frozen in liquid nitrogen. The cell pellets were thawed, resuspended in 3-5 volumes of buffer 1 (20 mM Tris-HCl pH=7.5, 4 mM EDTA, 1 mM DTT) supplemented with protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin A) and subjected to glass bead lysis by vigorous agitation on a Vortex mixer set on the highest setting six times for 30 s with 30 s incubations on ice between each lysis. Unbroken cells were pelleted by centrifugation at ~2800 x g for 10 min at 4°C and cell membranes were pelleted from the resulting supernatant by centrifugation at ~48,000 x g for 40 min at 4°C. The cleared lysate (~35 ml) was applied to a 30 ml DE52 column equilibrated in buffer 1 (flow rate 0.5-1.0 ml/min) at 4°C. The column was washed with 2 column volumes of buffer 1 and with 2 volumes of buffer 1 containing 50 mM KCl. Bound protein was eluted with a 30 x 30 ml gradient of buffer 1 to buffer 1 containing 1 M KCl, 2 ml fractions were collected and peak Hsc82p-containing fractions, as assessed by SDS-PAGE and Coomassie Brilliant blue staining, were pooled and dialyzed against 4 L of buffer 2 (20 mM Tris-HCl pH=7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol) overnight at

4°C. The dialysate (~10 ml) was loaded onto a ~5 ml high performance Q-Sepharose column equilibrated in buffer 2 and the column was washed with 6 column volumes of buffer 2 and 6 volumes of buffer 2 containing 50 mM KCl (flow rate ~1.0 ml/min) at 4°C. Bound protein was eluted with a 15 x 15 ml gradient of buffer 2 to buffer 2 containing 1 M KCl and 1 ml fractions were collected. Peak Hsc82p-containing fractions were pooled, diluted to ~1.5 mg/ml of protein and dialyzed against 4 L of buffer 3 (20 mM Tris-HCl pH=7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glyercol) for ~ 29 h at 4°C, with fresh buffer used after ~26 h. The final protein concentration was determined using the BioRad protein assay kit (BioRad Laboratories, Hercules, CA) with BSA as the standard, and the final purity of Hsc82p was determined to be ~80%. Protein aliquots were snap frozen in liquid nitrogen and stored at – 80°C.

GST-Hlj1p-6His-tagged (containing J-domain) was expressed in M15 *E. coli* and a 100 ml culture was grown in LB + KAN (25 μg/ml) to an OD_{600} ~2.4. A total of 10 ml of the culture was diluted into 1 l of LB + KAN (25 μg/ml) and grown to OD_{600} ~ 0.2 at 37°C, and then expression was induced by the addition of IPTG to a final concentration of 0.2 mM. Cells were incubated for ~7 h at 37°C until an OD_{600} of ~0.8 was reached, and were harvested and frozen at –80°C. The cells were thawed and resuspended in 10 ml of Buffer 88 (20 mM Hepes pH=6.8, 150 mM KOAc, 5 mM MgOAc, 250 mM sorbitol) containing 0.1% TritonX-100 and 1mM EDTA, and protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin A), and were broken using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA). The lysate was cleared by centrifugation at 10,000 rpm for 10 min at 4°C in an SS34 rotor (Sorvall, Newton, Ct), and the lysate was added to a 1 ml, ~50% slurry of gluthanione-agarose beads (Sigma, St. Louis, MO) and incubated for ~2 h with rotation at 4°C. The gluthanione beads were washed (10 min,

4°C) with 25 ml of Buffer 88 supplemented with 0.1% TritonX-100, 1mM EDTA, and protease inhibitors, followed by a second wash with the same buffer supplemented with 1 M KCl, and finally washed with Buffer 88 plus 0.1% TritonX-100. The bound Hlj1p was eluted by two 1 ml washes of 50 mM Tris, pH 8/5 mM reduced gluthanione. Protein concentration was assessed as described above. Only the GST-Hlj1p-6his fusion protein was evident on a Coomassie Brilliant Blue-stained SDS-PAGE gel.

2.2.4. Biochemical Assays

Sba1p pull-down assays using purified hexahistidine-tagged Sba1 and Ni-NTA resin were performed essentially as described (Fang *et al.*, 1998). Ssa1p-ATP complex formation and single-turnover ATPase assays were performed as published by incubating pre-formed Ssa1p-α^{32}P- ATP complex with the indicated protein (Hlj1p-J domain-GST chimera, Ydj1p, and GST) at a final concentration of 0.2 μM (Sullivan *et al.*, 2000).

For luciferase aggregation assays, firefly luciferase (Sigma, St. Louis, MO) at an initial concentration of ~0.65 μM was pre-incubated in the presence or absence of ~20 μM purified Hsc82p in 150 μl of refolding buffer (10 mM MOPS/KOH pH=7.2, 50 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 2 mM DTT) for 20 min at 25°C before 500 μl of refolding buffer was added at 45°C to yield a final concentration of 0.15 μM for luciferase and 2.4 μM for Hsc82p. Aggregation was measured by light scattering at a wavelength of 320 nm at 45°C in a 14DS UV-VIS-IR spectrophotometer (AVIV, Lakewood, NJ).

The ability of Hsc82p to prevent the aggregation of NBD1 was investigated as described (Strickland *et al.*, 1997). Our NBD1 construct spans amino acids G404 to L644 (from helix H1b to helix H9), and includes the F1-type core ATP-binding subdomain (Lewis *et al.*, 2004). This permits the measurement of early folding intermediates (Strickland *et al.*, 1997). The assay was performed using the purified hexahistidine-tagged NBD1 diluted ~100-fold out of 6 M guanidine-HCl buffer, 20 mM Hepes, pH 7.5, and into 650 μl of refolding buffer (100 mM Tris-HCl pH=7.4, 0.385 M L-Arginine, 10 mM DTT, 200 mM KCl, 20 mM $MgCl_2$) to a final concentration of 2 μM. Protein aggregation was measured over time at a wavelength of 400 nm at 37°C in a 14DS UV-VIS-IR spectrophotometer (AVIV, Lakewood, NJ) in the absence or presence of the indicated concentrations of Hsc82p and other indicated reagents. Results of aggregation experiments were plotted as the relative amount of aggregation, normalized to the NBD1 control at 10 min, versus time, and fitted to a single exponential using KaleidaGraph software version 3.0.4 (Abelbeck software, Reading, PA) to determine initial rates. Macbecin I/II was kindly provided by the Drug Synthesis & Chemistry Branch in the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, at the National Cancer Institute.

2.3. Results

2.3.1. Hsp40 Co-chaperones Function Redundantly during CFTR, but not CPY* Degradation

We previously reported that a cytoplasmic Hsp70 in yeast, Ssa1p, facilitates CFTR degradation (Zhang *et al.*, 2001), and in mammalian cells Hsc70 cooperates with Hdj2, an Hsp40 homologue, during CFTR biogenesis (Meacham *et al.*, 1999). Ydj1p is the yeast Hdj2p homologue, is tethered to the ER membrane via a farnesyl moiety, and interacts with Ssa1p based on genetic and biochemical studies (Caplan *et al.*, 1992; Cyr *et al.*, 1992; Becker *et al.*, 1996). However, CFTR degradation was unaffected in yeast containing a temperature-sensitive allele of *YDJ1* (Zhang et al., 2001), suggesting either that there are inherent differences between CFTR biogenesis in yeast and mammals, or that more than one functionally redundant Hsp40 in yeast cooperates with Ssa1p to facilitate CFTR degradation.

There are >20 J-domain containing proteins in yeast and at least 14 of these reside in the cytoplasm (Costanzo *et al.*, 2001; Walsh *et al.*, 2004a). Recently, an Hsp40 homologue, Hlj1p, was revealed in a search for tail-anchored membrane proteins (Beilharz *et al.*, 2003). The NH_2-terminal domain of Hlj1p is 58% identical to the J-domain of Ydj1p (Figure 15) and the Hlj1p J domain was predicted to reside in the cytosol (Beilharz *et al.,* 2003). A GFP-tagged form of Hlj1p co-localizes with the tail-anchored SNARE Slt1p (Burri and Lithgow, 2004) and Sec12p, an integral ER membrane protein required for COPII-mediated vesicle budding (Barlowe,

2003)(Youker *et al.*, 2004). Hlj1p is localized to both the nuclear and peripheral ER and the punctate peripheral staining pattern is reminiscent of Sec63p, a component of the translocon (Voeltz *et al.*, 2002; Prinz *et al.*, 2000). Additional localization and biochemical studies are needed to determine if indeed Hlj1p interacts with the translocon. Trypsin digestion of isolated ER microsomes showed that Hlj1p is accessible to exogenous trypsin (Youker *et al*., 2004). Furthermore, Hlj1p associated with ER microsomes is resistant to sodium carbonate extraction (Youker *et al.*, 2004)[1]. Together, these results indicate that Hlj1p is tethered to the ER membrane via a COOH-terminal anchor and, like Ydj1p, contains a cytoplasmically-oriented J domain.

To test whether Hlj1p is involved in CFTR degradation, I expressed an HA-epitope-tagged form of CFTR under the control of a constitutive promoter in an *hlj1* deletion mutant and in an isogenic wild type yeast strain and performed cycloheximide chase analyses (see materials and methods in section 2.2). I found that the rates of CFTR degradation were identical (Fig. 16). Consistent with previous results (Zhang *et al.*, 2001), I also found that CFTR degradation was unaffected in the *ydj1-151* strain compared to an isogenic wild type (Fig. 17). However, in a double mutant, *hlj1Δ ydj1-151*, CFTR degradation was slowed relative to the wild type strain (Fig. 18). This effect on CFTR degradation was strongest when using fresh transformants that were less then 3 weeks old and was not seen in transformants streaked from frozen stocks. The degradation of Ste6p*, a mis-folded yeast ABC transporter and ERAD substrate, is also attenuated in a *hlj1Δ ydj1-151* double mutant (Huyer *et al*., 2004). These data suggest that

[1] The Hlj1p trypsin digestion and localization data was obtained by the work of Peter Walsh, a student in our collaborator's lab (Dr. Trevor Lithgow at the University of Melbourne)

Ydj1p and Hlj1p function redundantly to facilitate the degradation of CFTR and at least one other integral membrane ERAD substrate.

Unique chaperone requirements for the ERAD of soluble versus integral membrane proteins have been observed (Fewell *et al*., 2001). To examine this distinction further, we measured the degradation of CPY*, a soluble ERAD substrate (Hiller *et al.*, 1996), in the *hlj1Δ ydj1-151* double mutant and in the isogenic wild type strains by pulse-chase analysis (see materials and methods section 2.2). We found nearly identical rates of degradation in the two strains (Figure 19), indicating that Ydj1p and Hlj1p are dispensable for the degradation of CPY*.

Based on the localization of Hlj1p to the ER membrane, we predicted that the effect of Ydj1p/Hlj1p during CFTR degradation is through their interaction with Ssa1p, the Hsp70 that in turn catalyzes CFTR degradation in yeast (Zhang *et al.*, 2001). The functional interaction between J domain-containing proteins and their cognate Hsp70's is best examined by measurements of Hsp70 ATP hydrolysis in the presence and absence of an Hsp40/J- domain-containing protein. For example, a GST fusion protein containing the J-domain of Sec63p, a membrane protein whose J-domain faces the ER lumen, stimulates BiP's ATPase activity *in vitro* (Corsi and Schekman, 1997). To examine whether Hlj1p interacts functionally with Ssa1p, I purified a GST-tagged fusion protein that contains the Hlj1p J-domain (Figure 20), and incubated the purified protein with pre-formed α^{32}P-ATP-Ssa1p complex. Hlj1p stimulated Ssa1p in this single-turnover ATPase assay to a similar extent as equimolar amounts of Ydj1p (Figure 21), indicating that both Hlj1p and Ydj1p interact with the yeast cytoplasmic Hsp70. Further evidence to support this proposition is shown in Figure 22 in which the abilities of full-length

Hlj1p ("GFP-Hlj1p") and a form of Hlj1p lacking the transmembrane domain ("GFP-Hlj1pΔTMD") to suppress a slow-growth phenotype of *ydj1-151* mutant cells were examined. We observed a suppression of the growth defect of the *ydj1-151* mutant upon Hlj1p over-expression, suggesting that Hlj1p partially supplants Ydj1p function. We also noted in this experiment that the soluble Hlj1p derivative is somewhat more effective at improving the growth of the mutant strain than full-length Hlj1p, suggesting that over-expression of the membrane anchor might be somewhat toxic; consistent with this hypothesis, we have observed toxicity derived from the over-expression of other stable, wild type ER membrane proteins in yeast (our unpublished observations). In any event, protein expression was confirmed by assessing GFP fluorescence of each fusion protein (see Figure 23).

Figure 15: Alignment of Hlj1p and Ydj1p J-domains.

The sequences of the J-domains are depicted and asterisks denote regions corresponding to the four predicted alpha-helices. Amino acids shaded in red are identical between Hlj1p and Ydj1p and the functionally essential HPD motif is bold-faced

J = J-domain, G/F = glycine/phenylalanine-rich region, G = glycine-rich region, TM = transmembrane domain.

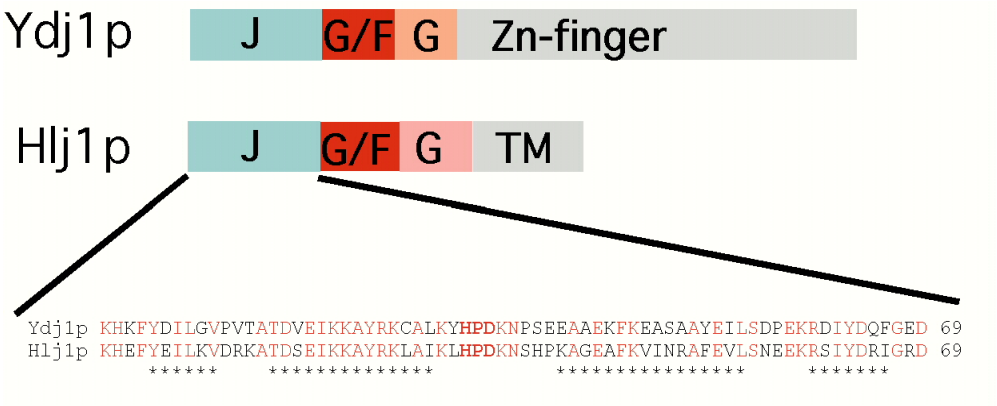


Figure 15: Alignment of the Hlj1p and Ydj1p J-Domains

Figure 16: CFTR Degradation is Similar in Wildtype and an *hlj1Δ* Mutant Yeast Strain.

Wild type and mutant yeast strains expressing CFTR were subjected to cycloheximide chase analysis as described in Materials and Methods (section 2.2). The relative amount of CFTR remaining in wild type (open circles) and *hlj1Δ* (closed circles) yeast strains versus time was plotted and the amount of CFTR at time zero was set to 1.0. The data represent the means of three independent experiments +/-SEM.

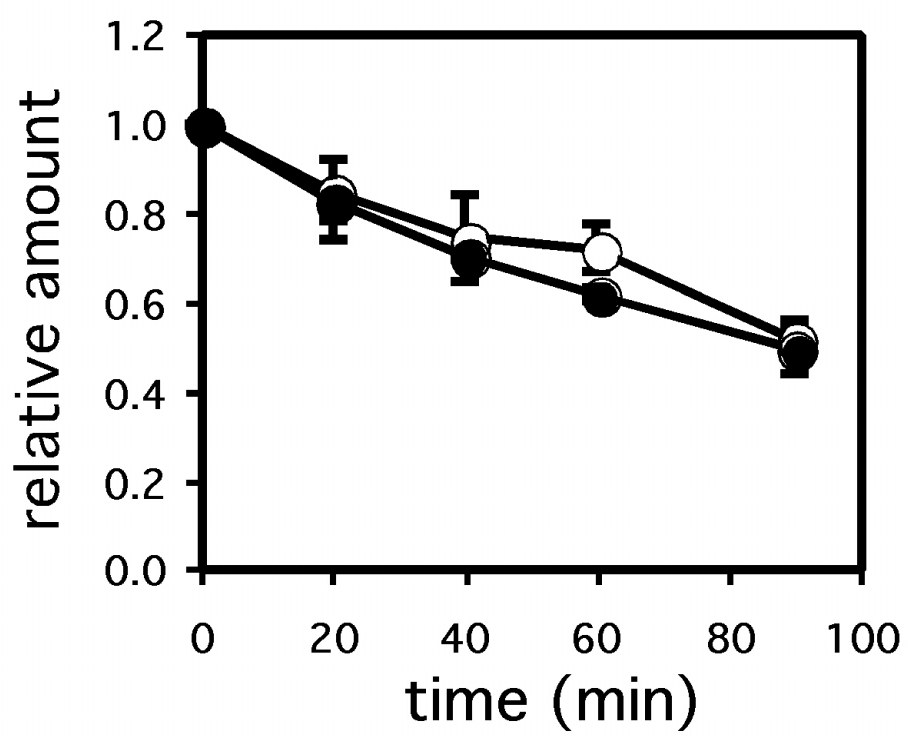


Figure 16: CFTR Degradation is Similar in Wildtype and an *hlj1Δ* Mutant Yeast Strain

Figure 17: CFTR Degradation is Identical in a Wildtype and an *ydj1-151* Mutant Yeast Strain. Wild type and mutant yeast strains expressing CFTR were subjected to a cycloheximide chase analysis as described in Materials and Methods (section 2.2). The relative amount of CFTR remaining in wild type (open circles) and *ydj1-151* (closed circles) yeast strains versus time was plotted and the amount of CFTR at time zero was set to 1.0. The data represent the means of three independent experiments +/-SEM.

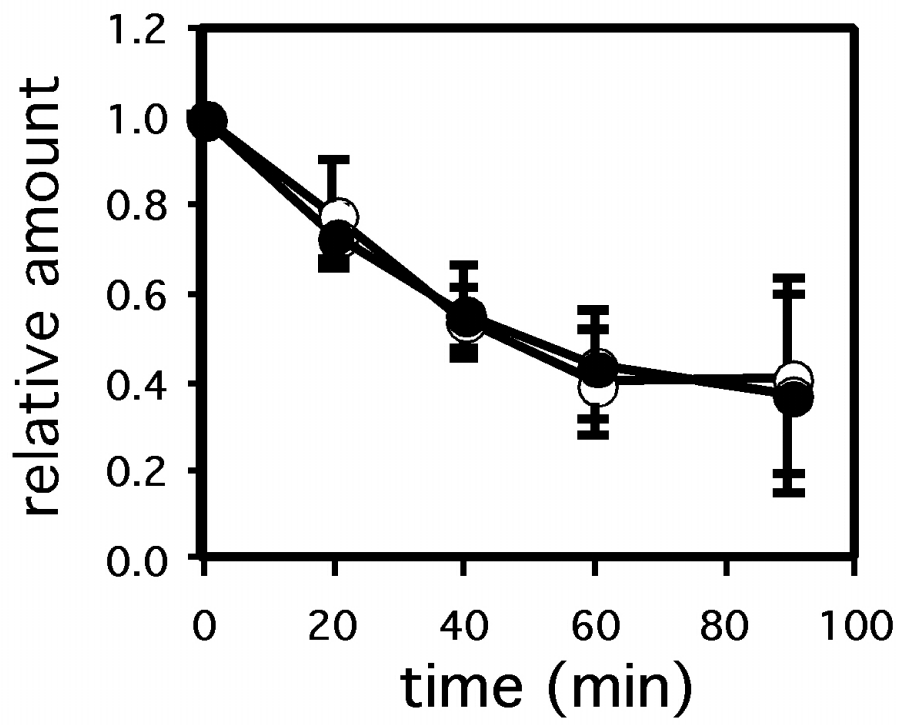


Figure 17: CFTR Degradation is Identical in a Wildtype and an *ydj1-151* Mutant Yeast Strain

Figure 18: CFTR Degradation is Attenuated in an *hlj1Δ ydj1-151* Mutant Yeast Strain.

Wild type and mutant yeast strains expressing CFTR were subjected to cycloheximide chase analysis as described in Materials and Methods (section 2.2). The relative amount of CFTR in wild type (open circles) and *hlj1Δ ydj1-151* (closed circles) yeast strains versus time was plotted and the amount of CFTR at time zero was set to 1.0. The data represent the means of three independent experiments +/-SEM. Two-tailed P-values were < 0.05 except where indicated: * = 0.0655, ** = 0.116.

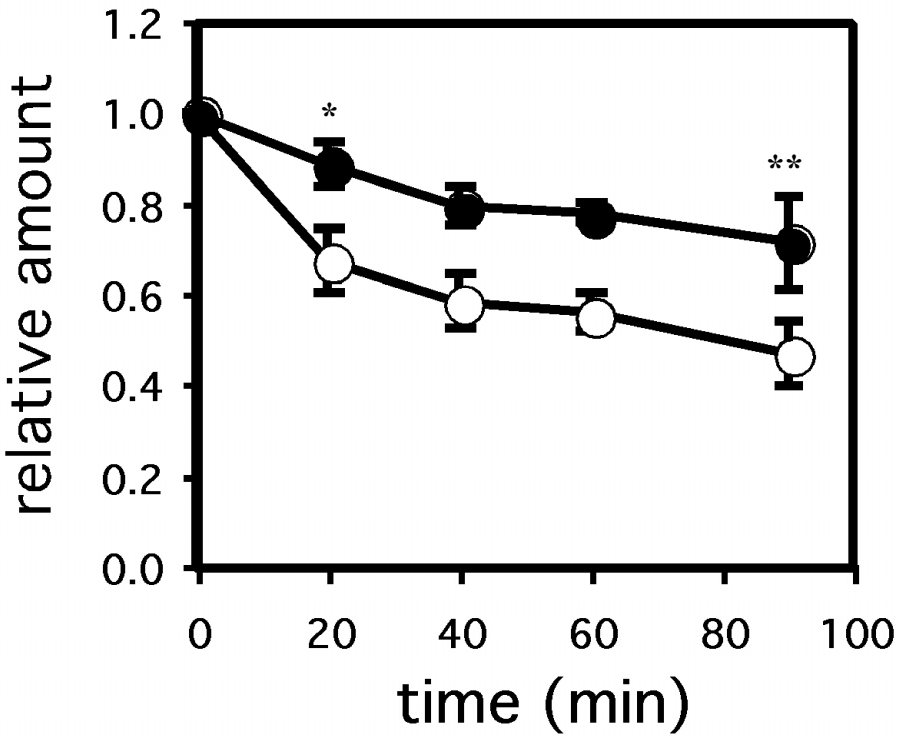


Figure 18: CFTR Degradation is Attenuated in an *hlj1Δydj1-151* Mutant Yeast Strain

Figure 19: CPY* Degradation is Unaffected in an *hlj1Δ ydj1-151* Mutant Yeast Strain.

CPY* degradation was assessed by pulse-chase analysis as described in the materials and methods (section 2.2) in wild type (open circles) and the *hlj1Δ ydj1-151* mutant (closed circles) yeast. Data represent the means of three independent experiments +/- SEM.

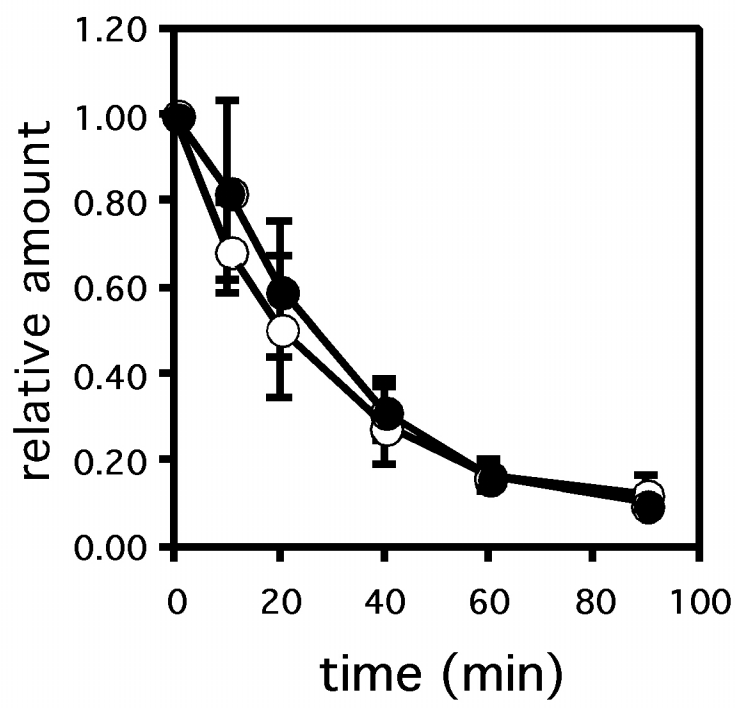


Figure 19: CPY* Degradation is Unaffected in an *hlj1Δydj1-151* Mutant Yeast Strain

Figure 20: Purification of GST-Hlj1-6his.

GST-Hlj1-6his was purified from *E. coli*, (see Materials and Methods, section 2.2). Coomassie Brilliant blue stained gel of fractions collected from gluthanione resin.

M = molecular weight markers

L = lysate

F = flow through

E = Elution

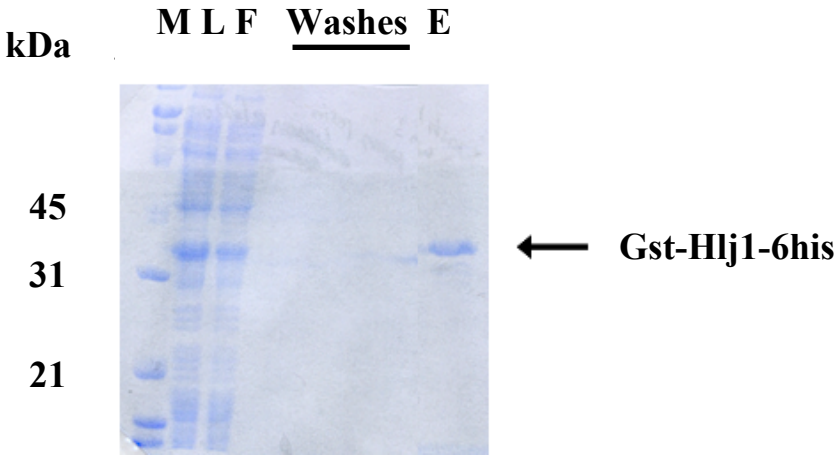


Figure 20: Purification of Gst-Hlj1-6his

Figure 21: Hlj1p Stimulates the ATPase Activity of Ssa1p.

The ATPase activity of Ssa1p is enhanced by purified Ydj1p and by a fusion protein containing the Hlj1p J domain. Incubations were established to pre-form a α^{32}P-ATP-Ssa1p complex (Fewell *et al.*, 2004), which was then incubated at 30°C for the indicated times with equimolar amounts of the Hlj1p J domain fusion protein (closed circles), Ydj1p (open circles), GST (closed triangles), or buffer (open triangles), and the extent of ATP hydrolysis was assessed as described in the Materials and Methods (section 2.2).

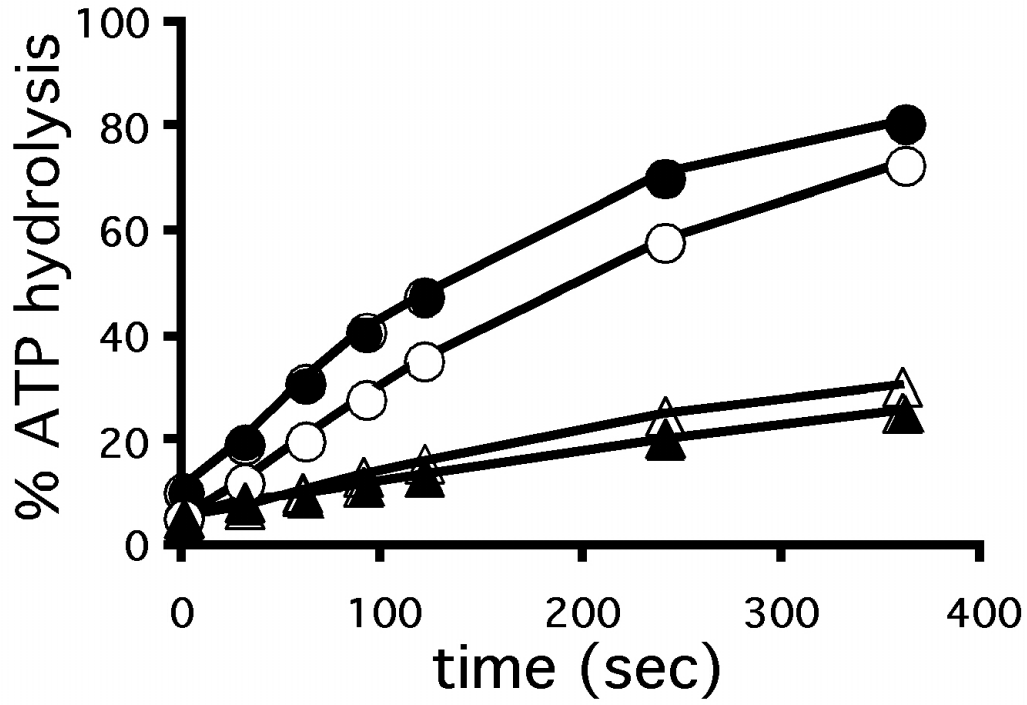


Figure 21: Hlj1p Stimulates the ATPase Activity of Ssa1p

Figure 22: Overexpression of GFP-Hlj1p or GFP-Hlj1pΔTMD Partially Rescues the Growth Defect at 30°C of the ydj1-151 Yeast Strain.

Wild type and ydj1-151 yeast either lacking or containing a MET17-driven GFP-Hlj1p or GFP-Hlj1pΔTMD expression construct were grown overnight and serial dilutions were plated on selective media containing 500 μM methionine at 30°C for 2 days.

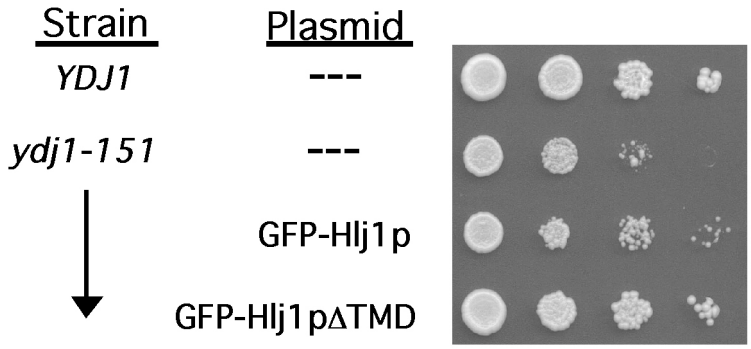


Figure 22: Overexpression of Hlj1p Partially Rescues the Growth Defect of the *ydj1-151* Yeast Strain

Figure 23: Fluorescence Microscopy of Cells Expressing GFP-Hlj1p or GFP-Hlj1p-ΔTMD.

Wildtype yeast cells transformed with **A)** GFP-Hlj1p or **B)** GFP-Hlj1p-ΔTMD expression vectors were grown on selective media containing 500 μM methionine for 3 days at 30°C. Cells were scraped off the plate, resuspended in water and observed under an Olympus Bx60 fluorescence microscope with an excitation λ= 450-480 nm and an emission λ = 515 nm LP (Long Pass).

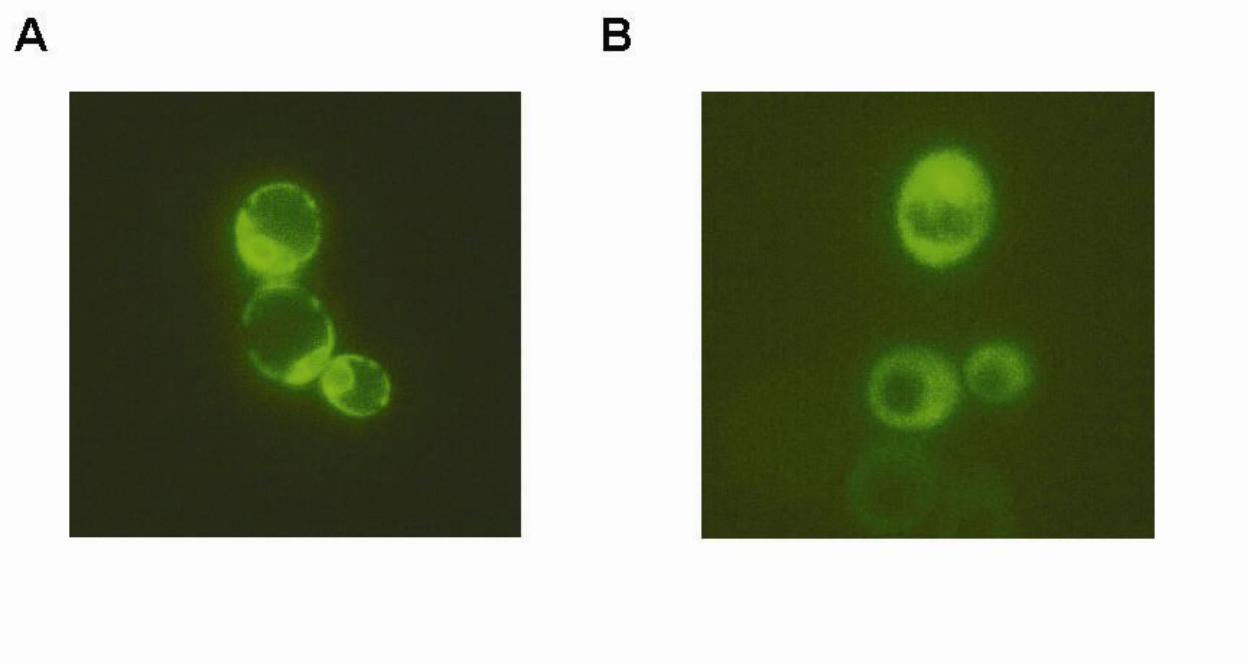


Figure 23: Fluorescence Microscopy of Cells Expressing GFP-Hlj1p or GFP-Hlj1p-ΔTMD

2.3.2. Mutations in Hsp90 Enhance CFTR Degradation but Have no Effect on CPY* Turn-over

The role of the Hsp90 molecular chaperone during CFTR maturation in mammals is controversial (Loo *et al.*, 1998; Fuller and Cuthbert, 2000). To better define the action of Hsp90 during CFTR biogenesis, I expressed CFTR in yeast deleted for the genes encoding the constitutive (*HSC82*) and heat-inducible (*HSP82*) Hsp90s. Yeast Hsp82 and Hsc82p are ~97% identical at the amino acid level and are functionally interchangeable, but at least one homologue must be expressed to maintain viability (Borkovich *et al.*, 1989). Therefore, the wild type strain for this experiment contains a plasmid-borne copy of *HSP82*, and the mutant strain contains a temperature-sensitive allele, $hsp82^{ts}$ (G313N). The mutant protein is extremely unstable when cells are shifted to the non-permissive temperature of 37°C and is rapidly degraded (Bohen and Yamamoto, 1993; Fliss *et al.*, 2000). This results in the equivalent of a null phenotype immediately after temperature shift. After cells were grown at a permissive temperature, CFTR degradation in these strains was monitored at 37°C by cycloheximide chase analysis. As shown in Figure 24, the rate of CFTR degradation was significantly higher in the mutant strain. Similar results were obtained when CFTR degradation was examined in the G170D *hsp82* mutant (Data not shown), which also rapidly loses activity at the non-permissive temperature (Nathan and Lindquist, 1995). Because only the immature, unfolded form of CFTR is an ERAD substrate (Gelman *et al.*, 2002) these data suggest that Hsp90 is important to maintain the stability of CFTR.

Hsp90 associates with several co-chaperones to form a macromolecular complex required for the folding and activation of select client proteins (Caplan, 1999; Richter and Buchner, 2001; Young *et al.*, 2001). Although the role of Hsp90 in mammalian cells can be assessed using ansamycin antibiotics, such as GA (see above), examining the functions of Hsp90 co-chaperones is more challenging. In yeast, mutations in Hsp90 co-chaperones are readily available. We therefore examined the roles of three well-defined Hsp90 co-chaperones (Sti1p, Sba1p, Sse1p) on CFTR stability in yeast. Sti1p is the yeast Hop homologue, and both Hsp70 and Hsp90 can dock onto the TPR domains of Sti1p (Johnson *et al.*, 1998). Sba1p is the p23 homologue that stabilizes Hsp90 substrate binding (Fang *et al.*, 1998). Sse1p is a yeast Hsp110 homologue that resides in the Hsp90 complex (Liu *et al.*, 1999; Goeckeler *et al.*, 2002). Deletion of the genes encoding each of these factors compromises Hsp90 complex-mediated processes in yeast (Chang *et al.*, 1997; Fang *et al.*, 1998; Liu *et al.*, 1999; Cox and Miller, 2002). When CFTR degradation was examined in isogenic wild type strains and in *sti1Δ*, *sba1Δ*, or *sse1Δ* yeast we detected no statistically significant differences in the rates of CFTR degradation in the mutants compared to wild type yeast (Fig. 25, Fig. 26). Furthermore, CFTR degradation was unaltered in a *sti1Δ sse1Δ* double mutant (Fig. 27) that has a severe growth defect at 37°C (Liu *et al.*, 1999), indicating that the accelerated degradation observed in the *hsp82* mutant (see Figure 24, above) was not simply the result of shifting temperature-sensitive cells to the non-permissive temperature. Together, these results demonstrate that Hsp90, but not the Hsp90 complexes tested, participates in CFTR biogenesis in yeast. Moreover, the action of Hsp90 during CFTR Biogenesis is not via an indirect effect on cellular signaling pathways because the same signaling pathways are compromised in strains mutated for the co-chaperones (Bohen *et al.*, 1998; Lee *et al.,* 2004b).

Figure 24: CFTR Degradation is Accelerated in an Hsp90 Mutant Yeast Strain.

Yeast strains expressing CFTR were subjected to a cycloheximide chase and immunoblot analysis as described in Materials and Methods (section 2.2). (A) The degradation of CFTR in wild type (open circles) and *hsp82* (closed circles) strains are plotted as the relative amount of CFTR remaining versus time. The amount of CFTR at time zero was set to 1.0. Data represent the mean of four independent experiments +/-SEM. Two-tailed P-values were < 0.05 except where indicated: * = 0.147, ** = 0.111, *** = 0.332. Bottom: representative western blot. Sec61p is an integral protein of the ER and serves as a loading control.

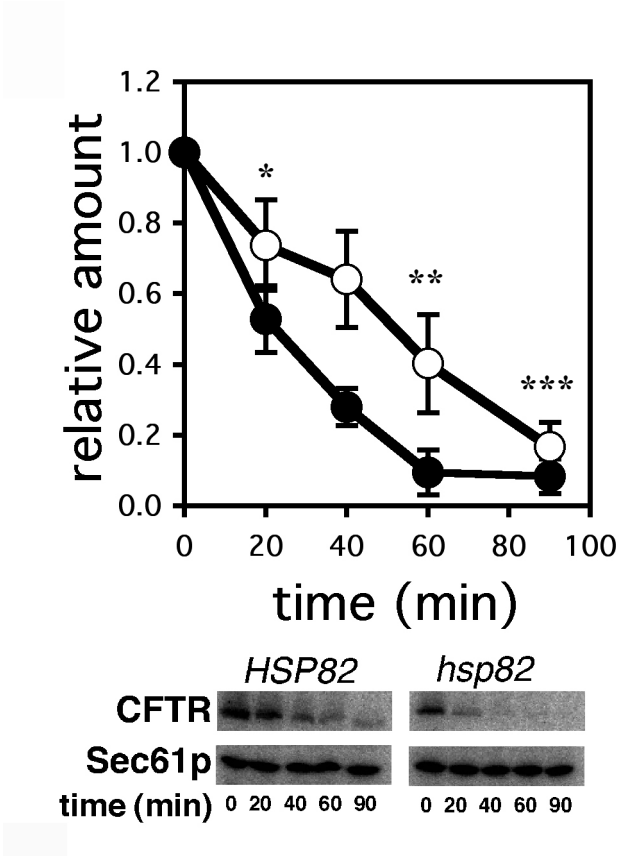


Figure 24: CFTR Degradation is Accelerated in an Hsp90 Mutant Yeast Strain

Figure 25: CFTR Degradation is Unaffected in *sti1Δ* and *sba1Δ* Mutant Strains.

The degradation of CFTR in wild type (open circles), *sti1Δ* (closed circles) and *sba1Δ* (open triangles) yeast strains are plotted as the relative amount of CFTR remaining over time. Data represent the mean of 3-4 independent experiments +/-SEM.

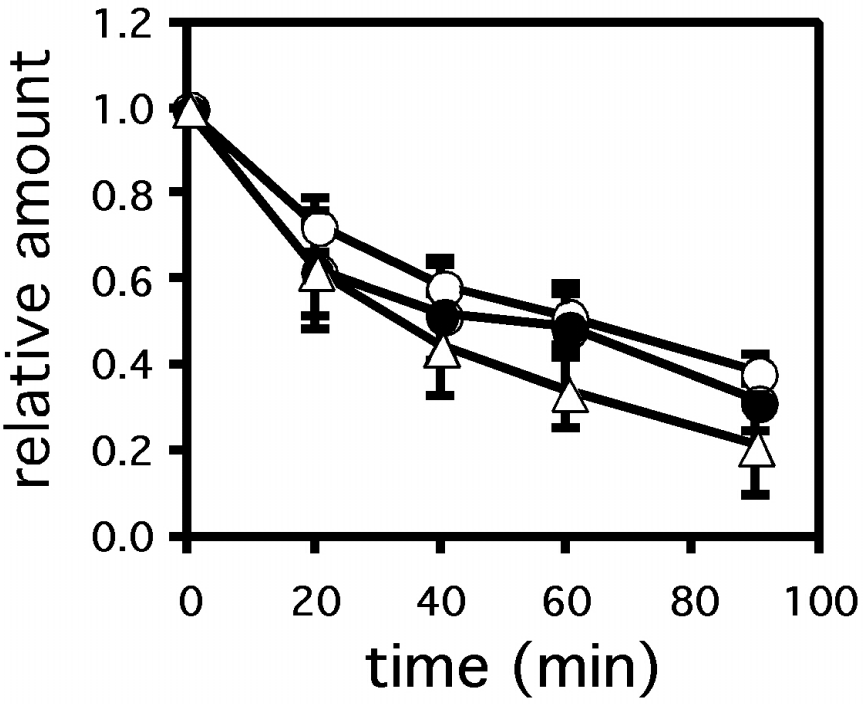


Figure 25: CFTR Degradation is Unaffected in *sti1Δ* and *sba1Δ* Strains

Figure 26: CFTR Degradation is Unaffected in the *sse1Δ* Strain.

CFTR degradation in wild type (open circles) and *sse1Δ* (closed circles) yeast strains are plotted as the relative amount of CFTR versus time. The amount of CFTR at time zero is set to 1.0. Data represent the mean of five independent experiments +/-SEM.

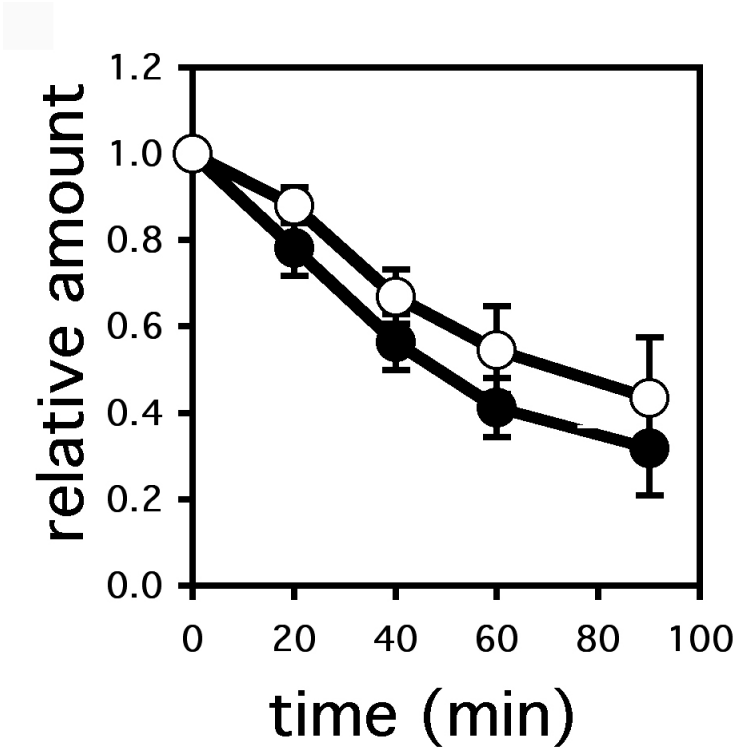


Figure 26: CFTR Degradation is Unaffected in the *sse1Δ* Strain

Figure 27: CFTR Degradation is Unaffected in an *sti1Δ sse1Δ* Double Mutant Strain.

CFTR degradation in wildtype (open circles) and *sti1Δ sse1Δ* (closed circles) yeast strains is plotted as the relative amount of CFTR over time. Data represent the mean of two independent experiments +/-STD.

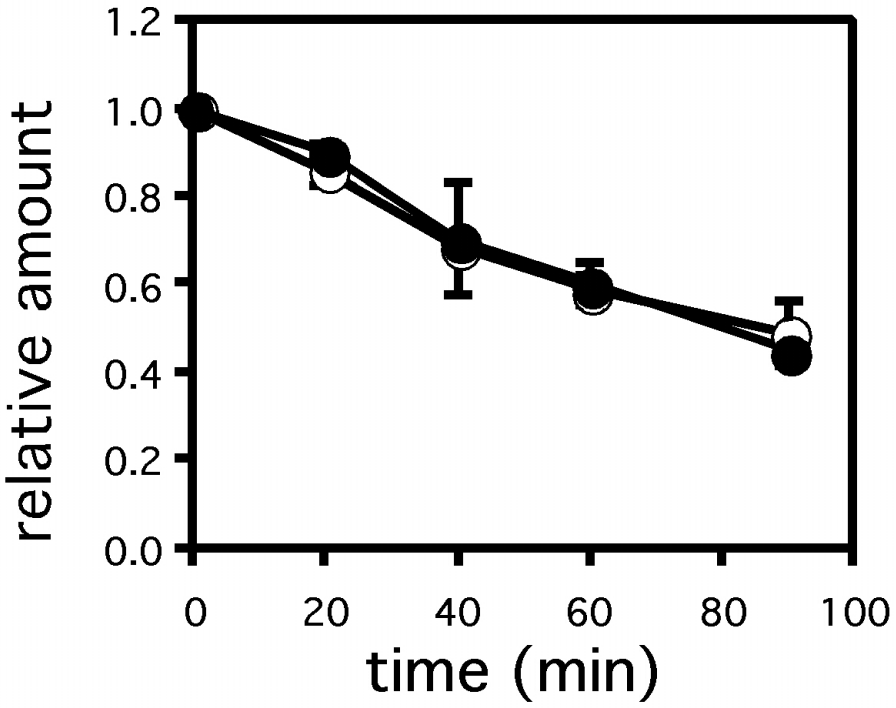


Figure 27: CFTR Degradation is Unaffected in an *stiΔ sse1Δ* Double Mutant Strain

As discussed above, distinct chaperone requirements for the degradation of soluble and membrane proteins have been noted, and this distinction was supported further by the data presented in Figs. 18 and 19. Therefore, I examined CPY* degradation in the *hsp82* mutant and in the isogenic wild type strain but found that there was no statistically significant difference in the rate or extent of degradation (Fig. 28). I conclude that the ERAD of CPY* is Hsp90-independent.

Figure 28: CPY* Degradation is Unaffected in an *hsp82* Mutant Yeast Strain.

CPY* degradation was assessed by pulse-chase analysis as described in the materials and methods (section 2.2) in wild type (open circles) and Hsp90 mutant (closed circles) yeast. Data represent the means of three independent experiments +/- SEM.

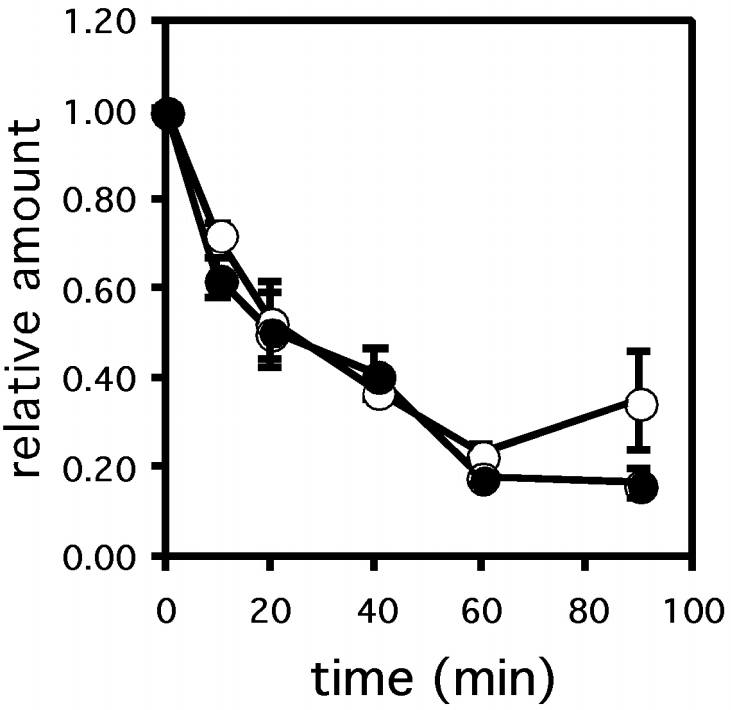


Figure 28: CPY* Degradation is Unaffected in an Hsp90̣ Mutant Yeast Strain

2.3.3. Yeast Hsp90 Prevents the Aggregation of the First Nucleotide Binding Domain (NBD1) of CFTR

The data presented in Figure 24 suggest that Hsp90 helps protect CFTR from degradation, and might therefore be important to maintain CFTR in its folded conformation, or a non-ERAD compatable form. In general, a loss of structural integrity can be accompanied by protein aggregation, and it is well-known that the first NBD1 in CFTR is aggregation-prone. Moreover, the low efficiency or slow rate of NBD1 folding directly determines the efficacy of CFTR maturation, and maintaining NBD1 solubility prevents the formation of off-pathway aggregates (Qu and Thomas, 1996; Strickland *et al.*, 1997; Zhang *et al.*, 1998). To test directly whether yeast Hsp90 maintains NBD1 solubility I purified Hsc82p (see Materials and Methods section 2.2) (Figure 29). First, to confirm that the purified protein was active, I examined the purified chaperone's ability to suppress the aggregation of firefly luciferase because mammalian Hsp90 was previously shown to slow the aggregation of this substrate (Wiech *et al.*, 1992). Luciferase aggregation was suppressed by ~65% when a 16:1 molar ratio of Hsc82p to luciferase was used (Figure 30), consistent with previous data (Minami *et al.*, 2001). Next, I examined whether Hsc82p associated with the Sba1p co-chaperone, as previously published (Fang *et al.*, 1998). Proficient interaction between Hsc82p and Sba1p was observed by pull-down assay, and importantly the degree of association decreased ~6-fold in the presence of Macbecin II (Figure 31), an ansamycin antibiotic that inhibits yeast Hsp90 function both *in vivo* and *in vitro* (Bohen, 1998; Fang *et al.*, 1998; Donze and Picard, 1999; Liu *et al.*, 1999). Finally, I assessed Hsc82p prevention of NBD1 aggregation at a 5:1 molar ratio and found that aggregation was suppressed

by ~60% (Figure 32), indicating that yeast Hsp90 maintains NBD1 in solution. BSA at a 2:1 molar ratio partially suppressed NBD1 aggregation, but there was ~24 fold less aggregated NBD1 protein after 60 minutes in the Hsp90 reaction compared to the BSA control (2.4% versus 58%; Data not shown), the reactions were performed using slightly different reaction conditions (buffer lacked KCl and $MgCl_2$). The ability of yeast Hsp90 to maintain NBD1 in solution was reduced somewhat if Macbecin was added to a final concentration of 50 μM (Data not shown). This partial effect may be due to the fact that Hsp90 contains two polypeptide binding sites, only one of which is sensitive to ansamycin antibiotics (Young *et al.*, 1997; Scheibel *et al.*, 1998). These data are also consistent with previous work in which a partial effect of ansamycin antibiotics on Hsp90-dependent activities was noted (Minami *et al.*, 2001). In any event, these results suggest that Hsp90 stabilizes CFTR by binding NBD1.

In summary, I have identified a novel redundancy of function for Hsp40 chaperones in the degradation of CFTR in yeast. Disruption or mutation of the genes encoding Ydj1p or Hlj1p alone has no effect on the degradation rate of CFTR, but inactivation of both chaperones leads to a marked stabilization of CFTR. Furthermore, degradation of the soluble ERAD substrate CPY* is unaltered in an *hlj1Δ ydj1-151* double mutant, suggesting that the two Hsp40 chaperones act specifically on membrane proteins. Thus, I have further defined the ERAD requirements for membrane versus soluble proteins. I have confirmed that yeast Hsp90 aids in the stabilization/folding of CFTR, but the Hsp90 co-chaperones Sba1p, Sti1p, and Sse1p do not, suggesting that Hsp90 but not the Hsp90 complex is important in the biogenesis of CFTR. Finally, Hsp90 can prevent the aggregation of NBD1 *in vitro,* suggesting one mechanism for its action during CFTR folding (also see Chapter 4: Discussion).

Figure 29: Purification of Hsc82p.

Hsc82p (Yeast Hsp90) was purified from ECUpep4 cells as described in the Materials and Methods (section 2.2). Coomassie Brilliant Blue stained gel of fractions from the Q-sepharose column.

MWM = molecular weight markers

F.T. = flow through

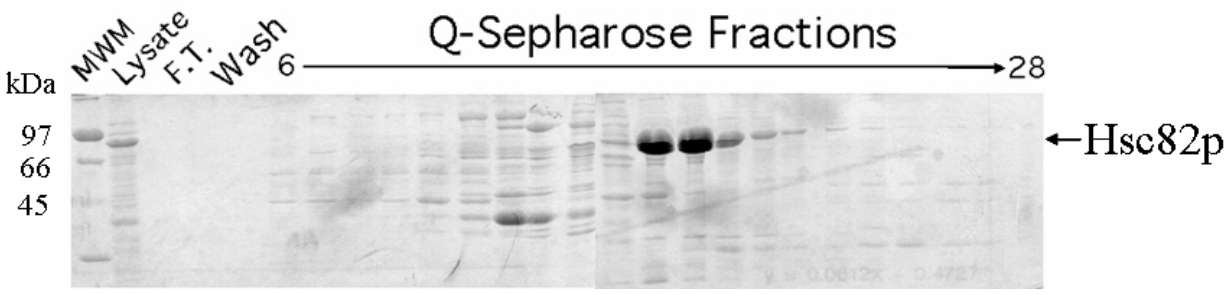


Figure 29: Purification of Hsc82p

Figure 30: Yeast Hsp90 Suppresses the Aggregation of Firefly Luciferase.

Luciferase was pre-incubated at a ~30:1 molar ratio of Hsc82p:Luciferase (red line) or in the presence of Hsc82p dialysis buffer (black line) for 20 minutes at 25ºC and then diluted ~4 fold into refolding buffer containing ATP at 45°C, resulting in a 16:1 final molar ratio of chaperone to substrate. Luciferase aggregation was measured by light scattering at 320 nm and 45ºC. Data at 10 sec intervals were recorded and line tracings are presented. For details see, materials and methods (section 2.2).

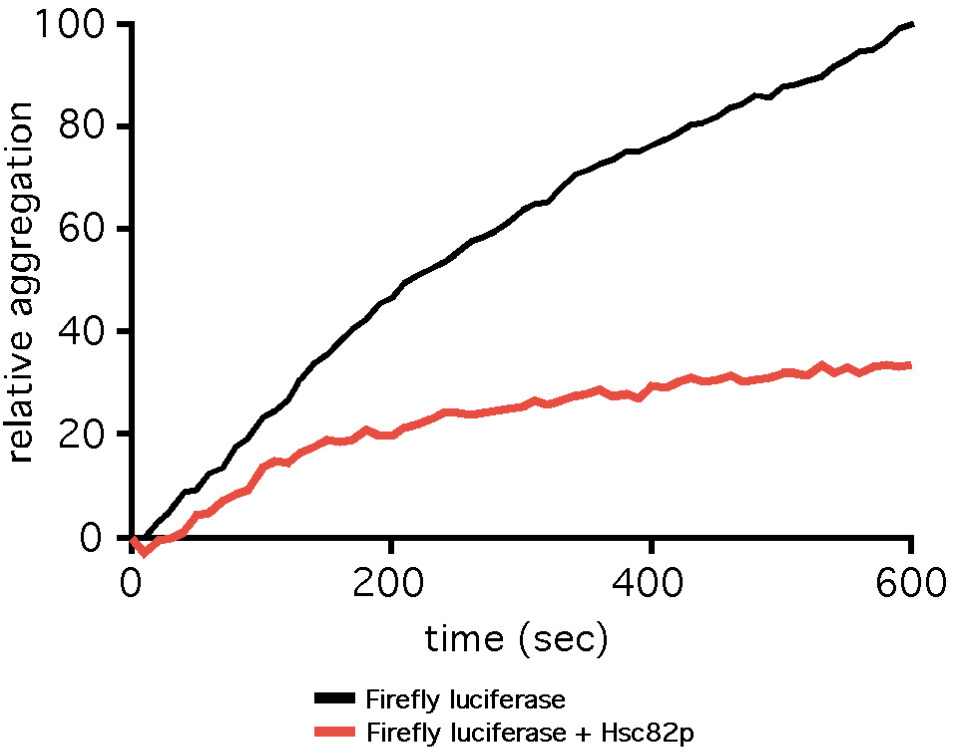


Figure 30: Yeast Hsp90 Suppresses the Aggregation of Firefly Luciferase

Figure 31: Yeast Hsp90 Binds the Sba1p/p23 Co-chaperone.

Hexa-histidine tagged Sba1p was pre-bound to nickel-linked resin and then incubated in either the absence (lane 1) or presence (lanes 2-4) of highly enriched Hsc82p. Bound protein was eluted, resolved by SDS-PAGE and visualized by silver staining. The reaction shown in lane 2 lacks pre-bound Sba1p and the reaction shown in lane 4 was supplemented with Macbecin II to a final concentration of 50 μM. Asterisk denotes contaminants in the Sba1p protein preparation.

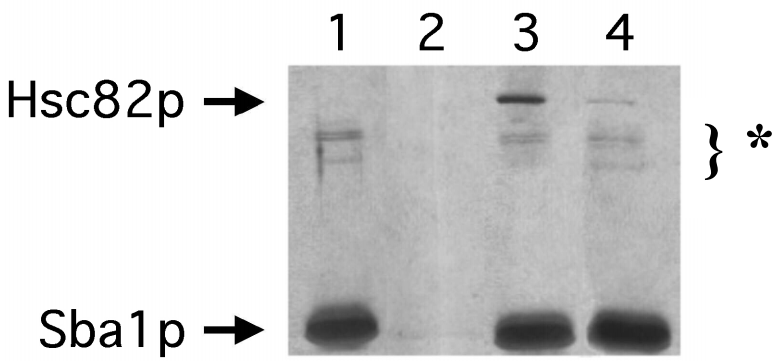


Figure 31: Yeast Hsp90 Binds the Sba1p/p23 Co-chaperone

Figure 32: Yeast Hsp90 Suppresses the Aggregation of NBD1 Early-Folding Intermediates. CFTR-NBD1 was diluted out of denaturant into refolding buffer containing ATP at 37°C in the absence (closed circles) or presence of Hsp90 at a 2.5:1 (closed triangles) and 5:1 (open circles) molar ratio, and light scattering was measured as described in the materials and methods (section 2.2). Data at 50 sec intervals are shown.

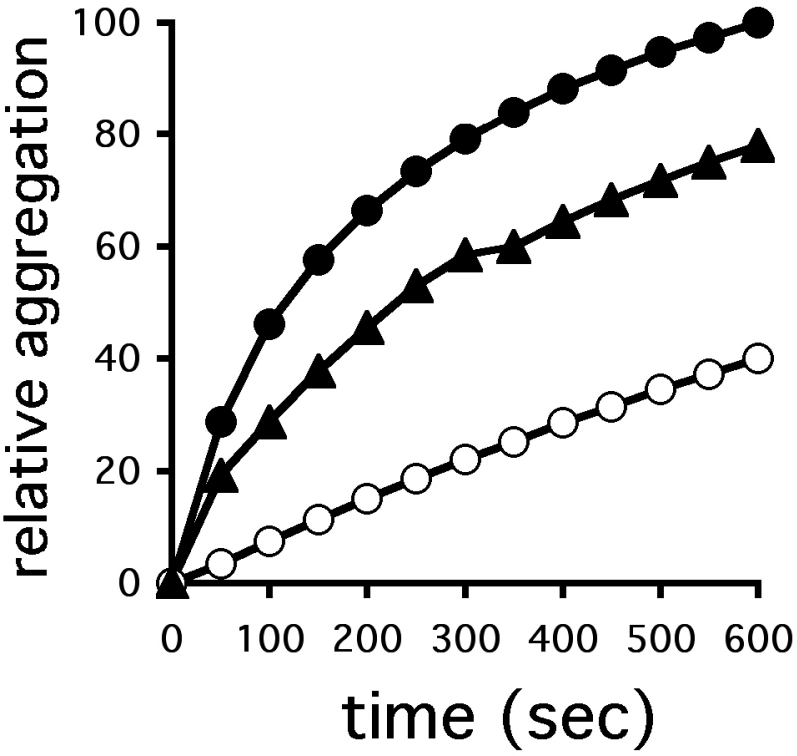


Figure 32: Yeast Hsp90 Suppresses the Aggregation of NBD1 Early-Folding Intermediates

3. The Mammalian Co-chaperones Bag-3 and FKBP8 Affect CFTR Degradation in *S. cerevisiae*

3.1. Introduction

In yeast, 100% of wildtype CFTR is retained in the ER (Figure 33; Sullivan *et al.*, 2003) and degraded by the proteasome compared to ~80% in mammalian cell lines. There are several mechanisms to account for this phenomenon: First, the ER-to-Golgi transport machinery in yeast may not "recognize" CFTR, which is a human protein. Second, CFTR is a slowly-folding protein and may not fold in time to be recognized by the yeast's ER-to-Golgi transport machinery. Third, additional factors may be needed for efficient transport of CFTR out of the ER that are only found in humans. Recently, the Balch laboratory has identified CFTR-interacting proteins in mammalian cells by performing mass spectroscopy on CFTR-containing complexes (W. Balch, personal communication). They have determined that several of these proteins are Hsp70 or Hsp90 co-chaperones that when overexpressed in mammalian cells partially stabilize and aid in CFTR trafficking. Two co-chaperones that exhibited the greatest effect on CFTR biogenesis in mammalian cells were Bag3 and FKBP8.

Bag3 is a member of the Bag family of co-chaperones that are negative regulators of Hsc/Hsp70 (see section 1.3.1.1) (Takayama *et al.*, 1999). Bag-3/CAIR-1 (CAI stresses cells-1) is a 75 kDa protein that binds to phospholipase-C-γ and Hsc/Hsp70 in a ternary complex that is regulated by epidermal growth factor (EGF) and CAI (inhibitor of calcium influx) (Doong *et al.*, 2000). Based on these observations, Bag3 has been proposed to act as a signaling protein that connects

Hsc/Hsp70 with the EGFR signaling pathway. Bag3 can also bind to the anti-apoptotic factor Bcl-2 and prevent Fas-induced and Bax-mediated apoptosis (Lee *et al.*, 1999), suggesting a role in regulating apoptotic pathways (Lee *et al.*, 1999) (Romano *et al.*, 2003; Bonelli *et al.*, 2004).

Figure 33: CFTR Resides in the ER in Yeast.

Extracts from CFTR expressing wildtype cells were subjected to sucrose gradient centrifugation (see Materials and Methods, section 3.2). The gradient was fractionated and the migrations of CFTR, Sec61p (ER membrane protein), BiP (ER lumen chaperone) and Pma1p (Plasma membrane ATPase) were determined by immunoblot analysis. Fraction 1 represents the top of the gradient (adapted from (Sullivan *et al.*, 2003)). The majority of CFTR is present in fractions 2-5 which corresponds to 25-38% sucrose (w/v). Pma1p is present in fractions 11-13 which corresponds to 58-65% sucrose (w/v). Note, CFTR is absent from fractions containing the plasma membrane ATPase Pma1p.

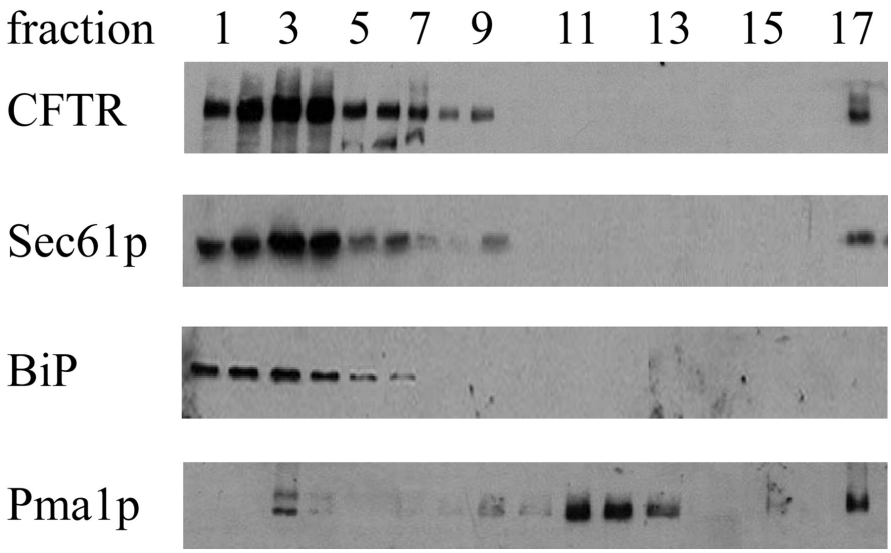


Figure 33: CFTR Resides in the ER in Yeast

A second CFTR-interacting protein identified by the Balch laboratory is FKBP8 that also binds to Bcl-2 and inhibit apoptosis (Shirane and Nakayama, 2003). FKBP8 is a member of the immunophilin class of proteins, which are the molecular targets of the immunosuppressive drugs FK506 and cyclosporin A (CsA). FK506 binds to FK506 binding proteins (FKBPs) and CsA binds to cyclophilins (CyPs). The FK506-FKBP or CsA-CyPs complex binds and inhibits the calcium-regulated phosphatase calcineurin, thus blocking a critical step in T-cell activation and in suppressing the immune response (Friedman and Weissman, 1991; Liu *et al.*, 1991; Clipstone and Crabtree, 1992; O'Keefe *et al.*, 1992). FKBP8/FKBP38 (FKBP of 38 kDa) contains an FKBP-like domain, a leucine zipper repeat, three TPR domains, and a COOH-terminal transmembrane domain (Lam *et al.*, 1995). FKBP8 has the highest similarity to a subfamily of immunophilins that include FKBP52 and CyP40 (Lam *et al.*, 1995; Shirane and Nakayama, 2003). FKBP52 also contains a TPR motif, binds to Hsp90 and is required for proper trafficking of the glucocorticoid receptor to the nucleus (Galigniana *et al.*, 2001). In general, FKBPs possess peptidyl-prolyl *cis/trans*-isomerase (PPIase) activities that catalyze conversion of proline from the *cis*- to the *trans*-isomer, a rate-determining step in protein folding. However, FKBP8 lacks several conserved amino acids that are required for binding of FK506 and PPIase activity, suggesting that it lacks those activities (Lam *et al.*, 1995). In fact, FKBP8 inhibits calcineurin by a FK506-independent mechanism, and as stated above targets Bcl-2 and Bcl-X_L to the mitochondria to prevent apoptosis. Recently, a longer form of FKBP8 has been identified in adult mouse brain (Nielsen *et al.*, 2004). Mouse has two isoforms, 44 kDa and 46 kDa, and humans possess one, 45 kDa. Previous studies used the NH_2-terminal-truncated 38 kDa human FKBP8 protein to determine PPIase activity (Lam *et al.*, 1995). Identification of the full length

45 kDa human isoform requires that these previous studies be re-visited, in order to confirm the lack of PPIase activity.

The Brodsky lab has entered into collaboration with the Balch lab to determine if Bag3 or FKBP8 affect CFTR stability/maturation in yeast. In this chapter, I show that overexpression of either Bag3 or FKBP8 slows the degradation of CFTR, but does not appear to affect CFTR trafficking in yeast.

3.2. Materials and Methods

3.2.1. Yeast Strains, Plasmids, and Molecular Methods

Yeast strain RSY620 (*Mat **a**, ade2-1, trp1-1, leu2-3,112, ura3-1, his3-11,15, PEP4::TRP1*) was grown at 26°C unless indicated otherwise and standard methods for growth, preparation of media, and transformation of yeast cultures were used (Adams, 1997). RSY620 cells expressing HA-epitope-tagged CFTR were transformed with pCu-415-CUP1-FKBP8, pCu-425-CUP1-FKBP8, or pCu-425-CUP1-Bag-3 and were grown to early logarithmic phase (OD600 ~ 0.20) in synthetic complete medium lacking uracil and leucine, but supplemented with glucose to a final concentration of 2% (SC –ura –leu). These plasmids contained the indicated genes and their expression was regulated by the CUP1 promoter (Labbe and Thiele, 1999). Cells were incubated with 1mM copper sulfate for 4 hr to induce expression of FKBP8 or Bag-3, and cycloheximide was added to a final concentration of 50 or 100 μg/ml. A total of 2-2.5 ODs of cells were

removed (t=0), the culture was shifted to 37°C, and aliquots were removed at the indicated timepoints. The cells were washed and TCA precipitated as described (Zhang *et al.*, 2002b). Proteins were resolved on 10% SDS-polyacrylamide gels, transferrd to nitrocellulose, and probed with mouse monoclonal anti-HA antibody (12CA5, Roche Molecular Biochemicals, Indianapolis, IN), polyclonal anti-Bag-3 (1:500 dilution) (ab5898, Abcam, Cambridge, MA), polyclonal FKBP8 (1:5000 dilution)(W. Balch, Scripps Research Institute), polyclonal anti-Sec61p (Stirling 1992), polyclonal anti-Hsp90 (A. Caplan, Mount Sinai School of Medicine), and Polyclonal anti-Ssa1p was prepared by immunizing rabbits against an ~27 kDa C-terminal fragment of Ssa1p (J. L. Brodsky, unpublished results). Blots for Bag-3 were blocked in TBST supplemented with 2% donkey serum instead of 2% milk to reduce background. Signals were visualized using horseradish peroxidase-conjugated secondary antiserum and the results were quantified using the Kodak 440CF Image Station and the associated Kodak 1D (V. 3.6) software (Rochester, NY).

3.2.2. Subcellular Fractionation of Membranes

Membranes from CFTR-expressing cells were separated using a protocol adapted from the Hollenberg and Kaiser laboratories (Kolling and Hollenberg, 1994) (Roberg *et al.*, 1997). Briefly, 100 ml of CFTR-expressing cells (OD_{600}= 0.5-1.0) were harvested, and washed with 1 volume of cold 10 mM NaN_3, and then with 1 volume of cold STED10 buffer (10% sucrose, 10 mM Tris-HCl (pH = 7.6), 1 mM EDTA, 1 mM DTT) before they were resuspended in 0.5 ml STED10 + protease inhibitors (1 μg/ml leupeptin, 0.5 μg/ml pepstatin A, and 1 mM phenylmethylsuflonylfluoride). Glass beads were added to the meniscus and the cells were

vortexed for 2 min at the highest setting. An additional 0.5 ml of STED10 buffer was added, unbroken cells were pelleted, and 300 µl of crude lysate was loaded onto an 11 ml 30-70% sucrose gradient prepared by layering STED70, STED60, STED50, STED40, and STED30 buffers sequentially. Membranes were separated by centrifugation for ~ 18 hr at 100,000 g. A total of 700 µl fractions were collected starting at the top of the gradient. Total protein was TCA-precipitated from each fraction; proteins were resolved on 6% or 10% SDS-polyacrylamide gels, transferred to nitrocellulose, probed with mouse monoclonal anti-HA antibody (12CA5, Roche Molecular Biochemicals, Indianapolis, IN), polyclonal anti-Sec61p (Stirling *et al.*, 1992), polyclonal anti-Pma1p (C. Slayman, Yale University), or polyclonal anti-Gas1p (T. Doering, Washington University School of Medicine). For FKBP8 and Bag-3 experiments cells were grown to early-log phase, ande were incubated with 1 mM $CuSO_4$ for 4 hr before the cells were harvested and membranes were resolved on sucrose gradients.

3.3. Results

3.3.1. CFTR Degradation is Reduced in Yeast Expressing FKBP8

Mutant forms of CFTR that escape ERAD are degraded by the lysosome in the late secretory pathway in mammalian cells (see section 1.4.4). To increase the likelihood of detecting CFTR that has trafficked out of the ER in yeast due to expression of FKBP8 or Bag-3, cells deleted for the gene encoding the vacuolar protease carboxypeptidase Y (Pep4) were chosen for all subsequent experiments. Yeast cells (*pep4Δ*) expressing CFTR were grown to early-log phase

and $CuSO_4$ was added to the medium to induce FKBP8 expression from a low-copy (pCu-415-CUP1-FKBP8), or high-copy (pCu-425-CUP1-FKBP8) copper inducible plasmid and a timecourse was performed (see Materials and Methods, section 3.2). For cells containing the high-copy plasmid, FKBP8 protein could be detected even at 1 hr of induction (Figure 34). FKBP8 protein could not be detected by western blotting after 4 hr of induction for yeast containing the low-copy plasmid (Figure 34). Therefore, the high-copy plasmid was used for subsequent cycloheximide chase and subcellular fractionation experiments (see Materials and Methods, section 3.2). Yeast cells expressing FKBP8 from the high-copy plasmid exhibited no growth defects compared to the empty vector control (Figure 35), thus expression of FKBP8 is not toxic to yeast. In agreement with studies conducted in mammalin cells (W. Balch, personal communication), the rate of CFTR degradation was diminished upon overexpression of FKBP8 compared to control cells (Figure 36), suggesting that this mammalian immunophilin may aid in the folding/maturation of CFTR. The effect of FKBP8 on CFTR degradation was most prominent using fresh transformants that were never older then ~2.5 weeks. The levels of Hsp90 and Hsp70 chaperones were similar in FKBP8 expressing cells compared to control cells, suggesting that the effect on CFTR degradation is not simply due to altered cytosolic chaperone levels (Figure 37). Extracts made from the same colonies used for cycloheximide chase experiments were fractionated on sucrose gradients to determine if stabilized CFTR could traffic beyond the ER compartment upon FKBP8 expression (see Materials and Methods, section 3.2). The majority of CFTR was present in early fractions (1-7), which are typically enriched for ER membranes (Figure 33), and significant amounts of CFTR were not detected in denser plasma membrane enriched fractions (>10) that typically contain the mature form of Gas1p, a plasma membrane marker (Figure 38). A small quantity of CFTR could be detected in fraction 10 for

both FKBP8 and control gradients, however this is most likely due to ER-membrane contamination of Golgi/plasma membranes, evident by the presence of the immature ER-form of Gas1p. Additional experiments will be required to confirm if this population of CFTR resides in a post-ER compartment. Nevertheless, these results suggest that FKBP8 stabilization of CFTR in yeast is not sufficient to allow efficient export of CFTR from the ER.

Figure 34: Induction of FKBP8 Expression in Yeast.

pep4Δ cells expressing CFTR and containing pCu-415-CUP1, pCu-415-CUP1-FKBP8, pCu-425-CUP1, or pCu-425-CUP1-FKBP8 were grown to mid-log phase (OD_{600} = 0.3-0.5) and $CuSO_4$ was added to a final concentration of 1mM. A total 2ml of cells were removed at 1, 2, or 4 hr and total protein was TCA precipitated, resolved on a 10% gel, transferred to nitrocellulose and probed for the presence of CFTR, FKBP8 or Sec61p (blot not shown) (see Materials and Methods, section 3.2). Two representative colonies of pCu-415-CUP1-FKBP8 and pCu-425-CUP1-FKBP8 are shown.

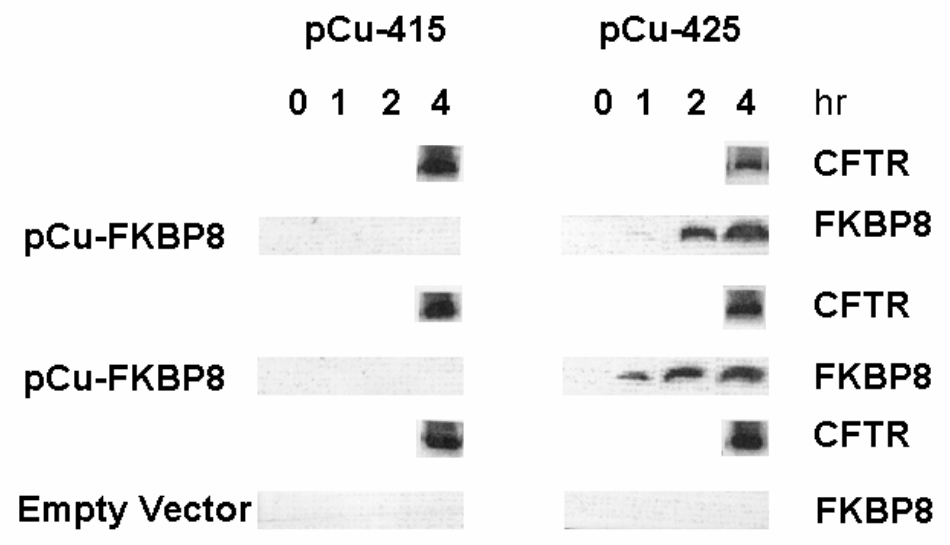


Figure 34: Induction of FKBP8 Expression in Yeast

Figure 35: Yeast Expressing FKBP8 Exhibit Normal Growth.

pep4Δ cells expressing HA-epitope-tagged CFTR and containing one of the following plasmids (see below) were grown to an OD_{600} = 0.3-0.5, serial dilutions were performed, and cells were plated on selective medium with/without 20 μM $CuSO_4$. Cells were grown at 26°C or 30°C for 4.5 days.

1 & 2) pCu-415-CUP1-FKBP8

3 & 4) pCu-425-CUP1-FKBP8

5) pCu-415-CUP1-Empty

6) pCu-425-CUP1-Empty

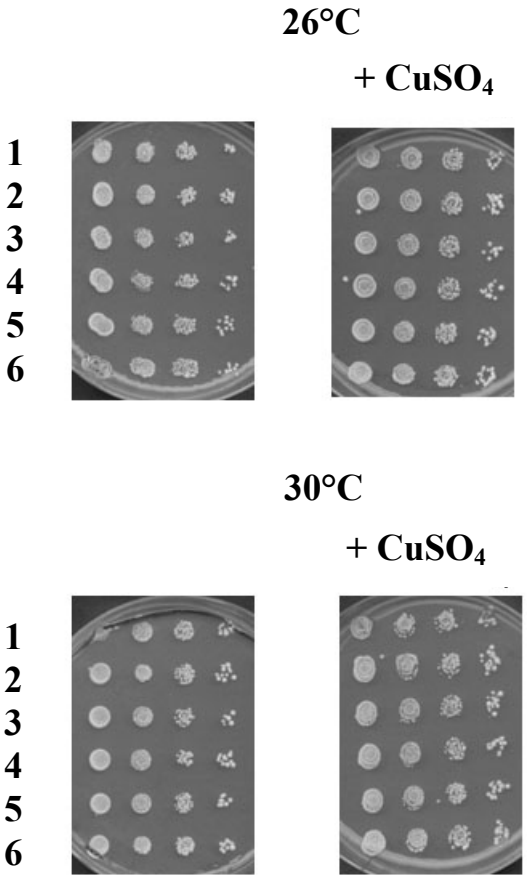


Figure 35: Yeast Expressing FKBP8 Exhibit Normal Growth

Figure 36: CFTR Degradation is Reduced in Yeast Expressing FKBP8.

pep4Δ yeast expressing CFTR and pCu-CUP1-425 or pCu-425-CUP1-FKBP8 were incubated for 4 hr with 1 mM $CuSO_4$ and subjected to cyloheximide chase analysis as described in Materials and Methods (section 3.2). The degradation of CFTR in the absence (closed black circles) or presence of FKBP8 (open pink circles) are plotted as the relative amount of CFTR remaining versus time. The amount of CFTR at time zero was set to 1.0. Data represent the means of 5 (FKBP8, except 90 min timepoint N=4) or 3 (Empty vector) independent experiments +/- SEM. Two-tailed P-values are < 0.05, except were indicated: * = 0.15, ** = 0.14. Bottom: representative western blot. Sec61p serves as a loading control.

.

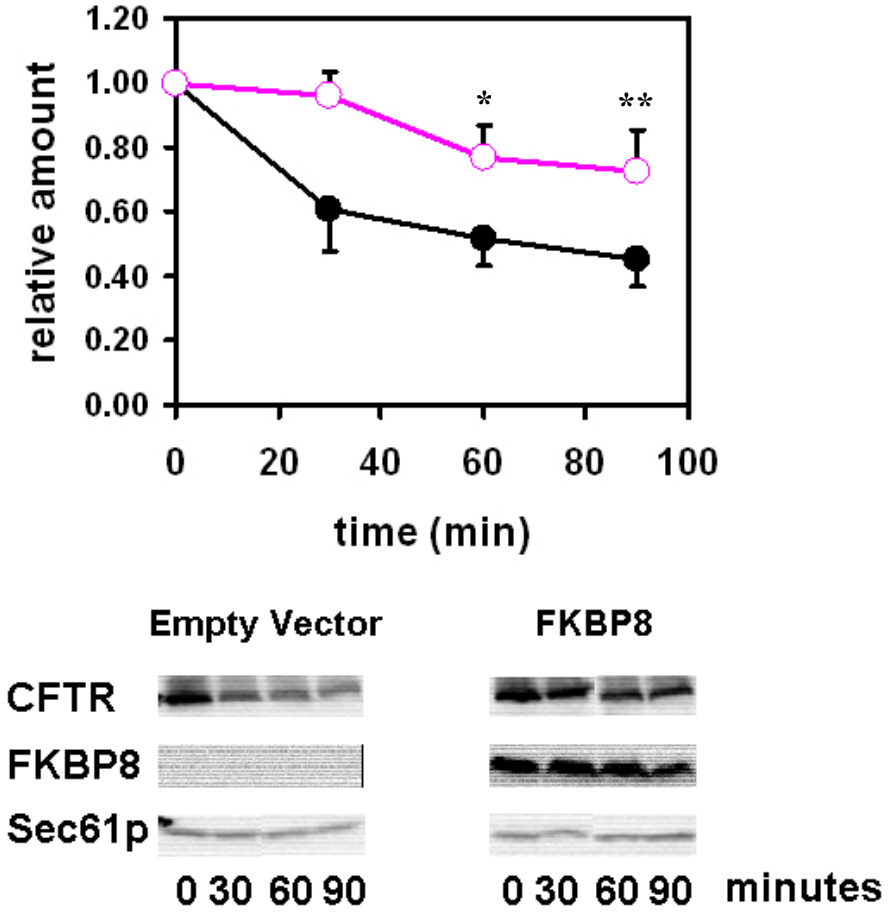


Figure 36: CFTR Degradation is Reduced in Yeast Expressing FKBP8

Figure 37: Hsp90 and Hsp70 Chaperone Levels are Normal in Yeast Cells Expressing FKBP8.

Duplicate samples (1-2 independent colonies) of TCA-precipitated protein from timepoint zero of the FKBP8 cycloheximide chase was resolved on a 10% SDS-polyacrlyamide gel, transeferred to nitrocellulose, and probed with anti-Hsp90 antibody, anti-Hsp70 antibody, or anti-Sec61p antibody (see Materials and Methods section, 3.2). Proteins were visualized using horseradish-peroxidase conjugated secondary anti-serum and the data were scanned using the Kodak 440CF Image Station and quantified using Kodak 1D (version 3.6) software (Rochester, NY). The Hsp90 and Hsp70 signals were normalized to Sec61p to calculate the relative amount of chaperone. Top: Representative immunoblot. Bottom: Bar graph of normalized chaperone levels. Data represent the mean +/- range.

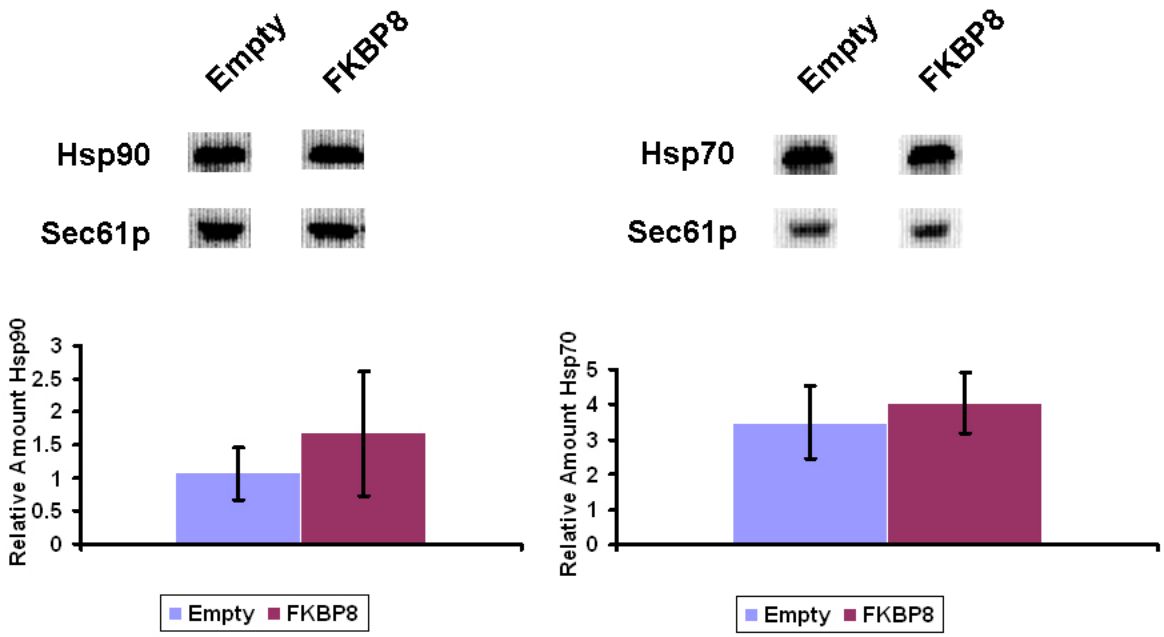


Figure 37: Hsp90 and Hsp70 Chaperone Levels are Normal in Yeast Expressing FKBP8

Figure 38: Subcellular Fractionation of Yeast Cells Expressing FKBP8.

Extracts from CFTR-expressing *pep4Δ* cells containing pCu-425-CUP1 or pCu-425-CUP1-FKBP8 were subjected to sucrose gradient centrifugation. The gradient was fractionated and the migration of CFTR and Gas1p were determined by immunoblotting analysis (see Materials and Methods section 3.2). Fraction 1 represents the top of the gradient: m = mature GPI form of Gas1p that resides in the Golgi/Plasma membrane (~125 kDa); i = immature form that resides in the ER (~105 kDa).

Bracket = Position of the plasma membrane marker Pma1p in the gradient, which corresponds to 58-65% sucrose (w/v) or fractions 11-13.

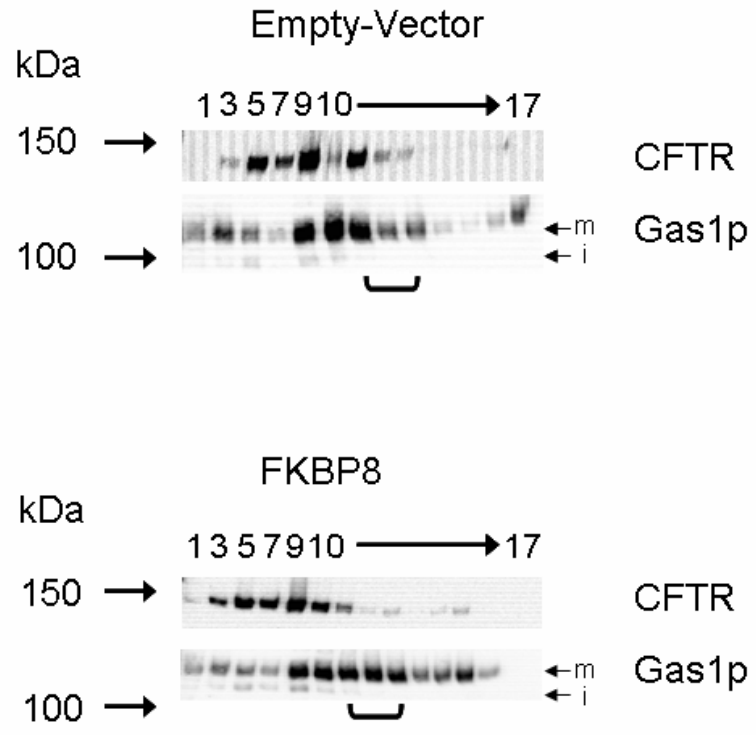


Figure 38: Subcellular Fractionation of Yeast Expressing FKBP8

3.3.2. CFTR Degradation is Reduced in Yeast Expressing Bag-3

To test whether Bag-3 affects the degradation rate of CFTR, I transformed CFTR-expressing yeast cells with a Bag-3 high copy number plasmid under the control of a copper inducible promoter, or with an empty vector. Bag-3 expression was induced and cycloheximide chase analyses were performed (see Materials and Methods, section 3.2). Expression of Bag-3 diminshed the rate of CFTR degradation compared to control cells (Figure 39). The levels of the cytoplasmic chaperones Hsp90 and Hsp70 did not change upon expression of Bag-3 (Figure 40), suggesting that CFTR stabilization is not due simply to changes in cellular chaperone concentrations. Next, the contribution of Bag-3 on CFTR trafficking was determined by subcellular fractionation of CFTR containing cells expressing Bag-3 (see Materials and Methods, section 3.2). Expression of Bag-3 did not grossly alter the distribution of CFTR and the bulk of CFTR resided in earlier (ER) fractions (1-7) (Figure 41). However, there was some CFTR present in later fractions (11-13), which could be due to the greater amount of CFTR present in the membranes compared to control membranes. Additional experiments will be required to confirm if this population of CFTR actually is in a post-ER compartment (see Discussion Chapter 4).

In summary, I have shown that expression of FKBP8 or Bag-3 reduces CFTR degradation, but does not appear to significantly facilitate the trafficking of CFTR to the plasma membrane in yeast. This reaffirms that stabilization is not sufficient for ER export of CFTR (Zhang *et al.*, 2001). The concentration of Hsp90 and Hsp70 chaperones are not changed upon expression of either mammalian protein, suggesting that the reduced CFTR degradation observed is not due to

alteration of cytoplasmic chaperone levels, but could be through modulation of Hsp70 or Hsp90 activity (see Discussion Chapter 4).

Figure 39: CFTR Degradation is Reduced in Yeast Expressing Bag-3.

pep4Δ yeast expressing CFTR and containing pCu-CUP1-425 or pCu-425-CUP1-Bag-3 were incubated for 4 hr with 1 mM $CuSO_4$ and subjected to a cyloheximide chase analysis as described in the Materials and Methods (section 3.2). The degradation of CFTR in the absence (closed black circles) or presence of Bag-3 (open pink circles) are plotted as the relative amount of CFTR remaining versus time. The amount of CFTR at time zero was set to 1.0. Data represent the means of 3 independent experiments (Bag-3) +/- SEM or 2 independent experiments +/- STD (Empty-vector). Two-tailed P-values are < 0.05, except were indicated: * = P-value < 0.10. Bottom: representative western blot. Sec61p serves as a loading control.

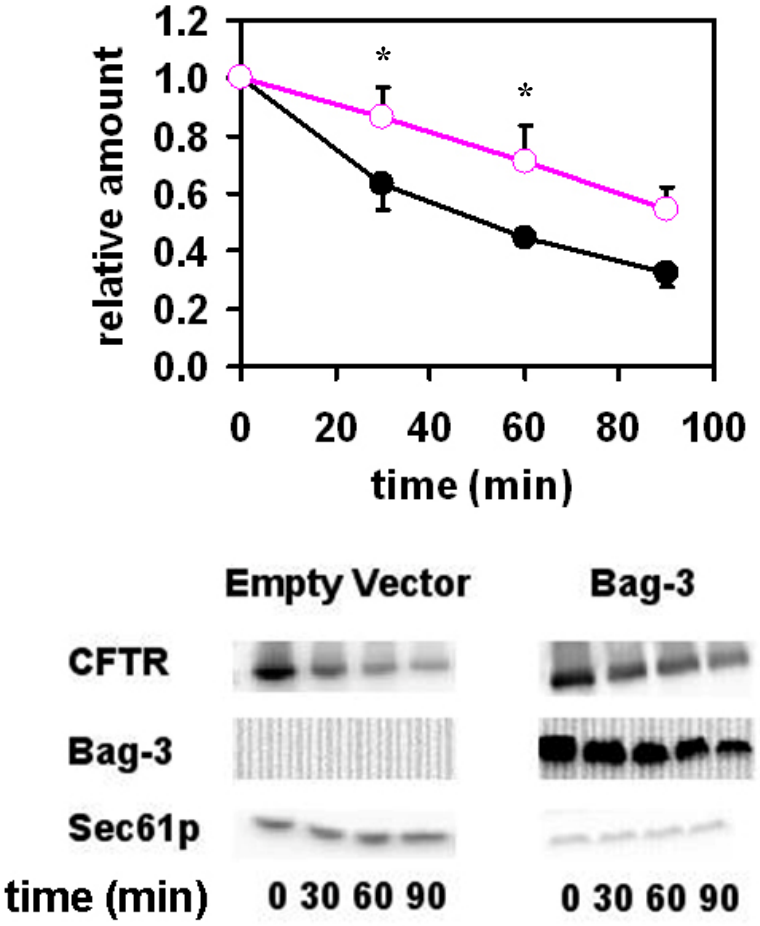


Figure 39: CFTR Degradation is Reduced in Yeast Expressing Bag-3

Figure 40: Hsp90 and Hsp70 Levels are not Significantly Altered in Bag-3-Expressing Yeast. Duplicate samples (1-2 independent colonies) of TCA-precipitated protein from timepoint zero of the Bag-3 cycloheximide chase was resolved on a 10% SDS-polyacrlyamide gel, transeferred to nitrocellulose, and probed with anti-Hsp90 antibody, anti-Hsp70 antibody, or anti-Sec61p antibody (see Materials and Methods section, 3.2). Proteins were visualized using horseradish-peroxidase conjugated secondary anti-serum and the data were scanned using the Kodak 440CF Image Station and quantified using Kodak 1D (version 3.6) software (Rochester, NY). The Hsp90 and Hsp70 signals were normalized to Sec61p to calculate the relative amount of chaperone. Top: Representative immunoblot. Bottom: Bar graph of normalized chaperone levels. Data represent the mean +/- range.

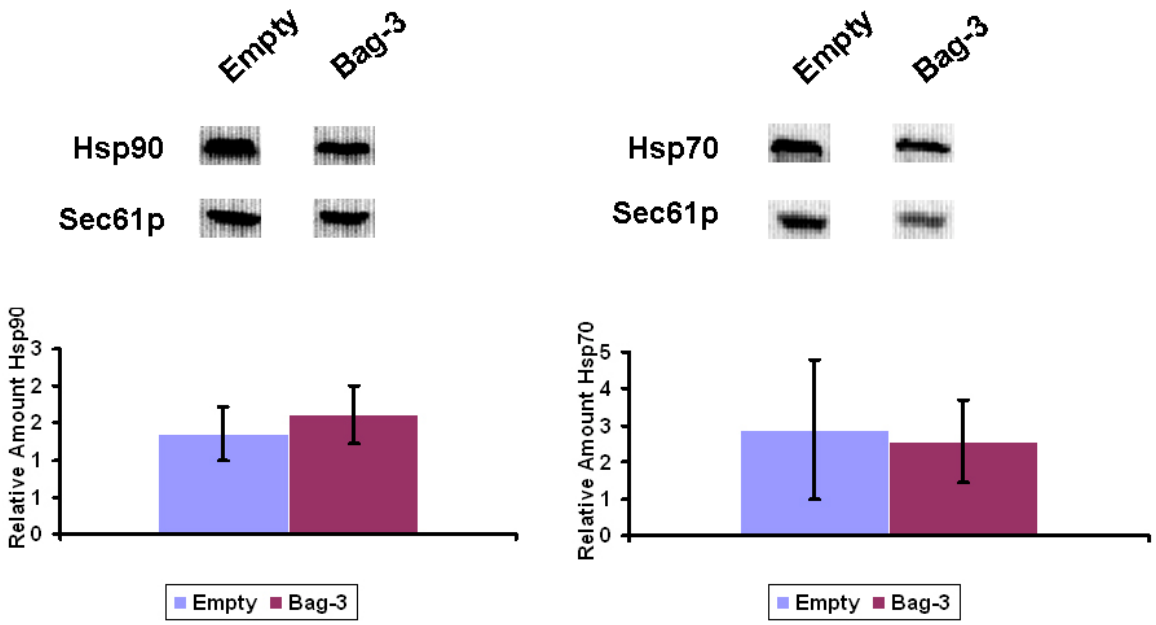


Figure 40: Hsp90 and Hsp70 Levels are not Significantly Altered in Bag-3-Expressing Yeast

Figure 41: Subcellular Fractionation of Yeast Expressing Bag-3.

Extracts from CFTR-expressing *pep4Δ* cells containing pCu-425-CUP1 or pCu-425-CUP1-Bag-3 were subjected to sucrose gradient centrifugation. The gradient was fractionated and the migration of CFTR and Gas1p were determined by immunoblot analysis (see Materials and Methods, section 3.2). Fraction 1 represents the top of the gradient: m = mature GPI form of Gas1p that resides in the Golgi/Plasma membrane (~125 kDa); i = immature form that resides in the ER (~105 kDa).

Bracket = Position of the plasma membrane marker Pma1p in the gradient, which corresponds to 58-65% sucrose (w/v) or fractions 11-13.

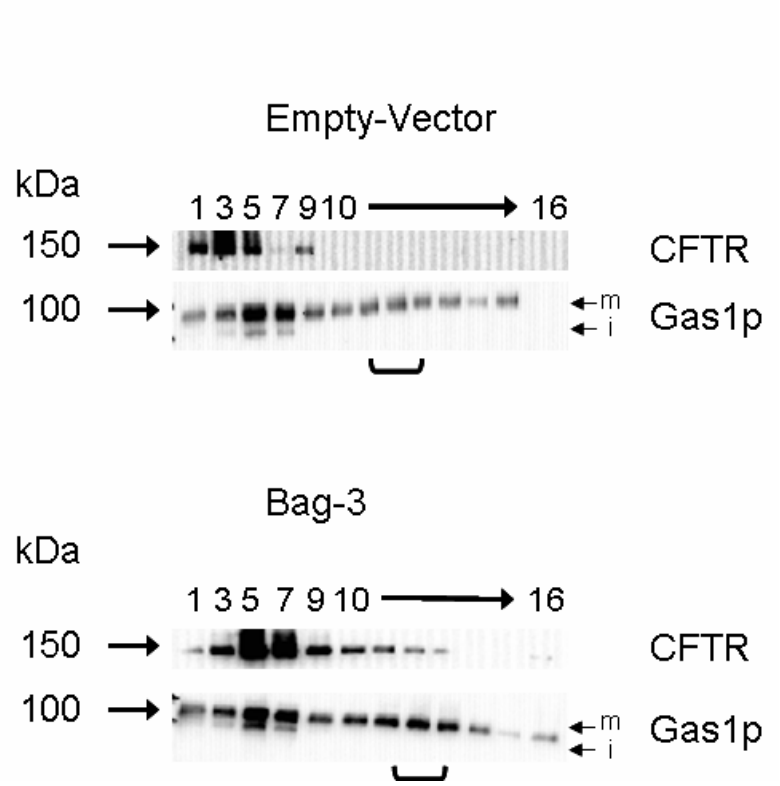


Figure 41: Subcellular Fractionation of Yeast Expressing Bag-3

4. Discussion

My analysis of CFTR biogenesis in yeast has uncovered several novel aspects of molecular chaperone function and of the ERAD pathway for integral membrane proteins. First, I have identified the Hsp40 homologues in yeast, Ydj1p and Hlj1p, that facilitate CFTR degradation and that act most likely in conjunction with an Hsp70, Ssa1p. These results suggest that Hlj1p and Ydj1p function redundantly. Both chaperones enhance Ssa1p ATPase activity and the extent of CFTR stabilization in the *hlj1 ydj1* mutant strain (Figure 18) is similar to that observed in the *ssa1* mutant (Zhang *et al.*, 2001).

It is important to note that the experimental technique employed (i.e. cycloheximide chase analysis) to determine the extent of CFTR degradation in wildtype and mutant yeast does not define the conformational state of the protein. Stabilization in this context refers simply to increased levels of CFTR remaining in the mutant cells compared to wildtype. The stabilization or accumulation of CFTR in the *hlj1 ydj1* mutant strain could arise because of favorable conformational changes, aggregation, and/or uncoupling of CFTR from the ubiquitin-proteasome degradation pathway. Additional experiments such as limited proteolysis, protein solubility measurements, or biophysical techniques would need to be employed to ascertain the conformational state of "stabilized" CFTR in the *ssa1* and *hlj1 ydj1* mutant strains.

These data are reminiscent of the reported functional redundancy and interactions between BiP and two redundant ER luminal Hsp40 chaperones, Scj1p and Jem1p, during the ERAD of soluble proteins (Nishikawa *et al*., 2001). The functional redundancy displayed by Ydj1p and Hlj1p is

not limited to CFTR turn-over because yeast mutated for the genes encoding these co-chaperones also exhibit slowed degradation of Ste6p* (Huyer *et al.*, 2004), another integral membrane ERAD substrate in yeast. Other recent studies have also hinted at a role for Hlj1p in protein quality control. For example, *hlj1Δ* mutants grew poorly when they expressed a Huntingtin fragment (HD53Q) (Willingham *et al.*, 2003) and the degradation of a synthetic, integral membrane ERAD substrate was mildly suppressed in an *hlj1Δ* strain (Taxis *et al.*, 2003). The functional overlap of *YDJ1* and *HLJ1* reported here might explain the weak phenotype observed in the *hlj1Δ* single mutant.

Second, I suggest that the yeast Hsp90 chaperone, Hsp82, is required to maintain the folded state of CFTR because *hsp82* mutant yeast degrade CFTR faster than isogenic wild type yeast, and because highly enriched Hsp82p prevents the aggregation of NBD1, a domain whose folding is critical and possibly rate-limiting during CFTR maturation (Qu and Thomas, 1996; Qu *et al.*, 1997; Zhang *et al.*, 1998). A significant prevention of aggregation was achieved at an Hsp82:NBD1 molar ratio of 5:1, an amount that is not unreasonable given the high concentration (1-3%) of cellular Hsp90 (Buchner, 1996). The more rapid degradation of CFTR in Hsp90 mutant yeast is not due to the well-characterized impact of the Hsp90 complex on cellular signaling pathways because no effect on CFTR degradation was observed in yeast mutated for Hsp90 co-chaperones either individually (Sti1p, Sba1p, Sse1p) or in combination (Sti1p/Sse1p). I therefore conclude that CFTR is one of several cellular proteins that require Hsp90 for efficient folding, a list that includes p53, Src and steroid hormone receptors (Richter and Buchner, 2001). In contrast, it is important to note that Hsp90 facilitates the ERAD of Apolipoprotein B (Gusarova *et al.*, 2001) and an insulin receptor mutant (Imamura *et al.*, 1998). These data

indicate that some substrates utilize Hsp90 for protection, whereas other substrates engage Hsp90 *en route* to degradation. In some cases, Hsp90 is involved in both events, acting first to promote folding, and then if folding cannot proceed, targeting the substrate to the proteasome (Schneider *et al.*, 1996). A role for Hsp90 in degradation is also supported by connections between Hsp90 and the ubiquitin-proteasome machinery: For example, Hsp90 binds to the 19S cap of the yeast proteasome (Verma *et al.*, 2000), and in mammals Hsp90 function is linked to the E3 ubiquitin ligase CHIP (Connell *et al.*, 2001).

Third, I found that Hsp90 co-chaperones do not impact CFTR biogenesis in yeast. To our knowledge, this is the first investigation of the relative contributions of Hsp90 versus Hsp90 complex members in membrane protein biogenesis. In contrast, a recent study demonstrated that deletion of individual Hsp90 co-chaperones had differential effects on the activity of the yeast MAP kinase, Ste11p (Lee *et al.*, 2004b), suggesting that interactions between client proteins and Hsp90/Hsp90 co-chaperones are likely to be complex. In addition, McClellan and colleagues have identified distinct chaperone-mediated folding and degradation pathways for the von Hippel-lindau tumor suppressor protein (McClellan *et al.*, 2005). In the best-characterized example, an Hsp90 folding pathway has been proposed based on in-depth studies of the progesterone and estrogen receptor folding pathways. Two distinct Hsp90 complexes are evident in this pathway: an early complex containing Hop (Sti1p), Hsp40 and Hsp70, and a mature complex containing p23 (Sba1p) and cyclophilins. The transition from the early to the late complex involves conformational changes in Hsp90 upon ATP binding and hydrolysis, and upon p23 binding (Smith, 1998; Pratt and Toft, 2003)(section 1.3.2). It is unknown, however, whether other Hsp90 sub-complexes exist and how additional Hsp90 co-chaperones impact this

pathway. I therefore cannot rule out the possibility that a novel Hsp90 sub-complex might be important for CFTR biogenesis in yeast.

I also cannot rule-out the possibility that Hsp90 co-chaperones might be required for CFTR biogenesis in mammals. Formally, the ability of GA to induce more rapid degradation of CFTR in mammalian cells (Loo *et al.*, 1998) might have occurred through direct inhibition of Hsp90 function or through an effect on Hsp90 complex maturation. In fact, it was noted in the published study that p23 associates with CFTR. Until individual Hsp90 co-chaperones can be disabled in mammalian cells, this issue cannot be resolved.

Fourth, my data further define the unique chaperone requirements for the degradation of soluble and integral membrane proteins in the ER. Ssa1p impacts the ERAD of integral membrane substrates (Hill and Cooper, 2000; Zhang *et al.*, 2001) and the Ydj1p/Hlj1p pair (this study) acts similarly. Precisely how these chaperones facilitate membrane protein turn-over is not completely clear. In mammals the Hsp70-Hsp40 complex is directly linked to the ubiquitin-proteasome degradation machinery through its association with CHIP (Meacham *et al.*, 2001). An Hsp70 co-chaperone, BAG-1, might augment Hsp70-catalyzed degradation by transferring substrates from Hsp70 to the proteasome; BAG-1 is an Hsp70 nucleotide exchange factor that promotes substrate release, contains a ubiquitin-like element, and binds to the proteasome (Hohfeld, 1998). Therefore, the effect of Bag-3 over-expression on CFTR degradation in yeast (Chapter 3 and see below) might have resulted from its impact as a dominant negative. In yeast the cytoplasmic Hsp70-Hsp40 chaperone complex (Ssa1p-Ydj1p/Hlj1p) might similarly link the

selection of integral membrane ERAD substrates to one or more of the E3 ligases that play a role in ERAD (Bays *et al.*, 2001; Deak and Wolf, 2001; Swanson *et al.*, 2001).

Fifth, expression of the mammalian co-chaperones FKBP8 or Bag-3 attenuates the degradation, but apparently not the trafficking of CFTR in yeast. FKBP8 and Bag-3 may interact directly with CFTR, or they may indirectly affect CFTR folding by modulating the activities of Hsp90 and Hsp70. There is evidence to support both a direct or indirect mechanism of action, and these are not mutually exclusive. For example, the immunophilin FKBP52, which is in the same subfamily as FKBP8, can bind to non-native proteins and suppress the aggregation *in vitro* of several soluble proteins (Bose *et al.*, 1996; Pirkl and Buchner, 2001). Furthermore, the chaperone activity of FKBP52 is independent of its PPIase activity because the drug rapamycin does not disrupt chaperone activity (Bose *et al.*, 1996). The regions of FKBP52 that bind substrate and Hsp90 are distinct (Pirkl and Buchner, 2001). All three immunophilins display different degrees of chaperone activity and appear to compete for binding to Hsp90, which is based on affinities between the immunophilin's TPR domain and the EEVD at Hsp90's COOH-terminus (Pirkl and Buchner, 2001). In fact, in mammalian cells all three of the large immunophilins—FKBP52, FKBP51, or cylophilin 40—can form a ternary complex with Hsp90 and a client substrate depending on the identity of the protein (Nair *et al.*, 1997; Barent *et al.*, 1998). These data suggest that immunophilins have the capability to bind directly to unfolded substrates and deliver them to Hsp90 for folding. However, FKBP8 might indirectly act on the CFTR degradation pathway because it might bind with greater affinity to Hsp90 than the endogenous large immunophilins in yeast, Cpr6 and Cpr7. To test this hypothesis, co-immunoprecipitation experiments could be performed to determine if FKBP8 binds to CFTR

and/or Hsp90, and the effect of deleting *CPR6* and/or *CPR7* on CFTR degradation in yeast could be examined. In addition, *in vitro* NBD1 aggregation assays in the absence or presence of purified FKBP8 could be performed to determine if FKBP8 has endogenous chaperone activity.

As stated above, Bag-3 suppresses Bad- and Bax-mediated apoptosis, but recently Bag-3 has also been implicated in modulating chaperone function and the cellular stress response. The overexpression of Bag-3 in human breast cancer cells partially protects Hsp70/Hsp90 client proteins (i.e., Akt, Raf-1, Cdk4 and EGFR) from GA-mediated degradation (Doong *et al.*, 2000). Deletion of the Bag domain of Bag-3 abrogates protection, suggesting that binding to Hsc/Hsp70 is required to mediate the effect. Interestingly, polyubiquitinated forms of the client proteins accumulate upon overexpression of Bag-3 similar to when cells are incubated with proteasome inhibitors, suggesting that Bag-3 inhibits Hsc/Hsp70 at a step after client-protein ubiquitination (Doong *et al.*, 2000). Furthermore, Bag-3 localizes to the ER in mammalian cells that are stressed due to high temperature or to an increase in heavy metal concentrations (Pagliuca *et al.*, 2003). Expression of Bag-3 in yeast may disrupt delivery of polyubiquitnated CFTR to the proteasome by stimulating substrate-release from Hsp70, thus slowing the rate of degradation. This seems unlikely given that no high molecular weight CFTR species (i.e. ubiquitinated material) could be observed in immunoblots of resolved proteins from cycloheximide chase samples (data not shown). Nevertheless, it would be interesting to determine if Bag-3 localizes to the ER in yeast cells and if a greater amount of poly-ubiquitnated CFTR can be co-immunoprecipitated with anti-ubiquitin antibodies from Bag-3-expressing cells compared to control cells.

Finally, neither FKBP8 nor Bag-3 could enhance the export of CFTR from the ER in yeast; however, there are several additional CFTR-interacting proteins in mammalian cells that are known to regulate its trafficking, such as the SNAREs. Thus, one reason for the ER export defect in yeast could be the lack of a requisite CFTR-modulating SNARE that also facilitates membrane fusion between the donor ER vesicle and the target Golgi membrane. Specifically, the SNARE proteins syntaxin-8 and syntaxin-1A have been shown to regulate the activity and trafficking of CFTR in mammalian cells (Naren *et al.*, 1997; Cormet-Boyaka *et al.*, 2002) (Peters *et al.*, 1999; Bilan *et al.*, 2004). To address this hypothesis, I have tried with little success to express the human syntaxin 1A in yeast to determine its effect on CFTR trafficking (data not shown).

Besides the SNAREs, other proteins might be lacking in yeast but that are required for the ER-to-Golgi trafficking of CFTR. For example, the 14-3-3 family of are acidic proteins are ~ 30 kDa and are expressed ubiquitiously throughout the eukaryotic kingdom (Aitken *et al.*, 1992). The 14-3-3β isoform is known to bind to di-basic retrieval sequences in the COOH-terminal tails of membrane proteins and block the binding of beta-COP, thus disrupting Golgi-to-ER retrieval and allowing the release of membrane proteins from the ER (O'Kelly *et al.*, 2002). Notably, CFTR is known to harbor multiple retrieval signals through its sequence (Chang *et al.*, 1999). The interaction between the 14-3-3β protein and its substrate depends on the phosphorylation state at the COOH or NH_2-terminus of the protein (O'Kelly *et al.*, 2002; Yuan *et al.*, 2003). Therefore, it would be interesting to express 14-3-3β in yeast cells to determine its effect on CFTR trafficking, although this experiment might be difficult to interpret if phosphorylation is

absent. PACS proteins must also be phosphorylated at the COOH-terminus to interact with membrane protein substrates, but instead of enhancing trafficking out of the ER (PACS-2) or Golgi (PACS-1) they keep the protein localized to that compartment (Crump *et al.*, 2001; Crump *et al.*, 2003; Kottgen *et al.*, 2005). Yeast lack PACS homologs, and so expressing these factors in CFTR-containing yeast might also be informative if the phosphorylation event can take place in yeast.

The Balch laboratory recently identified five co-chaperones (FKBP8, Bag-3, HOP, p23, Cyclophilin B) that reduce CFTR degradation and enhance trafficking of the protein when over-expressed in mammalian cells (W. Balch, personal communication). I tested the effects of expressing FKBP8 and Bag-3 on CFTR biogenesis in yeast (Chapter 3), but the effects of the remaining three mammalian proteins on CFTR degradation/maturation in yeast remain to be elucidated. I am especially interested in the effect of over-expression of p23 and HOP because I observed no effect on the degradation of CFTR in yeast disrupted for Sba1p (p23) or Sti1p (HOP) function (Figure 25).

APPENDIX

Analysis of CFTR-ΔS489 Mutant

Introduction

Yeast contain 31 ABC transporters (Bauer *et al.*, 1999), including several that are structurally similar to CFTR. One such transporter is encoded by the gene *YOR1*. Yor1p is a plasma membrane ABC transporter in yeast that confers oligomycin resistance. Deletion of phenylalanine at position 670 (ΔF670), analogous to the disease-causing ΔF508 mutation in CFTR, causes Yor1p to be retained in the ER and display a degradation half-life similar to CFTR (Katzmann *et al.*, 1999). Interestingly, the spacing between functional motifs (Walker A and LSGGQ motif) in the NBD1 of Yor1p is shorter by one amino acid compared to CFTR. Katzmann *et al.* inserted an alanine at position 652 to change this spacing difference and observed that Yor1p containing the additional amino acid was now trapped in the ER and also degraded rapidly (Katzmann *et al.*, 1999). I propose that the deletion of the "additional" amino acid will facilitate CFTR trafficking to the plasma membrane in yeast.

To test this hypothesis, I have deleted serine 489 in CFTR (CFTR-ΔS489) to match the spacing of the amino acids found in Yor1p, which as indicated above, trafficks to the plasma membrane. I have found that the CFTR-ΔS489 mutant protein has the same stability as wildtype CFTR and the majority of CFTR-ΔS489 is retained in the ER.

Materials and Methods

Yeast Strains and Growth Conditions

Yeast strains RSY620 (*Mat* ***a****, ade2-1, trp1-1, leu2-3,112, ura3-1, his3-11,15, PEP4::TRP1*) or W3031b (*Mat* α*, ade2, his3, leu2, ura3, trp1, can1-100*) were grown at 26°C unless indicated otherwise and standard methods for growth, preparation of media, and transformation of yeast cultures were used (Adams, 1997).

Construction of CFTR Mutant

Serine 489 of CFTR was deleted (CFTR-ΔS489) using the Strategene Quickchange XL mutagenesis kit (Strategene,) with plasmid PSM1152 (Zhang *et al.*, 2002b) and the primers, (forward- GCACAGTGGAAGAATTTTCTGTTCTCAGTTTTCCTGG, Reverse- CCAGGAAAACTGAGAACAGAAAATTCTTCCACTGTGC) according to the manufacturers instructions. The CFTR DNA was sequenced to verify the deletion of S489.

Cycloheximide Chase Assay and Subcellular Fractionation

Yeast strains expressing HA-CFTR or HA-CFTR-ΔS489 were grown to mid-logarithmic phase at 26°C in synthetic complete medium lacking uracil, but supplemented with glucose to a final concentration of 2% (SC –ura) and a cycloheximide chase was performed as described in Materials and Methods (section 2.2). Membranes from an 100 ml culture of logarithmically

growing yeast cells expressing HA-CFTR or HA-CFTR-ΔS489 were resolved on a 30-70% sucrose gradient as described in the Materials and Methods (section 3.2.2). A total of 700 µl fractions were collected starting at the top of the gradient, total protein was TCA-precipitated and resolved on 6% or 10% gels. Immunoblots were probed for CFTR, Sec61p and Gas1p (see Materials and Methods, section 3.2.2).

EndoH Digestion

Fractions from the CFTR-ΔS489 sucrose gradient were treated with endogylcosidase H (EndoH) to determine if the mutant protein trafficked to the Golgi compartment. A total of 15 µl of TCA-precipitated protein from sucrose gradient fraction 7 (ER membranes), or fraction 11 (Golgi/plasma membrane) were mixed with 50 µl of 0.1 M sodium citrate pH=5, 3 µl of PMSF (100mM), 22 µl of ddH_2O, and 2 µl of water or EndoH (0.005 U/µl). Samples were incubated for 24 hr at 37°C. Next, total protein was TCA-percipitated, resuspended in 15 µl of sample buffer, resolved on 6% polyacrylamide gels (mini, or large), and transferred to nitrocellulose. Blots were probed with anti-HA antibody to determine the size of CFTR-ΔS489.

Results

First, I performed cycloheximide chase experiments to determine the stability of CFTR-ΔS489 comapred to wildtype. There was no significant difference in the degradation rate of the mutant compared to wildtype CFTR (Figure 42). This result suggests that the conformation of the

CFTR-ΔS489 protein is not drastically less-stable compared to wildtype. Second, I performed subcellular fractionation on sucrose gradients to determine if the CFTR-ΔS489 protein exits the ER and trafficks to the plasma membrane. There was no significant change in the distribution of CFTR-ΔS489 compared to wildtype CFTR in the *pep4Δ* strain (Figure 43 A & B). Results were similar for both proteins in the wildtype yeast strain W3031b (Data not shown). There appeared to be a small population of the mutant protein in fraction 11 that migrated more slowly compared to wildtype (Figure 44 A) and was in a denser "Golgi/plasma membrane" fraction. This could represent mutant CFTR that has trafficked to an post-ER compartment, or simply could be ER membrane contamination of Golgi/plasma membrane fractions. I incubated mutant CFTR protein from ER-membranes (fraction 7), or Golgi/plasma membranes (fraction 11) with endoglycosidase H (EndoH) to determine if the apparent shift in the mutant CFTR was due to modifications of its two NH_2-linked glycans. I could detect no shift in the migration of the mutant protein, from either fraction 11, or fraction 7 (Figure 44 B, and Data not shown). EndoH removes NH_2-linked glycans at their Asn attachment, therefore there should be a shift in the wildtype and mutant CFTR that resides in the earlier ER fractions, representing the conversion from core-glycosylated to non-glycosylated protein. There are several possibilities why I did not detect a shift in mobility, which are addressed in the following discussion (see Appendex Discussion). It appears that the change in mobility for the mutant CFTR is not due to glycan processing in the Golgi. Together, these results suggest that CFTR-ΔS489 is not more stable than wildtype, and the majority of the mutant protein remains in the ER.

Discussion

The Riordan laboratory has demonstrated that CFTR is indeed glycosylated and a shift in mobility can be detected, that corresponds to the removal of two NH_2-linked glycans (Kiser *et al.*, 2001). However, no shift in either the ER form of the wildtype, or the mutant could be detected on EndoH treatment. Although, the EndoH enzyme is active because a shift in mobility of the gylcosylated protein apolipoprotein B (kind gift from Stacy Waksmonski) could be detected (Data not shown). These results suggested that the linkages between the glycans and CFTR were not accessible for cleavage, or that CFTR is not gylcosylated in yeast as it is in mammalian cells.

Immunofluorescence was employed to try to resolve these ambiguities. Wildtype CFTR appeared as perinuclear punctuate dots (2-3) that co-localized with the ER marker BiP. CFTR-ΔS489 also gave a similar staining pattern, except there were a couple extra punctuate structures (4-5) compared to wildtype, and a small portion of the protein did not appear to colocalize with BiP (Data not shown). My current hypothesis is that a small population of CFTR-ΔS489 may localize to a denser sub-domain of the ER where BiP is excluded. ER membranes (5-7), or plasma membrane (13-14) could be subjected to immuno-electron microscopy to definitely prove if a subpopulation of the mutant CFTR resides at the plasma membrane. This technique was employed previously by the Brodsky lab to confirm that wildtype CFTR was not present at the plasma membrane in yeast (Sullivan *et al.*, 2002). A second method to determine if a fraction of CFTR-ΔS489 exits the ER would be to monitor the co-localization of GFP-CFTR-ΔS489 (GFP attached to the NH_2-terminus of CFTR does not disrupt folding (Moyer *et al.*, 2002)) with the

vital dye FM464, which stains the plasma membrane and endosomal membranes. Nevertheless, the majority of the mutant CFTR appears to be retained in the ER similar to wildtype, suggesting there are fundamental differences in the folding of YOR1p and CFTR.

Figure 42: The Degradation of CFTR and CFTR-ΔS489 are Similar in Yeast.

Cultures of *pep4Δ* yeast expressing wildtype CFTR, or CFTR-ΔS489 were grown to mid-logarthimic phase and subject to cycloheximide chase analysis as described in Materials and Methods (Appendex A). The degradation of wildtype CFTR (closed black circles) or CFTR-ΔS489 (open pink circles) are plotted as the relative amount of CFTR remaining versus time. The amount of CFTR at time zero was set to 1.0. Data represent the means of 3 independent experiments +/- SEM.

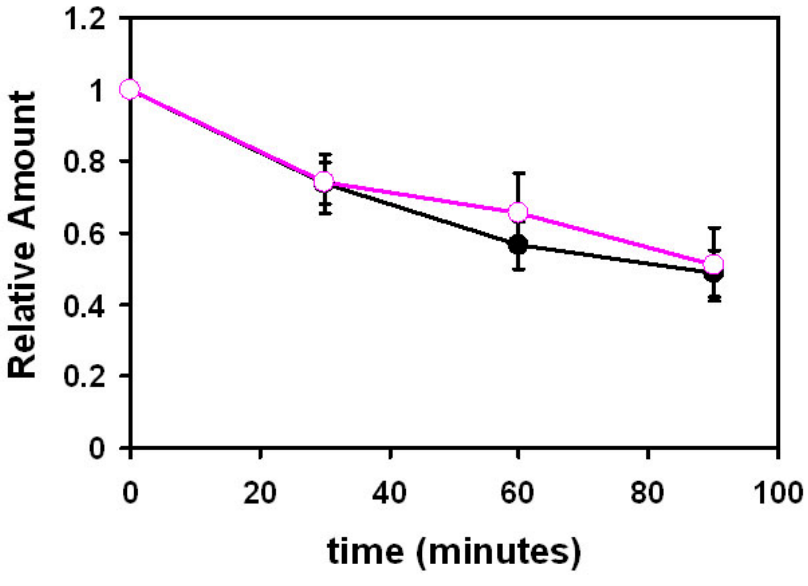


Figure 42: The Degradation of CFTR and CFTR-ΔS489 are Similar in Yeast

Figure 43: Subcellular Fractionation of Yeast Expressing wildtype CFTR or CFTR-ΔS489.

Extracts from CFTR, or CFTR-ΔS489 expressing cells were subjected to sucrose gradient centrifugation. The gradient was fractionated and the migration of CFTR, Sec61p and Gas1p were determined by immunoblotting analysis (see Materials and Methods, section 3.2). Fraction 1 represents the top of the gradient: m = mature GPI form of Gas1p that resides in the Golgi/plasma membrane (~125 kDa); i = immature form that resides in the ER (~105 kDa).

A) Top: representative immunoblots for CFTR (blue diamond), Sec61p (pink square) and Gas1p (orange triangle). Bottom: Quantification of protein bands in each lane graphed as the fraction of total protein in all lanes. X axis = gradient fraction #1, Y = fraction of total protein.

B) Top: representative immunoblots for CFTR-ΔS489 (blue diamond), Sec61p (pink square) and Gas1p (orange triangle). Bottom: Quantification of protein bands in each lane graphed as the fraction of total protein in all lanes. X axis = gradient fraction #1, Y = fraction of total protein.

Bracket = Position of the plasma membrane marker Pma1p in the gradient, which corresponds to 58-65% sucrose (w/v) or fractions 12-14.

Blacks lines on immunoblots indicate fractions were resolved on two different gels.

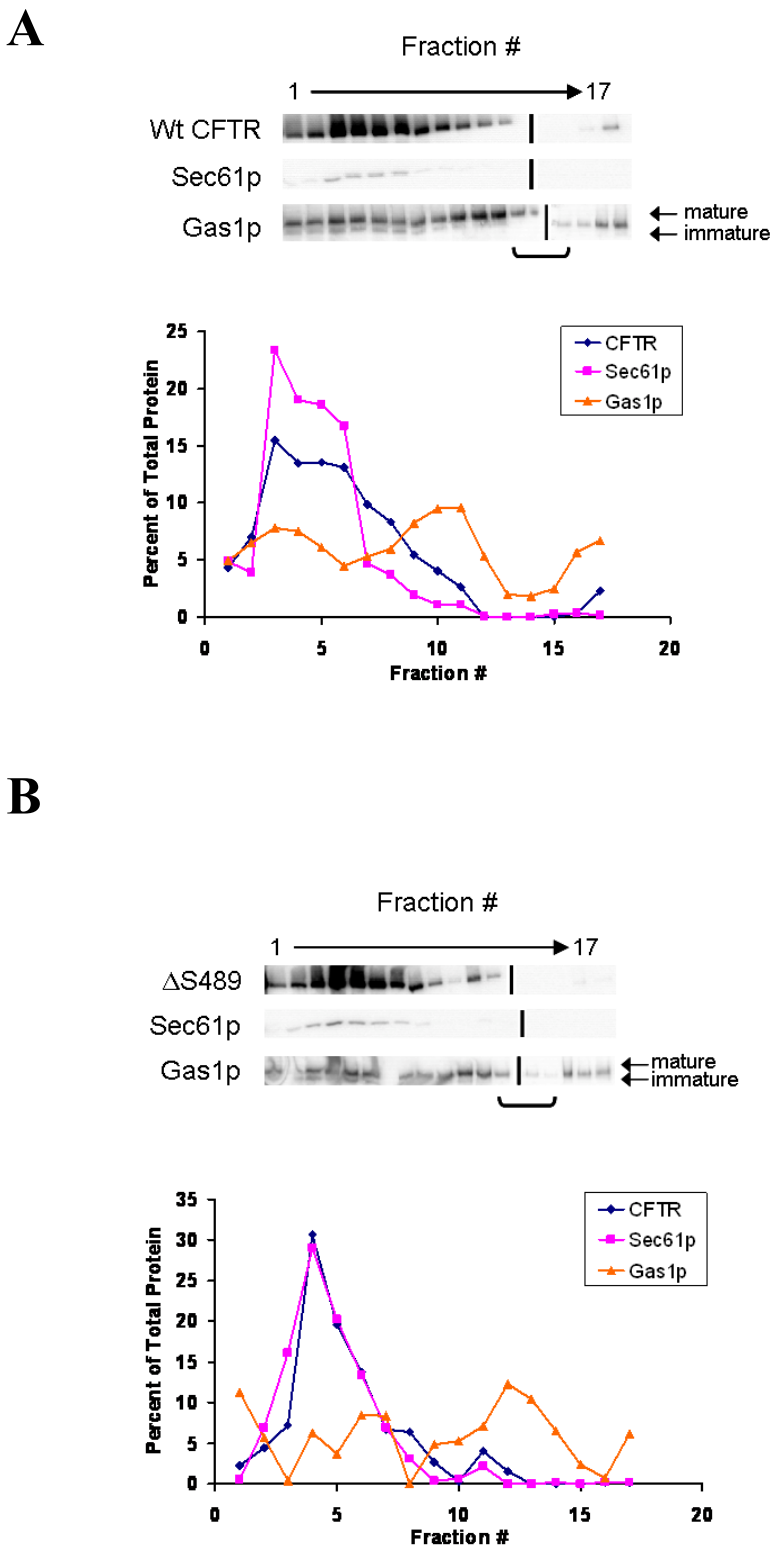


Figure 43: Subcellular Fractionation of Yeast Expressing wildtype or CFTR-ΔS489

Figure 44: A Small Population of CFTR-ΔS489 Migrates at a Higher Molecular Weight Compared to Wildtype.

A) Membrane fractions from CFTR, or CFTR-ΔS489 expressing cells were resolved on 6% polyacrylamide gels, transferred to nitrocellulose and probed with anti-HA antibody to detect CFTR. Note, * = the higher migrating CFTR band seen in mutant but not wildtype.

B) Protein from fraction 11 of CFTR-ΔS489 was incubated in the absence (-), or presence (+) of EndoH. Protein was resolved on 6% polyacrylamide gel, transferred to nitrocellulose, and probed with anti-HA antibody (see Materials and Methods, Appendex A). Note, No change in the migration of CFTR-S489Δ in presence of EndoH. Similar results were obtained for wildtype CFTR fractions 7 and 11 (data not shown).

Bracket = Position of the plasma membrane marker Pma1p in the gradient, which corresponds to 58-65% sucrose (w/v) or fractions 12-14.

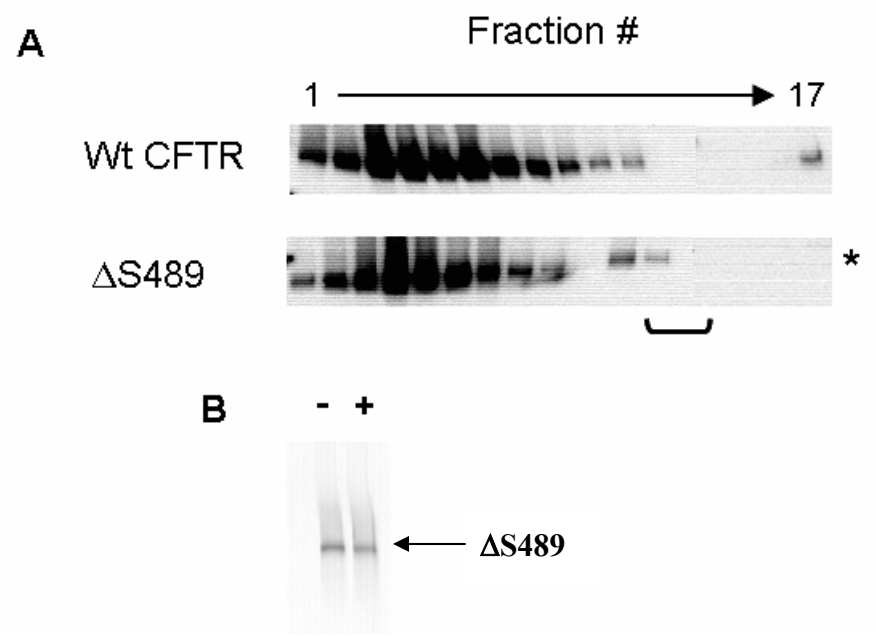


Figure 44: A Small Population of CFTR-ΔS489 Migrates at a Higher Molecular Weight Compared to Wildtype

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