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Sorting Signals, Domain Conformation and Interdomain Interactions in CFTR Misprocessing and Rescue

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SORTING SIGNALS, DOMAIN CONFORMATION AND INTERDOMAIN INTERACTIONS IN CFTR MISPROCESSING AND RESCUE

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DEDICATION

I would like to dedicate my dissertation to my dear parents, parents-in-law, brother, brother-in-law and my dear husband, Vipul.
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A loving thank you to my husband, Vipul for all the concern, support and patience during my graduate school and helping me in my dissertation preparation. All is much appreciated.
Cystic fibrosis (CF) is among the most common lethal inherited diseases in the US. The deletion of phenylalanine at residue 508 (∆F508) within cystic fibrosis transmembrane conductance regulator (CFTR) is the most common CF causing mutation. ∆F508 mutant is unable to exit the endoplasmic reticulum (ER). The precise mechanism of the defective ER-to-Golgi export of ∆F508 CFTR is not known. A di-acidic ER exit code (DAD) has been identified within the first nucleotide binding domain (NBD1) of CFTR. Disruption of this motif leads to the defective coupling of CFTR to the COPII machinery and the subsequent impaired export of CFTR from the ER. We performed a systematic analysis of the sorting signals, domain conformation and inter-domain interactions of CFTR in presence of ∆F508 mutation and during its rescue by low temperature or second site mutation (R555K). We found that rescue of ∆F508 CFTR depends on the DAD motif within the NBD1. Disruption of DAD (DAA) reduces the Sec24 association of ∆F508 CFTR whereas rescue mutation R555K increases it. Using in situ limited proteolysis we identified conformational defects in N-terminal region and NBD1 in ∆F508 CFTR. ∆F508 rescue is accompanied by global conformational reversion. Further domain-domain interactions within CFTR play an important role in both ∆F508 CFTR misprocessing and rescue. Our data firmly establish that while DAA is an exit code mutant ∆F508 CFTR has widespread conformational defect which compromises its export and stability. Restoration of its global conformation leads to restoration of its export as well as function.
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AFT</td>
<td>Arginine framed tripeptide</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane regulator</td>
</tr>
<tr>
<td>COP II</td>
<td>Coatomer complex II</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
</tr>
<tr>
<td>LDL-R</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>MSD</td>
<td>Membrane spanning domain</td>
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<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
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<tr>
<td>R (domain)</td>
<td>Regulator (domain)</td>
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<tr>
<td>Ub</td>
<td>Ubiquitin</td>
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<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus glycoprotein</td>
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INTRODUCTION

Cystic fibrosis (CF) is one of the most prevalent lethal, autosomal-recessive genetic diseases (Tsui, 1992). The root cause of CF is the defective cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan, 1993). CFTR is an ATP-binding cassette (ABC) transporter (Collins, 1992; Kartner et al., 1991). It regulates the chloride ion conductance of many tissues including lungs, intestines, pancreas, cardiac muscle as well as macrophages (Amaral, 2005; Riordan, 1993). It consists of two homologous modules linked together by a regulatory (R) domain that regulates channel gating (Ostedgaard et al., 1997). Each module is formed by a membrane-spanning domain (MSD) followed by a nucleotide binding domain (NBD) (Riordan, 1993; Riordan et al., 1989). CFTR enters the endoplasmic reticulum (ER) co-translationally (Kleizen et al., 2005). After synthesis, the protein leaves the ER, moves to the Golgi complex and finally to the plasma membrane (Farinha et al., 2002). This transport from the ER is mediated by coat protein complex II (COP II) vesicle budding proteins (Antonny and Schekman, 2001; Kuehn et al., 1998; Tang et al., 2005).

It is suggested that certain sequence motifs in different ER exiting proteins help them export from the ER (Nishimura and Balch, 1997). A conserved di-acidic motif is present in most ER exiting proteins such as vesicular stomatitis virus glycoprotein (VSV-G), epidermal growth factor receptor (EGFR), low density lipoprotein receptor (LDLR), etc. It has been demonstrated that disruption of the di-acidic motif, DAD, to DAA inhibits export of WT CFTR (Wang et al., 2004). This di-acidic code (DAD) is present within the first nucleotide binding domain (NBD1) of CFTR. It has been shown that disruption of this di-acidic code prevents CFTR from efficiently exiting ER (Wang et al.,
Misprocessing of CFTR protein results in CF (Amaral, 2005; Riordan, 1999). The most common CF mutation is a deletion of phenylalanine at position 508 of CFTR (ΔF508 CFTR) (Collins, 1992). ΔF508 CFTR has impaired conformational maturation during its biosynthesis, and is unable to exit the ER (Cui et al., 2007; Du et al., 2005; Lukacs et al., 1994). This results in a deficiency of chloride ion channel at the plasma membrane. Nevertheless, there is a residual export of ΔF508 CFTR to the plasma membrane which is also demonstrated to be functional. However, it is not sufficient to alleviate CF symptoms (Amaral, 2005). At low temperature it is well established that ΔF508 CFTR can escape from the ER to the plasma membrane and remains functional if cells are retained at reduced temperature (Bear et al., 1992; Denning et al., 1992a; Drumm et al., 1991).

A detailed biochemical analysis of this di-acidic motif and its mutation will present important insights into CFTR trafficking, its availability to export machinery in specific conformational defects of ΔF508 CFTR and the possible molecular mechanism underlying the rescue of ΔF508 CFTR. In this study, we hypothesize that defective ER-to-Golgi trafficking of ΔF508 CFTR is due to its inability to be packaged in COPII vesicles because it cannot present its di-acidic motif, DAD, to Sec24. In these studies we analyzed the relationship between CFTR folding and defective transport of ΔF508 CFTR from ER to plasma membrane. We also analyzed the role of the di-acidic motif, DAD, in the export of ΔF508 CFTR at low temperature.

It has also been informed that ΔF508 CFTR localizes to ER-Golgi intermediate compartment (ERGIC) (Gilbert et al., 1998). However, not much is known about the role of the di-acidic motif in aforementioned export of ΔF508 CFTR. It is widely believed that
ΔF508 CFTR conformation has exposed arginine framed tripeptide (AFT) (retention signals) (Chang et al., 1999; Hegedus et al., 2006; Sharma et al., 2004). Once it reaches the ERGIC its exposed retention signals are recognized, followed by its retrieval to the ER from the ERGIC. Mutation of these retention signals (R29 and R555 to lysine) in ΔF508 CFTR rescues the protein to the plasma membrane at 37ºC (Chang et al., 1999; Hegedus et al., 2006). However even WT CFTR has been demonstrated to localize in the ERGIC (Bannykh et al., 2000). At physiological temperature ΔF508 CFTR has been detected at the plasma membrane, albeit in very small amounts as compared to WT CFTR. Considering these facts we postulate that ΔF508 CFTR leaves the ER, but rather than being retrieved back to the ER, it reaches the plasma membrane. It has also been set forth that the deletion of phenylalanine at residue 508 changes CFTR protein folding in different domains (Cui et al., 2007; Du et al., 2005). This might result in disruption of inter-domain interactions of the protein.

Both export and retention signals have been identified within CFTR (Chang et al., 1999; Hegedus et al., 2006; Wang et al., 2004). We performed a systematic analysis of the potential ER-to-Golgi sorting signals within CFTR, and specifically examined the functional relationship between different sorting signals in the context of ΔF508 CFTR. Our results indicate that the di-acidic motif is the only functional ER export signal within ΔF508 CFTR. Deletion of phenylalanine at residue 508 leads to the impaired presentation of this motif to the COPII machinery. Reduced temperature or additional secondary mutation (R555K) corrects this mis-presentation of the exit code. We further studied the underlying mechanism of this mis-presentation by analyzing the conformation of immature DAA CFTR as compared to ΔF508 CFTR. Therefore it shows that in ΔF508
CFTR the inefficient interaction of the di-acidic motif DAD with the COP II machinery is attributed to the defective conformation of ∆F508 CFTR. Given the global nature of both misfolding and rescue, we observed the roles of NBD2 in CFTR processing, misprocessing and rescue, and found that while NBD2 is not essential for CFTR processing, it is important in the residual export and rescue of ∆F508 CFTR. To study the N-terminal processing of ∆F508 CFTR we analyzed the conformation of CFTR with deleted N-terminal region. Our data emphasize a crucial role for interdomain interactions in the mis-processing and rescue of ∆F508 CFTR. Taken together, correcting the interdomain interaction lies at the heart of ∆F508 CFTR trafficking correction, which may lead to the potential rescue of clinical phenotypes in CF patients.
Cystic Fibrosis

CF is the most prevalent autosomal recessive genetic disorder (Tsui, 1992) common among the Caucasian population. CF is a clinically complex disease mainly disturbing the epithelial ion transport system resulting in "multi-system" disease i.e., affecting several organ systems. Morbidity and mortality are mostly because of chronic bacterial infection and inflammation in the lung.

This disease is characterized by a large variety of clinical symptoms such as, bacterial colonization in the airway, especially, by *Pseudomonas aeruginosa*, elevated sweat electrolytes, pancreatic enzyme insufficiency and poor growth. The average normal life expectancy in CF patients is now more than 30 years of age, but with best treatment, patients have a greater than 80% chance of living into their late forties.

CF is caused by mutations in the ‘‘CF gene.’’ The product of this gene is a transmembrane chloride ion channel referred to as CFTR protein. There are nearly 1,500 different disease-causing CFTR mutations recognized. Nevertheless ∆F508 mutation is by far the most common. It is seen in at least one allele in 90% of CF patients and almost in 70% of all CF causing alleles (Collins, 1992). This deletion prevents normal transport of the CFTR protein from endoplasmic reticulum (ER) to the plasma membrane.

Other mutations of the CFTR gene such as G542X, G551D, N1303K, or W1282X are responsible for around 1.2% - 2.4% of CF (Human genome project database). These mutations lead to varying extents of loss of function. These CFTR mutations are functionally classified (Welsh and Smith, 1993; Zielenski and Tsui, 1995) as follows: Class I include mutations which abrogate protein production (Amaral, 2005; Zielenski
and Tsui, 1995). Class II includes mutations causing defective protein processing and trafficking. Class III and Class IV mutants involve defective protein regulation and defective Cl⁻ ion conductance, respectively. Lastly Class V mutants produce reduced amounts of protein, but with normal function (Amaral, 2005; Zielenski and Tsui, 1995). Although it has been shown that many symptoms of CF are dependent on other genetic and environmental influences, mutations in the CFTR gene are primary causal factors for CF.

**CFTR protein**

CFTR is an integral membrane protein and functions as a Chloride (Cl⁻) channel (Collins, 1992; Kartner *et al*., 1991) and plays an important role in secretion and reabsorption of ions in epithelial lining of the lungs, liver, pancreas, intestines, reproductive tract, and skin (Amaral, 2005; Riordan, 1993). It is a member of the ATP-binding cassette (ABC) transporter super family. In 1989, Riordan *et al*., isolated overlapping complementary DNA clones from epithelial cell libraries and found that CFTR gene is located on chromosome number 7. The CFTR protein has 1480 residues, with two MSDs each consisting of six α-helical loops, two cytosolic NBDs and an R domain (Riordan, 1993; Riordan *et al*., 1989) (Fig 1). The MSDs form the channel through which Cl⁻ ions are transported. The three cytoplasmic domains regulate the ion channel. It has been shown that folding of R domain influences the activity of NBDs (Ostedgaard *et al*., 1997). The two NBDs bind to ATP and hydrolyze it to ADP thereby providing energy, inducing conformational change and leading to a closer interaction with each other (Vergani *et al*., 2005a; Vergani *et al*., 2005b).
∆F508 CFTR

∆F508 belongs to the NBD1 domain of the CFTR protein (Cheng et al., 1990; Riordan et al., 1989). ∆F508 mutation prevents CFTR from attaining its native global conformation (Qu and Thomas, 1996; Zhang et al., 1998) which leads to ER retention of the protein (Sheppard and Welsh, 1999). The retained mis-folded protein undergoes ER-associated degradation (ERAD) through the ubiquitin-proteasome pathway (Denning et al., 1992a; Denning et al., 1992b; Riordan, 1999) thereby preventing ∆F508 CFTR from reaching the apical membrane of epithelial cells. This mutation is thus included in Class II (Amaral, 2005; Cheng et al., 1990). It is also included in Class III since it causes major defects in channel regulation that greatly hinder channel opening (Wang et al., 2000).

Figure 1. Features of CFTR representing the membrane spanning domains MSD1 and MSD2, the nucleotide binding domains NBD1 and NBD2 and the regulatory domain R. The glycosylation sites at residues N894 and N900 are shown in the MSD2.
**Effect of ΔF508 mutation on NBD1 structure of CFTR**

Deletion of F508 causes change in NBD1 domain conformation of CFTR (Qu et al., 1997b; Qu and Thomas, 1996). It was demonstrated by studying the thermodynamic stability of the NBD1 domain between WT and ΔF508 CFTR.

In 2004, a number of studies are published on the structure of WT CFTR. The low resolution crystal structure of full length CFTR was first assessed in vivo through heterologous expression in *Xenopus* oocytes and then followed by atomic force microscopy analysis (Schillers et al., 2004). Further by two-dimensional (2D) electron crystallography the structure of CFTR is solved (Rosenberg et al., 2004).

In a separate study high resolution crystal structure of NBD1 from mouse CFTR is solved (Lewis et al., 2004). Phenylalanine at residue 508 is reported to be located on the outer surface of the NBD1 domain crystal structure. Later it was presented by structural and biophysical studies on complete human NBD1 domains that ΔF508 mutation does not generate significant difference of *in vitro* stability and folding kinetics (Lewis et al., 2005). Additional second-site mutations (F494N/Q637R or F429S/F494N/Q637R) are included to facilitate purification of the NBD1 domain of ΔF508 CFTR. Considerable changes in local surface topography at the 508 site are demonstrated suggesting that ΔF508 mutation causes disruption of proper interdomain interactions at this site in CFTR.

Later in a separate study it is confirmed that the additional mutations (F494N/Q637R or F429S/F494N/Q637R) rescue trafficking as well as gating defect in ΔF508 CFTR (Pissarra et al., 2008). It is suggested that these mutations partially correct the solubilization and crystallization of NBD1 domain of ΔF508 CFTR.
Folding dynamics and kinetics of the NBD1 are affected by ΔF508 resulting in misfolding of the complete protein followed by retention and early degradation (Serohijos et al., 2008b). Critical interactions, near residue 508, among Q493/P574 and F575/F587 localized in intercellular loops S7-H6, are predicted to be important structural elements which influence the folding kinetics of CFTR protein. This is confirmed by NBD1s with rescue mutations in the S7-H6 loop which are capable of generating higher folding efficiency than the NBD1 of ΔF508 CFTR.

**Effect of ΔF508 mutation on interdomain interactions of CFTR**

Folding of CFTR is mostly co-translational, i.e. domain by domain (Kleizen et al., 2005). This is demonstrated by studying the early folding events of CFTR examining the nascent chain elongation by in vitro translation in semipermeabilized cells. By the use of protease susceptibility assay it is stated that most of the CFTR protein folds during synthesis. It is also shown that formations of individual domains are independent of the C-terminal end of the protein.

Since phenylalanine at residue 508 lies within the NBD1 of CFTR, structural details of other CFTR domains was very helpful in understanding the folding relationships of CFTR. It was shown that ΔF508 CFTR has a globally misfolded protein conformation (Zhang et al., 1998). Comparative limited proteolysis of WT and the mutant CFTR demonstrated that the cytosolic domain conformations of ΔF508 CFTR are different from that of WT CFTR both at steady state as well as during their biosynthesis. Proteolytic cleavage patterns of ΔF508 CFTR are almost identical to that of the early folding intermediates of WT CFTR. It is suggested that ΔF508 mutation generates a form
of the protein which is similar to an intermediate form of the WT CFTR formed during its biogenesis, instead of a non-native variant.

Proper CFTR folding is a consequence of complex interactions among the NBDs, MSDs and/or the R domain (Pollet et al., 2000). A series of chimeric CFTRs are constructed where the NBD domains are exchanged or deleted. These chimeras are expressed in Cos-1 or CHO cells. It is demonstrated by Western blotting and iodide efflux assays that deletion of NBD1 domain prevented transport of CFTR to the plasma membrane. It is also demonstrated that NBD2 domain deletion did not prevent the export of CFTR from the ER; however it resulted in an inactive chloride channel. Substitution or inversion of the NBD domain in CFTR molecule is also shown to impair CFTR processing.

In a separate study it is notified that ∆F508 causes disruption in NBD2 domain folding and its interaction with NBD1 (Du et al., 2005). This is shown by in situ limited proteolysis. The proteolytic susceptibility for NBD2 domain increases by 100 fold with ∆F508. It is also shown that the side chain interactions of phenylalanine at 508 are essential for NBD2 domain folding and domain-domain assembly of CFTR. Substitution of the phenylalanine with cysteine, methionine, aspartic acid, asparagine, lysine, leucine, alanine, or glycine always demonstrated a decrease in the folding efficiency. Taken together it is suggested that proper domain-domain assembly of CFTR is strictly required for ensuing a correct co- and post- translational folding of the protein.

It is revealed that the peptide backbone of phenylalanine at 508 contributes greatly to the proper folding of the NBD1 domain of CFTR (Qu et al., 1997b; Qu and Thomas, 1996; Thibodeau et al., 2005). The side chain of the phenylalanine is suggested
to interact with the other domains during CFTR maturation (Thibodeau et al., 2005). This is confirmed by demonstrating that replacing this phenylalanine with many other amino acids disrupted the folding of full length protein but not that of purified NBD1 domain. Substituents with charged and bulky residues are also shown to disrupt the folding of full length CFTR.

Disruption of MSD1 folding with the ΔF508 is also demonstrated in a separate study by proteolytic digestion assays (Cui et al., 2007). In a different study 3D structure of CFTR constructed by molecular modeling is presented showing the tertiary interaction between the NBD1 and cytoplasmic loop 4 in the C-terminal end of MSD2, mediated by Phenylalanine 508 (Serojijos et al., 2008a). ΔF508 mutation is informed to impair interaction between the transmembrane segments of CFTR protein (Chen et al., 2004).

Potential contact points in WT CFTR are mapped and mutated to cysteine; M348C(TM6) / T1142C(TM12), T351C(TM6) / T1142C(TM12), and W356C(TM6) / W1145C(TM12). Disulphide crosslinking is demonstrated in the cysteine mutants in WT CFTR background, but interestingly, with the introduction of ΔF508 mutation, crosslinking is eliminated.

Yor1p, a yeast ABC transporter is a pleiotropic drug pump. It shows homology to human CFTR, deletion mutant Yor1p-ΔF670 is equivalent to ΔF508 CFTR. Proper interdomain interactions are required for functionally active Yor1p (Pagant et al., 2008). Interactions between the two NBDs and between the NBDs and the intracellular loops of the MSDs are addressed. These interactions are demonstrated by chemically cross-linking cysteine residues of different domains in proximity. Cross linking in Yor1p-ΔF670 shows increased aggregations which are not observed in WT Yor1p. Thereby it is suggested that
the increase in aggregations in ΔF670 are due to non-specific cross linking of exposed cysteine residues which remains buried in properly folded Yor1p structure.

It is stated that cooperative post-translational folding of CFTR further helps in domain stabilization (Du and Lukacs, 2009). This is demonstrated by the use of missense mutations in MSD1, NBD1, MSD2 or NBD2 and by domain deletions in full length CFTR. Missense mutations in any of the domains caused defective folding in multiple domains of CFTR. Truncated CFTR showed that the smallest folding unit capable of exporting to the plasma membrane is MSD1-NBD1-R-MSD2. Channel gating of CFTR is dependent on interaction of the two NBDs (Zhou et al., 2006). Based on the 3D structure of CFTR, it is presented that ΔF508 disrupts interfaces between domains (He et al., 2008). Interactions between the NBDs and MSDs are shown to have different effects on channel gating.

**CFTR - nucleotide binding and channel gating**

The R-domain has multiple consensus phosphorylation sites. They are phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) with a rise in intracellular cyclic-AMP (Collins, 1992; Gadsby et al., 1998; Tabcharani et al., 1991). CFTR channel gating is strictly dependent on the phosphorylation of the R domain. The channel activity increases by a 100 fold when R domain is phosphorylated (Csanady et al., 2005). The combined activation of the NBD1, NBD2 and R domains allows Cl⁻ ion to pass through the channel (Cui et al., 2007; Du et al., 2005). CFTR also controls other ion channels like epithelial Na⁺ channel (ENaC), voltage gated channel KVLQT 1), K+channels (ROMK) and also a water channel (Greger et al., 1996; Hopf et al., 1999; Kunzelmann et al., 1997; Mall et al., 1996a; Mall et al., 1996b; Schreiber et al., 1999).
The NBDs of CFTR as in all ABC family transporters encompass Walker A and Walker B motifs. ABC family transporters including CFTR have a highly conserved sequence (LSGGQ) motif, also known as C motif or ‘signature’ motif. Additionally, CFTR has other functional features like Q-loops (glutamine residues) responsible for ATP-recognition and H- loops (histidine residues) responsible for ATP hydrolysis (Borths et al., 2002; Hung et al., 1998; Karpowich et al., 2001; Lewis et al., 2004; Locher et al., 2002).

**Effect of ΔF508 on CFTR protein folding, phosphorylation and channel gating**

The folding efficiency and intracellular turnover of WT and ΔF508 CFTR are found to be different according to pulse-chase labeling assays, (Ward and Kopito, 1994). ΔF508 changes the folding of NBD1 domain of CFTR but has very little effect on its thermodynamic stability (Qu and Thomas, 1996). Purified NBD1 are folded *in vitro* into soluble monomers which are able to interact with nucleotides. Thermodynamic stability

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**Figure 2:** Cartoon representing the effect of CFTR phosphorylation on R domain interactions with other CFTR domains. (Chappe et al., 2005). PKA – protein kinase A.
of NBD1 with ΔF508 is comparable to that of the WT protein NBD1, whereas the folding yield is reduced with ΔF508 at different temperatures. It is later demonstrated that inter-domain interactions between the two MSDs is necessary for the connection between the two halves of the protein (Ostedgaard et al., 1997). Either half of the protein is co-immunoprecipitated with the other in immunoprecipitation assays using domain specific antibodies. Lack of NBDs or R or N-terminal region did not hinder the co-immunoprecipitate however only the two MSDs are capable of associating. ΔF508 mutation affects the folding of NBD1 domain prior to formation of ATP binding sites because stabilizing the mutant protein in native state by ATP could not rescue the folding efficiency, unlike observed by treating with glycerol or low temperature or second site mutation like R553M (Qu et al., 1997a; Qu et al., 1997b). ΔF508 CFTR is also noted to cause functional defect due to slower rate of PKA-dependent phosphorylation but not defective in ATP-dependent channel gating (Prat et al., 1999; Zeltwanger et al., 1999). This is suggested because once activated with PKA, the steady state values and the ATP dose response effects are similar to the WT CFTR.

**Effect of ΔF508 on CFTR protein degradation**

In addition to proper domain folding, proper inter-domain interactions contribute to the formation of the protein acceptable by the ERAD machinery for further transport to the plasma membrane (Cui et al., 2007). Nascent polypeptides that are destined for the secretory pathway translocate through the ER membrane through a pore protein complex known as the translocon. The soluble proteins move into ER lumen, whereas membrane proteins get integrated into ER membrane. Nascent CFTR polypeptide chains are similarly targeted to the ER (Lu et al., 1998; Skach, 2000). Since these nascent proteins
are in an unfolded state, folding into their native conformation occurs within the ER. When proteins fail to fold properly they are directed to proteosomal degradation through ERAD. Role of ubiquitin (Ub)-proteosome pathway in the degradation of improperly folded CFTR is confirmed by using potent proteosome inhibitors (Ward et al., 1995). In addition, it is also demonstrated that the degradation of CFTR is also inhibited by coexpression of dominant negative Ub mutant and as well as in cells expressing a mutant Ub-activating enzyme. This indicates ER quality control mechanism prevents transport of newly synthesized CFTR polypeptides from reaching their destination until they attain their native conformation. Degradation does not occur in ER lumen, ubiquitination occurs in the cytosol as the E2 and E3 enzymes are localized in the cytosol. Proteins to be degraded are transported back to the cytosol from the ER via the same translocon complex used for import by retrograde transport and both the retained and the retrieved proteins, in a common ERAD system are ubiquinated and are subsequently degraded (Vashist et al., 2001).

**ER export of CFTR**

CFTR is a complex protein. It is core-glycosylated at residues N894 and N900 with 14 units of oligosaccharides at its Asn-X-Ser/Thr consensus sequences; represented by band B which is ~145 kDa (Amaral, 2004). The newly synthesized WT and ∆F508 CFTR remains bound to ER lectins such as calnexin and calreticulin (Pind et al., 1994). It is demonstrated that the mutant, ∆F508 CFTR, remains bound to calnexin longer than WT CFTR.
Extended association with calnexin target the polypeptide for proteosomal degradation (Dejgaard et al., 2004; Meacham et al., 2001). Since WT CFTR folds correctly it continues along the secretory pathway by exiting the ER and moving to the Golgi complex (Farinha et al., 2002). In the Golgi complex, the N-linked sugar moieties

Figure 3. CFTR maturation and trafficking from the ER to the plasma membrane via Golgi complex. (Welsh and Ostedgaard, 1998)
of CFTR are replaced and modified with complex glycosylation by Golgi mannosidase I and II and N-acetylglucosamine transferase I (band C, ~170 kDa; Figure 2). Monitoring glycosylation state of CFTR is a convenient way to follow intracellular processing of WT and mutant CFTR (Amaral, 2004; Amaral et al., 2004; Kalin et al., 1999). This transport from ER is mediated by COPII vesicles (Antonny and Schekman, 2001; Kuehn et al., 1998; Mancias and Goldberg, 2005; Tang et al., 2005; Wang et al., 2004).

During COPII vesicle formation, Sec12, a GEF protein, activates Sar1, by replacing GDP with GTP. Sar1-GTP recruits Sec23/24 cargo selection protein complex. CFTR within ER binds to the B-site of Sec24 which remains in complex with Sec23. Sec13/31-the coat assembly protein complex is then recruited, which helps in the assembly of vesicle.

**Figure 4. COP II vesicle machinery** summarizing vesicle formation and disassembly. (Mancias and Goldberg, 2005).
Hydrolysis of GTP attached to Sar1 results in the vesicle uncoating. Subsequently the vesicle membrane fuses with pre Golgi intermediate complex (Mancias and Goldberg, 2005). CFTR then moves to the Golgi complex and finally to the plasma membrane and functions as an ion channel. It is believed that specific sequence motifs in ER exiting proteins mediate cargo selection for ER export. A conserved di-acidic motif is required for exiting ER in many proteins like VSV G, EGFR, LDLR, etc. (Nishimura and Balch, 1997; Nishimura et al., 1999). A di-acidic code, DAD, has been demonstrated to be the exit code in WT CFTR as well (Wang et al., 2004).

**ΔF508 CFTR processing is temperature sensitive**

Most of the ΔF508 CFTR can not reach plasma membrane resulting in insufficiency of chloride ion channel at the plasma membrane. Physiologically a small amount of the mutant protein, ΔF508 CFTR, escapes ERAD and moves out to the plasma membrane (Kalin et al., 1999; Penque et al., 2000). The escaped ΔF508 CFTR has reduced function (Dalemans et al., 1991; Wang et al., 2000), reduced membrane stability (Lukacs et al., 1993; Sharma et al., 2001), and also deficient recruitment from the subapical membranes (Bradbury and Bridges, 1992; Bradbury et al., 1992; Wei et al., 1996).

However, chloride channel activity of the mutant CFTR is detected in *Xenopus* Oocytes (Drumm et al., 1991), Vero cells (Dalemans et al., 1991) and Sf9 insect cells (Bear et al., 1992). In 1992 Denning et al., suggested that at low temperature ΔF508 CFTR can escape from ER efficiently and move to the plasma membrane. This is shown by the appearance of mature complex-glycosylated band C of ΔF508 CFTR when 3T3
fibroblast cells expressing the mutant protein are cultured at lower temperatures. Similar results are also observed by the authors using C127 cells. The stability of ΔF508 is confirmed by pulse chase assay, where labeled ΔF508 CFTR at 37°C could not be chased into mature band, but when chased at 26°C mature form of the mutant protein is detected. The plasma membrane expression of ΔF508 CFTR is represented by the complex glycosylated band C. At lower temperature ΔF508 CFTR can not only reach the plasma membrane, but is also functionally active (Denning et al., 1992a).

![Figure 5. WT and ΔF508 CFTR export.](image)

Figure 5. WT and ΔF508 CFTR export. WT and mutant CFTR were transiently transfected into HEK cells. Export from the ER at 37°C and 30°C was checked by immunoblotting for CFTR. C – complex glycosylated band; B – core glycosylated band.

To confirm the functionality of ΔF508 CFTR at the plasma membrane the cAMP activated currents in the plasma membrane are measured. Large amounts of chloride currents are counted when C127 cells expressing ΔF508 CFTR are incubated at low 30°C. Interestingly once the temperature is brought back to 37°C the amount of chloride currents considerably decreased. Based on these results, it is suggested (Denning et al., 1992a) that functionally active ΔF508 in *Xenopus* Oocytes (Drumm et al., 1991) and Sf9 cells (Bear et al., 1992) is observed as both these cell lines are grown at lower temperatures. Whereas in case of Vero cells (Dalemans et al., 1991), it is suggested
(Denning et al., 1992a; Denning et al., 1992b) that the use of very high level expression system in the respective study might have produced functionally active ΔF508 CFTR.

**Arginine framed tripeptide motifs in CFTR**

ER resident proteins bearing –KDEL- sequence or type 1 membrane proteins exposing –KK- retrieval signals at the carboxy termini are retrieved back to the ER. Both the retained and retrieved improperly folded proteins are degraded by ERAD (Vashist et al., 2001). It has been published that RXR tripeptide sequences (Arginine framed tripeptide or AFT) are responsible for ER retention or retrieval of individual subunits of ion channels such as ATP sensitive K+ channels (Zerangue et al., 1999). A three amino acid-motif (RKR) is reported to determine intracellular trafficking of an ion channel complex. ATP-sensitive K+ channel (KATP) consists of two subunits; (Kir 6.1/2) and (SUR 1). RKR sequence on either SUR 1 or Kir6.2 is shown to inhibit surface expression by retention or retrieval of the complexes. Replacing any of the 3 basic residues with alanines is demonstrated to increase surface expression. It is proposed that proper co-assembly of the subunits masks the RKR sequences thus generating a properly functioning channel.

AFT sequence motifs are also present in CFTR (Chang et al., 1999). These sequences are supposedly hidden within the native folding of WT CFTR. It is believed that in ΔF508 CFTR these AFT sequences are exposed due to improper folding of the protein. Exposed AFT motifs are suggested to be recognized by the ER quality control machinery, thereby retrieving ΔF508 CFTR back to the ER from the ERGIC. Disruption of the AFT sequences facilitates export of ΔF508 CFTR to plasma membrane at physiological temperature and forms functional Cl- channels at the cell surface. There are
four such arginine (R) sequences in CFTR: a) at residues 29-31 in the N-terminal cytoplasmic domain, b) at residues 516-518 and 553-555 in NBD1, and c) in the R domain at residue 764-766. Combined substitution of all four arginines by lysine (4RK: R29K, R516K, R555K and R766K) or individual substitutions of R at 29 and of R at 555 residues, i.e., R29K and R555K, increases cell surface expression of ΔF508 CFTR in BHK cells (Chang et al., 1999). ΔF508 CFTR with 4RK showed extensive peripheral localization as observed by immunofluorescence analysis unlike ΔF508 CFTR alone which shows perinuclear localization. ΔF508 CFTR with 4RK also showed chloride efflux rates more than half of that of WT CFTR.

Further it is observed that simultaneous substitution of the R’s at residue 29 and 555 to K in BHK cells are sufficient to facilitate ΔF508 CFTR recovery (Hegedus et al., 2006). ΔF508/2RK CFTR is shown to localize near the cell periphery with immunofluorescence staining. Even in Cl- efflux assay ΔF508 CFTR with 2RK is functionally active. Interestingly, on limited proteolysis, the digestion patterns of the revertants showed limited WT CFTR like proteolytic pattern.

It was observed that in a particular patient with homozygous ΔF508 mutation, sweat chloride levels are surprisingly in normal range. Later studies demonstrated the existence of an additional mutation wherein arginine at 553 is mutated to glutamine in the same patient (Dork et al., 1991). Later it is shown that substitution of R553 with Q or M rescues ΔF508 CFTR phenotype in STE6-CFTR chimera in yeast (Teem et al., 1993). This mutation is also shown to partially correct the maturational and functional defects of human ΔF508 CFTR. Maturation and channel function of ΔF508 CFTR can also be rescued in the presence of glycerol (Brown et al., 1996; Sato et al., 1996). A separate
study has shown that G550E or I539T substitutions also facilitates proper ∆F508 CFTR plasma membrane localization and channel activity (DeCarvalho et al., 2002a; deCarvalho et al., 2002b). A STE6/CFTR∆F508 chimera system in yeast led to the isolation of the two second site mutations. The G at 550 and I at 539 lie within the conserved ABC signature motif of NBD1. Both the mutants G550E and I539T rescued ∆F508 CFTR to the plasma membrane as observed by immunoblotting. Combination of the two resulted in 38 fold increase in channel activity, demonstrated by an increase in chloride current level. G550E mutation is also demonstrated to double the current activity of ∆F508 CFTR when cells are cultured at reduced temperature (30ºC). These experiments demonstrated that the ∆F508 CFTR defects can be significantly repaired by introducing second-site mutations in the NBD1 region.

Further it is shown that both G550E and 4RK rescue another NBD1 trafficking mutant (A561E) in CFTR (Roxo-Rosa et al., 2006). It is suggested that these mutants rescue ∆F508 CFTR by distinct mechanisms. Effect of the revertants is checked on ∆F508 and three other mutations, R560T, A561E and V562I, present within the NBD1. 4RK shows higher steady state levels of band C of WT and also of V562I than G550E. R560T and A561E mutants demonstrated trafficking defect. G550E rescued A561E but not R560T. By iodine efflux assay the authors have proven that 4RK and G550E rescues ∆F508 CFTR at different efficiency.

Given the effects of AFT mutations on ∆F508 CFTR, it remains to be elucidated if AFT motifs in CFTR act as ER-retention or retrieval signals or, like G550E and I539T act in promoting conformational corrections. As observed at reduced temperature, or by use of small molecule correctors like glycerol and bisaminomethylbithiazoles which
rescue the trafficking of ΔF508 CFTR, the AFT mutations might also rescue the mutant CFTR protein by correcting its folding followed by proper maturation.

**C-terminal region in processing and stability of CFTR protein**

Earlier in 1999 it is shown that C-terminal truncation destabilizes CFTR (Haardt et al., 1999). CF patients with premature stop codon or frame shift mutation, that generates CFTR which lacks 70-98 residues from its C-terminus, have severe pancreatic deficiencies, lung infections and increased chloride amount in sweat. It is suggested that the C-terminal tail of CFTR plays a part in its proper functioning. Haardt et al, demonstrated by immunofluorescence that unlike ΔF508 CFTR, both WT and carboxyl terminal truncated CFTR are found in the ER as well as in the cell periphery. Similar results are observed in Cos-1 and BHK cells also. Further the C-terminus of CFTR is truncated successively producing, T26, T70, T82 and T98 (T stands for truncated and the respective number indicates the residue of truncation). cAMP activated Cl- efflux assay is performed for functional analysis in BHK cells stably expressing WT and the mutant CFTRs. T70, T82 and T98 chloride current density are 10% of that of WT CFTR. Whereas that of T26 is similar to WT CFTR, deletion of the last 26 amino acid residues has no effect on CFTR channel function or its cell surface expression. When the steady state levels of WT and the C-terminal truncation mutants of CFTR in BHK cells are measured, the steady state levels of the C-band of T26, is comparable to WT CFTR, unlike T70, T82 and T98 which are 90-95% less than that of WT CFTR. It is also demonstrated that except T98, all the other mutants had folding efficiency comparable to WT CFTR. This is shown by monitoring the conversion of core-glycosylated CFTR to complex glycosylated protein. T98 also showed a two fold increase in degradation rate of
its core-glycosylated form (band B). Interestingly, it is shown by pulse chase labeling that half life of the complex glycosylated (C) bands of T70, T82 and T98 are 5-6 folds less than that of T26 and WT CFTR. The authors are able to demonstrate similar results in transiently transfected Cos-1 cells. All these suggested that even though this C-terminal region is not absolutely necessary for biogenesis and Cl- channel functioning, truncation of C-terminal region affects the steady state level and the turnover of the mature form of CFTR. The authors believe that C-terminal deletion might facilitate lysosomal degradation by exposing lysosomal sorting signals or by avoiding recycling back to the plasma membrane from the endosomes. They also suggested that this truncation can cause structural misfolding followed by increase in proteolytic susceptibility.

It is established that turnover of the mature C-terminal truncated CFTR is six times faster than the WT CFTR (Benharouga et al., 2001). This has been established by showing that the mutant has increased proteosome susceptibility. This leads to faster degradation from post-Golgi compartments as concluded from the pulse chase analysis in addition to cell surface biotinylation.

A hydrophobic patch of four residues (1413-1416) within NBD2 is shown to be necessary for CFTR folding and stabilization (Gentzsch and Riordan, 2001). Stability of nascent CFTR truncated by 81 amino acids (1400X) is severely reduced. Deleting 61 amino acids from the C-terminus did not stop the protein from moving to the cell periphery but interestingly deleting additional 20 amino acids showed only perinuclear localization. This mutant is sensitive to proteasome inhibitors but is not influenced by Brefeldin A (BFA) suggesting that the degradation happens in the ER. Though not
absolutely indispensable, the C-terminal domain alters the biogenesis and maturation of CFTR.

This boundary between C-terminal end of NBD2 and the downstream sequences which are known to affect cellular localization and endocytic turnover is later characterized (Gentzsch et al., 2002). They demonstrated by photolabelling of the NBD2 with 8-azido-ATP that the shorter versions of NBD2 are capable of binding ATP but not hydrolyzing them. It is also demonstrated that C-terminal truncation reaching out to the NBD2 domain considerably reduces channel activity, showing its impact on channel gating. Substitution of the four amino acids in the hydrophobic patch with alanines in both NBD1 and NBD2 domains resulted in arrest of maturation of the protein. This hydrophobic patch is conserved in all NBDs of all ABC family of proteins. Substitution of the residues with alanines has been shown to have the same effect in other ABC transporters as in CFTR. Therefore the hydrophobic patch is predicted to be required for processing and/or stability.

**Internalization signals in CFTR**

Shuttling of proteins between the intracellular compartments is co-ordinated by the vesicular transport of the coat complexes. These coat complexes select proper cargo proteins based on the particular signals in the protein. Integral membrane proteins are recognized and internalized depending on peptide sequences in their cytoplasmic tail. Multiple studies have shown that tyrosine based motifs, dileucine motifs and acid cluster/casein kinase II based motifs are responsible for protein internalization (Chang et al., 1993; Collawn et al., 1993; Letourneur and Klausner, 1992). A tyrosine based signal (Tyr1424) is identified in the C-terminus of CFTR that is required for efficient
internalization of the protein (Prince et al., 1999). This is demonstrated by inhibition of protein internalization with the deletion of residues 1440-1476 of CFTR. Further mutation of tyrosine at residue 1424 had 40% reduction in internalization efficiency when compared with WT CFTR. It is also suggested that both the N-terminal and C-terminal tail regions of CFTR are capable of mediating rapid internalization of CFTR as demonstrated by using chimeras consisting of N-terminal (residues 2-78) region or C-terminal (1391-1476) region of CFTR fused to transmembrane and extracellular domains of transferin reporter (TfR). Both the chimeras are internalized efficiently.

It is set forth that CFTR includes a tyrosine-based internalization signal in its C-terminus, which couples to and interacts with the endocytic adaptor complex AP-2 to assist efficient recruitment of CFTR into clathrin-coated I (COP I) vesicles (Weixel and Bradbury, 2000). This mechanism is demonstrated by in vivo cross linking and in vitro pull down assays showing CFTR binding to AP-2. Fusion protein comprising of residue 1404-1480 are capable of binding to AP-2 but not AP-1. Substitution of Y1424 to alanine also showed reduced coupling to AP-2.

In 2001 the presence of multiple endocytic signals in the C-terminus of CFTR is mentioned (Hu et al., 2001). Based on functional and morphological assays, in addition to Tyr1424, a di-Leu based endocytic signal at 1430-1431 is identified in CFTR. It is suggested that the regulation of CFTR internalization happens by an additive role of multiple endocytic signals in its C-terminal.
ER export signals in CFTR

Like Tyr or di-lysine based motifs that direct proteins to efficiently enter into COP I vesicles for retrograde transport, proteins are selectively packaged into the COP II vesicles and move out of the ER through specific sequences in their cytosolic regions.

It is addressed that a di-acidic signal (DXE) on the cytoplasmic tail of vesicular stomatitis virus glycoprotein (VSV-G) or other ER-exiting proteins is required for proper recruitment into COP II vesicles (Nishimura and Balch, 1997). Earlier it is found that a 29 amino acid oligomer in the carboxyl terminal of the VSV-G protein is important for its folding, oligomerization and ER export (Rose and Doms, 1988). A series of truncations are prepared in VSV-G gene to locate the exact sequence responsible for the efficient export (Nishimura and Balch, 1997). Deletion of residues 14-24 reduced the VSV-G export efficiency by ~80%. To identify specific amino acids responsible for ER export, residues 14-24 are individually substituted with alanine and checked for ER export. Two acidic residues; aspartic acid and glutamic acid at position 21 and 23 respectively, are found to be essential for efficient export of VSV-G. Further deletion mutants lacking residues 25-29 or 22-29 in VSV-G protein also demonstrated ~80% reduction in ER export. It is suggested that for proper presentation of the ER export signal a spacer region between the membrane and the di-acidic signal is necessary. It is also shown that the DXE sequence is specific because substituting D21 with E or R and E23 with D or R caused reduced export. By immunofluorescence analysis it is demonstrated that unlike WT VSV-G with intact DXE, mutated VSV-G with AXA instead remained localized in the ER. It has also been shown that mutation of DXE did not affect the folding or maturation of VSV-G by monitoring the time the protein is released from calnexin and its
rate of trimerization. Both are observed to be similar or more in the mutant as compared to WT VSV-G. Thus it is clear from these experiments that the reduced export level is not due to defective folding or oligomerization. The α subunit of T cell receptor is not capable of leaving the ER in the absence of other subunits. A chimera containing the TCRαT (extracellular domain of the α subunit of T cell receptor) and the transmembrane and cytoplasmic domains of VSV-G is able to efficiently export from the ER and interestingly this export is abolished once the DXE is replaced with alanine. Similar results are also observed in case of lysosomal acid phosphatase (LAP). It has been proposed earlier that efficient assembly and recruitment of the COP II coat complexes indicates a systematic mechanism for selective cargo recruitment (Rowe et al., 1996; Wilson et al., 1994). Therefore it is suggested that the signal DXE or other signals ultimately determine the export rate of a protein. VSV-G is known to transport via COP II vescicles as it is shown earlier that in presence of mutated Sar I, which blocks the assembly of COP II vesicles, VSV-G is not able to move out of the ER (Nishimura and Balch, 1997). So it is also proposed that this signal decides the recruitment of VSV-G into COP II vesicles.

Later the mechanism by which the DXE signal directs efficient ER export is established (Nishimura et al., 1999). Two sequential steps are proposed to be necessary for proper recruitment into COP II vesicles; a) concentration in the ER export sites followed by b) interaction with the COP II machinery proteins. This is demonstrated by mutating DXE to AXA which prevented protein accumulation in the ER export sites before vesicle fission.
In 2004 it is shown that selection of CFTR by the COP II vesicles is dependent on a di-acidic signal (Wang et al., 2004). A conserved di-acidic code (DAD) within the NBD1 domain at residues 565-567 is identified in the CFTR sequence. According to the structural study of NBD1 domain of CFTR, it is suggested that the DAD sequence is present on the surface exposed loop of the NBD1 (Lewis et al., 2004). Substitution of one of the acidic amino acids inhibited ER export of CFTR (Wang et al., 2004). This is demonstrated by monitoring the maturation of band B of mutant and WT CFTR to band C by pulse chase analysis. Multiple Sar1 mutants are used to demonstrate the COP II dependent export of CFTR (Wang et al., 2004).

Figure 6: Crystal structure of NBD1-CFTR showing the phenylalanine at residue 508, and the di-acidic residues at 565 and 567. (Wang et al., 2004)
In another assay by treatment with Brefeldin A (BFA) it is proved that unlike WT CFTR but similar to ΔF508 CFTR, DAA CFTR is unable to get recruited in to the COP II vesicles. BFA treatment leads to collapse of Golgi apparatus, thus specifically affecting the transported pool of CFTR protein. WT CFTR is unable to mature to C band but showed partially stabilized band B. However, ΔF508 CFTR and DAA CFTR showed poor band B stabilization suggesting no packaging of the mutants into the transport vesicles. The reduced ability of DAA and ΔF508 CFTR to couple to the cargo selection complex, Sec23/24 is demonstrated by co-immunoprecipitation.

Interestingly ΔF508CFTR when incubated at reduced temperature had increased coupling to Sec23/24 complex (Wang et al., 2008). It is suggested that at reduced temperature ΔF508 CFTR is stabilized and its maturation is facilitated, which further helps it to present the di-acidic code to the COP II machinery. The di-acidic motif DAD (residues 565-567) is a part of NBD1 domain of CFTR. The residue F at 508 also belongs to the same domain. According to the X-ray crystallographic structure, both DAD and F508 remain at the outer surface (Lewis et al., 2004) (Fig. 6). Thus there is a good possibility that ΔF508 mutation leads to a conformational change such that the di-acidic motif is not properly presented to the Sec23/Sec24 complex and thereby prevents it from exiting the ER. These results suggest that for the protein to travel out of ER, it should be able to present its di-acidic motif to the cargo selection complex.

A yeast plasma membrane protein Yor1p, also an ABC transporter protein, is a drug pump that helps clear toxic substances from the yeast cytoplasm (Katzmann et al., 1999). Its arrangement of MSDs and NBDs makes it a homologue of human CFTR (Riordan et al., 1989). F at position 670 within its NBD1 domain is equivalent to F at 508
in CFTR because deletion of this F670 causes similar ER retention followed by proteosomal degradation as is seen in respect to ΔF508 CFTR (Katzmann et al., 1999). Unlike WT Yor1p, Yor1p with a ΔF670 is inefficiently packaged into the COP II vesicles (Pagant et al., 2007). This is verified by an in vitro vesicle budding assay. ER membranes are purified from cells expressing HA-tagged WT and mutant Yor1p. Incubation of these purified membranes with COP II components; Sar1p, Sec23p/Sec24p and Sec13p/Sec31p in the presence of GTP are sufficient to generate COP II vesicles (Barlowe et al., 1994). These newly formed vesicles can be isolated by differential centrifugation. The cargo proteins within the vesicles can be assessed by immunoblotting (Barlowe et al., 1994). The ΔF670 mutant protein folds and assembles in a distinct manner from that of WT Yor1p, as is demonstrated by non-denaturing gel electrophoresis (Pagant et al., 2007). WT Yor1p showed a band around 350kD whereas ΔF670 Yor1p is found as a smear near 670kD. It is suggested that the large aggregation can be formed by complex formation with other proteins from the cytoplasm as well as the ER. Even when subjected to limited proteolysis, mutant Yor1p generated a different proteosome sensitivity pattern than the WT. ΔF670-Yor1p is also suggested to have improperly assembled transmembrane domains (TMDs) as unlike the WT protein, the mutant is unable to form specific cross-linked di-sulphide bonds among the TMDs. Interestingly two different di-acidic codes are identified in Yor1p; a) N-terminal DXE at residues 71-73 or b) C-terminal DXE at residues 1472-1474. Mutating either one of the di-acidic codes blocked the transport of Yor1p from the ER to the plasma membrane.

Based on these studies we propose a possibility that ΔF508 mutation leads to a conformational change which prevents coupling of the di-acidic exit signal, DAD to the
COP II machinery. Therefore we hypothesized that defective ER-to-Golgi trafficking of ΔF508 CFTR is due to its inability to be packaged in COPII vesicles because it cannot present its di-acidic motif, DAD, to Sec24.

To verify our hypothesis we have studied:

1. The role of the di-acidic code in trafficking of ΔF508 CFTR at reduced and physiological temperature.

2. The conformational pattern of ΔF508 CFTR with mutation of AFT signals, (R29K and R555K) to examine if alteration of conformation facilitates exposure of di-acidic motif to COPII machinery resulting in increased export of ΔF508 CFTR to plasma membrane.

3. The effect of reduced temperature on the conformation of ΔF508 CFTR is also studied.

4. Comparative study of the conformation of ΔF508 CFTR to immature and mature CFTR by using DAA CFTR as a molecular tool.

A detailed biochemical analysis of this di-acidic motif and its mutation provided important insights into CFTR trafficking, its availability to export machinery in specific conformational defects of ΔF508 CFTR and the potential molecular mechanism underlies the rescue of ΔF508 CFTR.
MATERIALS AND METHODS

**Plasmids:** The pcDNA3.1(+) harboring human full-length WT or ΔF508 CFTR coding sequence were the basis for constructing CFTR mutants by overlapping polymerase chain reactions. DAA was replaced from pTM-1 vector into pCDNA3.1(+) vector using restriction sites Bspe1 (cleavage site at amino acid 333 towards the end of MSD1 domain) and Hpa1 (cleavage site after amino acid 777 in between R domain and MSD2). GAG was prepared by overlapping PCR using WT CFTR in pCDNA3.1(+) as template and cloned into pCDNA3.1(+)CFTR using restriction enzyme sites Hpa1 and Bspe1 (both restriction sites are within the CFTR coding region). ΔNBD1-R was prepared by overlapping PCR using WT CFTR in pCDNA3.1(+) as template and cloned into pCDNA3.1(+)CFTR using restriction enzyme sites Nde1 (in the vector pCDNA3.1(+) 5’ site upstream of CFTR) and Pml1 (within the CFTR). ΔF/2RK was prepared by replacing region with double mutant ΔF508 and R555K from pCDNA3.1(+)CFTR(ΔF/R555K) into plasmid pCDNA3.1(+)CFTR(R29K) with R29K mutation. ΔF/R29K was prepared by overlapping PCR using ΔF508 CFTR in pCDNA3.1(+) as template and cloned into pCDNA3.1(+)CFTR using restriction enzyme sites Nde1 (in the vector pCDNA3.1(+) 5’ site upstream of CFTR) and Bspe1 (within the CFTR). The AFT mutants in WT CFTR (WT/R29K, WT/R555K and WT/2RK) were prepared in a similar way as described above and using WT CFTR as template. Mutants ΔF/DAA and ΔF/R555K was prepared by overlapping PCR using ΔF508 CFTR in pCDNA3.1(+) as template and cloned into pCDNA3.1(+)CFTR using restriction enzyme sites Bspe1 and Hpa1. ΔF/R555K/DAA was prepared by overlapping PCR using ΔF/R555K-CFTR in pCDNA3.1(+) as template and cloned into pCDNA3.1(+)CFTR using restriction enzyme sites Bspe1 and Hpa1.
The NBD2 domain deleted constructs (∆NBD2, ∆F/∆NBD2, ∆F/2RK/∆NBD2, ∆F/R555K/∆NBD2, WT/2RK/∆NBD2) were prepared by restriction digestion at HpaI site (present within the CFTR) and XhoI site (in the vector pCDNA3.1(+) 3’ site downstream of CFTR). ∆2-26 was prepared by overlapping PCR using WT CFTR in pCDNA3.1(+) as template and cloned into pCDNA3.1(+)CFTR using restriction enzyme sites NdeI (in the vector pCDNA3.1(+) 5’ site upstream of CFTR) and BspE1 (within the CFTR).

**Antibodies:** Monoclonal antibodies MM13P4 against the amino terminus of CFTR and M3A7 against the carboxy terminal region of NBD2 were purchased from Millipore (Billerica, MA). Monoclonal antibody 24P1 against the carboxy terminus of CFTR was purchased from R&D Systems (Minneapolis, MN). Other antibodies used include anti-Sec24 polyclonal antibodies generated in Dr. William E. Balch’s laboratory (The Scripps Research Institute, La Jolla, CA) (Wang et al., 2004) and anti-actin monoclonal antibody C4 purchased from Millipore (Temecula, CA).

**Cell lines:** HEK cells stably expressing wild-type or ∆F508 CFTR (HEK-WT and HEK-∆F, respectively) are generated in Dr. Neil A. Bradbury’s laboratory (Rosalind Franklin University of Medicine and Science, North Chicago, IL) (Silvis, 2003, 12529365) and were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FBS), 150 µg/ml Hygromycin, and 100 units/ml each of penicillin and streptomycin. HEK cells stably expressing CFTR DAA, ∆F508/DAA and ∆F508/R555K were generated by transfecting HEK cells with the corresponding CFTR expressing plasmids.
and subsequent selection using G418. BHK cells stably expressing wild-type or ΔF508 CFTR (BHK-WT and BHK-ΔF, respectively) were generated in Dr. John R. Riordan’s laboratory (University of North Carolina, Chapel Hill, NC) (Seibert et al., 1995) and were cultured in DMEM supplemented with F12, 5% FBS, 500 mM methotrexate (Ben Venue Laboratories, Inc., Bedford, OH), and 100 units/ml each of penicillin and streptomycin. BHK cells stably expressing CFTR ΔF508/R555K were generated by transfecting BHK cells with the CFTR construct and selecting by G418.

**Cell lysis and quantitative immunoblotting:** Cells were lysed on ice in Triton X-100 lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 (v/v) and Complete protease inhibitor cocktail (PIC) (Roche Diagnostics GmbH, Mannhem, Germany) for 30 min. Insoluble material was removed by centrifugation at 16,000 g for 20 min. Protein concentration was determined by Bradford Assay. Equal protein amount of lysates were separated by SDS-PAGE, immunoblotted with relevant antibodies and visualized by ECL. Multiple exposures were taken to ensure that the intensity of bands is within the dynamic range.

**Quantitative CFTR co-immunoprecipitation**

HEK cells were transiently transfected with plasmids encoding different forms of CFTR. After incubation at 37°C for 20 hours, cells were lysed and CFTR was immunoprecipitated as described previously. The immunoprecipitates were then immunoblotted for Sec24 and CFTR.
In case of Brefeldin A (BFA) treatment, prior to co-immunoprecipitation, HEK cells were transiently transfected with WT and mutant CFTR for 16 hours followed by BFA (10µg/ml) treatment for another 24 hours. CFTR was immunoprecipitated using anti-CFTR monoclonal 13-1 (R domain specific) antibody.

**In situ limited proteolysis**

Microsomes were prepared from CFTR-expressing cells by homogenization and differential centrifugation. When necessary, microsomes were incubated in 2.5 M urea on ice as described to remove peripheral membrane proteins. In case of Braefeldin A (BFA) treatment, prior to homogenization, HEK cells were transiently transfected with WT CFTR. 16 hours post transfection at 37°C, BFA (10µg/ml) was added to the HEK cells and incubated for another 24 hours.

Limited proteolysis was performed largely as described by (Zhang et al., 1998). Intact microsomes at a final protein concentration of 6.8 mg/ml or equivalent amount of urea-washed microsomes were digested with increasing concentrations of trypsin (SIGMA, St. Louis, MO) for 15 minutes on ice. Then, 5 mM PMSF was added to terminate the reaction. Equal amount of digestion mixtures were analyzed by 12% SDS-PAGE and immunoblotted with CFTR domain-specific monoclonal antibodies.
RESULTS

1. ER exit code and domain conformation in CFTR misprocessing

1.1. MSD1, NBD1 and R domains in CFTR protein are essential for CFTR processing: For proteins to exit ER and reach plasma membrane a di acidic amino acid motif is required to interact with cellular COPII machinery proteins. CFTR also has a similar motif consisting of aspartic acid (D)-alanine (A)-aspartic acid (D) at positions 565-567 (Wang et al., 2004) required for its export. The DAD motif is present in the nucleotide binding domain1 (NBD1) of CFTR. To verify whether other domains of CFTR have a functional exit code motif, two CFTR truncation mutants were constructed; 1) wherein MSD1, NBD1 and R domains were deleted and the N-terminal region was attached to the MSD2 domain to make ∆MSD1PNBD1PR CFTR (Fig 7a). 2) NBD2 domain was deleted to make ∆NBD2 CFTR (Fig 7a). These truncated CFTR plasmids were transiently transfected into HEK-293T cells. Additionally, WT CFTR and ∆F508 CFTR were included which served as positive and negative controls, respectively, for the transport of CFTR to the plasma membrane. Glycosylation was assessed by western blotting wherein band B represented core glycosylation and band C represented complex glycosylation. Appearance of band C indicates export of protein from the ER. Band C was observed in ∆NBD2 CFTR (Fig. 7b. lane 4) thus indicating that deleting the last domain did not prevent export of CFTR. However, ∆MSD1-NBD1-R CFTR did not show a complex glycosylated band (C*) (Fig. 7b. lane 3) thereby representing its necessity to enable CFTR to exit from ER. In a separate study it has been demonstrated that deletion of NBD1 domain inhibited export of CFTR from the ER however deletion of the NBD2 domain did not prevent export of CFTR (Pollet et al., 2000).
Endo Glycosidase H (Endo H) removes core glycosylation of a protein, thereby lowering its molecular weight which can be detected by a band shift in Western analysis.

We performed Endo H digestion to verify that the ∆MSD1-NBD1-R signal in Fig. 7b, is not a degraded protein product. Endo H digested proteins were checked for decrease in molecular weight (Fig. 7c). ∆MSD1-NBD1-R showed a band shift. This provides evidence to indicate that the low molecular weight protein is not a degraded protein and suggested that the truncation did not prevent CFTR processing. These results suggest that the functional exit code motif resides in the MSD1-NBD1-R domains. Endo H digestions of the other proteins (WT and mutants) confirmed Endo H sensitivity (Fig. 7c).
1.2. Processing of ER exit code mutants: Requirement of NBD1 domain for export of CFTR as demonstrated in the previous experiment suggests that the exit code motif possibly lies in this domain of CFTR. CFTR has multiple di-acidic codes. DAD from 565-567 residues of NBD1 domain of CFTR (Fig. 8a) is a di-acidic code and is present in an open structure making it more accessible to COPII machinery. To confirm that DAD at 565-567 is indeed the exit code and its requirement for export of CFTR we mutated DAD motif to DAA or GAG, thus separately removing one or both acidic amino acids. WT CFTR (DAD) and CFTR mutants were then transiently transfected into HEK cells. Transfected cells were cultured at 37ºC and lysed after 24 hours.

**Figure 8. Processing of ER exit code mutants:** a. Diagram showing the di-acidic residues in CFTR protein. b. The two acidic residues within the DAD were substituted with alanine (DAA) or together with glycines (GAG). Wild-type and mutant CFTR were transiently transfected into HEK cells and lysed after 24 hours and immunoblotted for CFTR. c. CFTR levels in different glycoforms (bands B and C) were quantified by densitometry.
To observe the effect of the different mutations of the di-acidic code, protein lysates were resolved by SDS-PAGE and immunoblotted for CFTR (Fig. 8b). Mutation of DAD with any amino acid resulted in reduction of export of CFTR as indicated by decrease in C/B ratio (Fig. 8c). DAD at 565-567 was also mutated to NAN and AAD but in both cases CFTR protein was not able to process properly and resulted in a degraded protein product as observed by Western analysis (data not shown). These experiments confirm the role of DAD code in export of wild type CFTR protein from ER.

1.3. WT and ∆F CFTR have different conformation in NBD1 domain: In separate studies major conformational changes were noticed in NBD2 domain of ∆F508 CFTR by in situ proteolysis (Du et al., 2005). It was also demonstrated (Du and Lukacs, 2009) that similar conformational changes occur in N, MSD1 and NBD1 domains of various CFTR processing mutants. Limited proteolysis is a useful assay for studying the conformation of WT and mutant CFTR. An in situ protease susceptibility assay was performed to check the trypsin digestion pattern of the WT and ∆F508 CFTR. A diagram (Fig. 9) of CFTR protein representing the N-terminal (MM13-4) and C-terminal (M3A7) specific antibody epitopes was referred to speculate the region where the digested bands belong in CFTR protein. MM13-4 a monoclonal antibody recognizes the epitope after 26 amino acid residues from the N-terminal of CFTR. While the M3A7 antibody is approximately 100 residues prior to the C-terminal of CFTR. The diagram also represents approximate positions of bands of ~50 kDa and ~42 kDa when probed with N-terminal antibody MM13-4.
Microsomes were prepared from HEK cells stably expressing WT and ΔF508 CFTR. CFTR being a transmembrane protein is incorporated in the microsomes. The microsomes were isolated by differential centrifugation and then treated with increasing dilutions of trypsin. Digested microsomes were then resolved by SDS-PAGE and probed for N and C-terminal regions of CFTR (Fig. 10). ΔF508 generates a markedly different digestion pattern from that of the WT when probed with MM13P4 (N-terminal specific monoclonal antibody) (Fig. 10a and b). ΔF508 evidently shows an increase in trypsin susceptibility with increase in trypsin concentration as compared to WT CFTR. According to the MM13-4 epitope site and corresponding accessible trypsin sensitive sites in WT CFTR, the bands of approximately 50kDa are predicted to be within the NBD1 domain (Fig. 9). Upon trypsinization of ΔF508 CFTR there is a gradual increase in the intensity of these bands with increasing concentration of trypsin as compared to WT CFTR (Fig. 10b. lanes1-6 and Figs. 10g and h) however at highest concentration it is similar to WT CFTR (Fig. 10b. lanes7-8). The increase in band intensity at lower concentrations indicated increased resistance of ΔF508 CFTR to trypsin. In case of WT
CFTR the ~50 kDa fragments shows more sensitivity to increasing concentration of trypsin as indicated by decreasing band intensity (Fig. 10a. lanes 1-6 and Figs. 10g and h). This suggests a difference in the folding of NBD1 in ∆F508 CFTR as compared to WT CFTR. Additionally with ∆F508, band fragments of approximately 42 and 37 kDa become more sensitive to higher concentration of trypsin. The ~37 kDa band fragments as generated in WT CFTR (Fig. 10a. lanes 7-8) is almost not produced in ∆F508 CFTR (Fig. 10b. lanes 7-8). Difference in sensitivity of ∆F508 CFTR as compared to WT CFTR to proteolysis demonstrates a difference in folding of these proteins. However, unlike ∆F508 CFTR, WT CFTR is capable of forming complex glycosylated band (band C). To analyze if this maturation of the CFTR protein is attributed to the difference in proteolytic pattern between WT and ∆F508 CFTR, HEK cells were transently transfected with WT CFTR and treated with brefeldin A (BFA). BFA treatment helped us subtract the band C. Microsomes were then prepared and digested with trypsin as mentioned earlier. Western analysis using the N-terminal antibody, MM13P4, showed the bands of ~42 kDa and 37 kDa even with BFA treatment. Thereby we propose that the missing bands of ~37 kDa in ∆F508 CFTR is not due to the lack of complex glycosylated bands (band C). Increased trypsin susceptibility in ∆F508 CFTR compared to WT was also observed when probed with M3A7, NBD2 specific monoclonal antibody (Fig. 10d and e). Band patterns of ∆F508 CFTR at higher trypsin concentration appear to be different than WT CFTR. According to the M3A7 epitope site and CFTR trypsin sensitive sites, the band fragments of ~36, 28 and 25 kDa are predicted to be within MSD2 and NBD2. A band of ~30 kDa which is observed in WT CFTR is abruptly lost with ∆F508 (Fig. 10d and e, lane 7).
Figure 10. WT and ΔF CFTR have different conformation in NBD1 domain: Microsomes of HEK cells expressing WT and ΔF were subjected to limited proteolysis with varying dilutions of trypsin as mentioned for 15 min on ice. The digested samples were probed with anti-CFTR monoclonal MM13-4 (N-terminal specific) antibody (a - c) and anti-CFTR monoclonal M3A7 (C-terminal specific) antibody (d - f). g. upper and h. lower 50 kDa bands of WT and ΔF508 CFTR were quantified and normalized to the respective CFTR band at trypsin amount zero.
The ~28 kDa band observed in WT CFTR also disappears with highest trypsin concentration in the CFTR mutant (Fig. 10d and e. lane 8). In ΔF508 CFTR, the band of ~25 kDa decreases in intensity and an extra set of bands in between 25 and 22 kDa is also generated unlike WT CFTR (Fig. 10d and e. lanes 7-8). Proteolytic pattern of WT CFTR was similar between BFA treated (Fig. 10f) and untreated (Fig. 10d) extracts.

Taken together banding pattern of WT and ΔF508 CFTR probed with N terminal or C terminal antibody after limited proteolysis indicates that deletion of phenylalanine at 508 residue affects the folding of various domains of CFTR protein.

1.4. DAA but not GAG has similar domain conformations as WT CFTR: Mutations may possibly alter the native structure of a protein. ΔF508 CFTR, a deletion of a single amino acid is capable of causing a conformational as well as a trafficking defect. On account of this, prior to further study the exit code of CFTR it was necessary to analyze if any folding defects are caused by mutating DAD to DAA or GAG. To verify this possibility, we performed limited proteolysis assay on WT CFTR and exit code mutant CFTR proteins. We prepared clonally stable HEK cell lines expressing DAA CFTR or GAG CFTR. Microsomes isolated from these cells were digested with increasing concentration of trypsin. The digested microsomes were immunoblotted by probing with MM134 and M3A7 antibodies as earlier (Fig. 11). Mutation of DAD to DAA does not appear to severely alter conformation as compared to wild type CFTR. When checked with MM13-4, the ~50 kDa bands in DAA CFTR appear similar to WT CFTR in their trypsin sensitivity (Fig. 11a and 8a). Even the ~42 and 37 kDa bands were generated with higher trypsin concentration just as in WT CFTR (Fig. 11a. lanes 6-8, 8a lanes 6-8). The proteolytic band pattern after trypsin digestion of DAA-CFTR when probed for C-
terminal region, using M3A7 antibody, was also similar to that of WT (Fig. 10c). All the bands ~30, 28 and 25 kDa were noticed (Fig. 11c. lanes 7-8).

Collectively these results suggest that mutation of DAD to DAA does not cause folding defect (Fig. 11a and b as compared to Fig 10a and c) but generates a trafficking defect in CFTR (Fig. 8). However GAG mutation generates a band pattern which is different from that of the WT CFTR. The mutant when probed with MM13-4 seems to be

Figure 11. DAA but not GAG has similar domain conformations as WT CFTR: Microsomes of HEK cells expressing DAA and GAG were subjected to limited proteolysis with varying dilutions of trypsin as mentioned for 15 min on ice. The digested samples were probed with (a and b) anti-CFTR monoclonal MM13-4 (N-terminal specific) antibody and (c and d) anti-CFTR monoclonal M3A7 (C-terminal specific) antibody.

Collectively these results suggest that mutation of DAD to DAA does not cause folding defect (Fig. 11a and b as compared to Fig 10a and c) but generates a trafficking defect in CFTR (Fig. 8). However GAG mutation generates a band pattern which is different from that of the WT CFTR. The mutant when probed with MM13-4 seems to be
more sensitive to trypsin (Fig. 11a). The band pattern for C-terminal region of GAG mutant (Fig. 11d) was comparable to ΔF508 CFTR (Fig. 10d) but quite different from WT CFTR (Fig. 10d). It was observed that mutation of DAD to GAG along with trafficking mutant produces a folding mutant as well, whereas DAA mutation only affects the trafficking.

1.5. DAD motif is necessary for the export of ΔF508 CFTR: To determine if ΔF508 CFTR uses the di-acidic motif to exit ER at low temperature, we mutated the di-acidic motif DAD in ΔF508 CFTR backbone to DAA (Fig. 12a),

![Figure 12. Diagram representing DAD in ΔF508 CFTR mutated to DAA. The DAD motif in ΔF508-CFTR is replaced with DAA (ΔF/DAA). Its export from the ER at both physiological and reduced temperature is monitored by transient transfection followed by Western blotting. CFTR levels were quantified by densitometry. Mean ± SEM values are n = 3.](image-url)
The double mutant along with WT and ΔF508 CFTR were then transiently transfected into HEK cells. Export from ER was monitored at 37°C and 30°C (Fig. 12b). As expected, no band C was observed for ΔF508 CFTR as well as the double mutant ΔF508/DAA CFTR at 37°C. On the contrary, at 30°C also there was no band C observed in the double mutant (Fig. 12b). We repeated the experiment in triplicate and plotted the results (Fig. 12c).

We further confirmed trafficking of ΔF508 CFTR by using stable cell lines expressing ΔF508 CFTR or the double mutant ΔF508/DAA CFTR (Fig. 13).

Figure 13. DAD motif is necessary for the export of ΔF508 CFTR at low temperature: a. HEK cells stably expressing ΔF or ΔF/DAA CFTR were cultured at 37°C for over 24 hours followed by incubation at 30°C for indicated time. Equal amounts of cell lysates were immunoblotted for CFTR and actin. b. CFTR levels in different glycoforms (bands B and C) were quantified by densitometry.
These cells were grown at 37°C until cells were approximately 80% confluent. Cells were then shifted to 30°C and harvested every three hours (Fig. 13a). With increasing time at 30°C, ΔF508 CFTR showed an increase in the export from the ER (indicated by C/B ratio) whereas the double mutant did not (Fig. 13b). This indicated that the di-acidic code, DAD plays a role in export of ΔF508 CFTR at 30°C.

1.6. DAD mutation reduces coupling of ΔF508 CFTR with Sec24: Sec24 (a COP II machinery protein) selects newly synthesized proteins and interacts with their di-acidic motif to help them move out of the ER (Nishimura et al., 1999). To test if ΔF508 CFTR uses its di-acidic motif DAD for residual export from the ER, we performed an immunoprecipitation (IP) assay.

![Figure 14. DAA mutation reduces coupling of ΔF508 CFTR with Sec24: a. HEK cells were transiently transfected with ΔF or ΔF/DAA CFTR, 20 hours post-transfection, cells were lysed, and quantitative co-immunoprecipitation was conducted using CFTR monoclonal antibody M3A7. The immunoprecipitates were immunoblotted for Sec24 and CFTR. Mock transfected HEK cells (no CFTR) served as the negative control. b. The level of the associated Sec24 was first subtracted by that of no CFTR, and then normalized to the level of CFTR in band B. n = 4; **indicates p<0.01.](image)

HEK cell lines transiently transfected with ΔF508 CFTR or ΔF508/DAA-CFTR were lysed at 4°C 20 hours post transfection. CFTR was immunoprecipitated by using an
antibody against NBD2 domain (M3A7). Equal amounts of eluted products were separated on an SDS gel and probed for Sec24 (Fig. 14a). As observed from the result after quantification, the amount of Sec24 coupled to ΔF/DAA is reduced by roughly half from ΔF508 (Fig. 14b) suggesting again that even the residual export of ΔF508 CFTR at 37°C is dependent on the di-acidic motif.

1.7. DAA mutation does not induce additional conformational defect in ΔF508 CFTR: As observed previously (Fig. 11a and c) DAA mutation does not lead to any significant variability in proteolytic pattern as compared to WT CFTR. We then decided to verify if DAD to DAA mutation causes any conformational change in ΔF508 CFTR. Stable HEK cells lines expressing ΔF508/DAA CFTR were made and used for microsome preparation. The isolated microsomes were digested with increasing amounts of trypsin. The digested proteins were checked for band pattern by Western blotting (Fig. 15). Proteolytic banding pattern was confirmed by probing with both N-terminal (MM13-4) antibody (Fig. 15a) and C-terminal (M3A7) antibody (Fig. 15b). Inclusion of DAA mutation in ΔF508 CFTR does not generate any considerable divergence in band pattern as compared to ΔF508 CFTR (Fig. 10b and e). The bands of ~50 kDa like ΔF508 CFTR increase in intensity with increase in trypsin concentration (Fig. 15a). The signature bands of ΔF508 CFTR present in between 22 and 25 kDa with higher trypsin amounts were also generated in ΔF/DAA CFTR (Fig. 15b. lanes 7-8). The upper and lower ~50 kDa band signals of WT (Fig. 10a), ΔF508 (Fig. 10b) and ΔF/DAA CFTR (Fig. 15a) were measured by densitometry and quantified by normalizing individual band intensity to the respective total CFTR at zero trypsin concentration (Fig. 15c). Interestingly these ~50 kDa bands in ΔF/DAA CFTR (representing the NBD1 domain) like that of ΔF508
CFTR, showed an increase in resistance with increase in trypsin. However, the ~50 kDa bands in WT CFTR show an increase in sensitivity with increasing trypsin concentration. Overall from the results of Fig. 12, 13, 14 and 15 we conclude that mutating DAD to DAA inhibits residual as well as reduced temperature mediated export of ∆F508 CFTR. We also conclude that this is not due to additional conformational defect. Therefore it is probable for ∆F508 to use the di-acidic code for exiting the ER at 37°C as well as at 30°C.

Figure 15. DAA mutation does not induce additional conformational defect in ∆F508 CFTR: Microsomes of HEK cells expressing ∆F/DAA were subjected to limited proteolysis with varying dilutions trypsin as mentioned for 15 min on ice. The digested samples were probed with a. anti-CFTR monoclonal MM13-4 (NBD1 specific) antibody and b. anti-CFTR monoclonal M3A7 (NBD2 specific) antibody. c. 50 kDa band fragments of WT (Fig. 10a), ∆F508 (Fig. 10b) and ∆F/DAA (Fig. 15a) were quantified and normalized to the respective CFTR band at trypsin amount zero.
1.8. DAA misprocessing is not temperature sensitive: An interesting feature of ΔF508 CFTR is that it is capable of reaching cell surface at lower than physiological temperatures (30°C). We wanted to observe the effect of reduced temperature on mutant DAA CFTR. The ΔF508 or DAA mutants were transiently transfected into HEK cells and its export from ER at 37°C and 30°C was checked by Western analysis (Fig. 16a). As expected, no band C for ΔF508 was observed at 37°C while at 30°C export of ΔF508 was higher. In case of DAA, both the C and B band intensities were higher at 30°C than that at 37°C, but the level of export for DAA did not increase. The experiment was done in duplicate and the C/B ratio was quantified (Fig. 16b). This demonstrates that unlike ΔF508, DAA is not temperature sensitive.

![Figure 16. DAA misprocessing is not temperature sensitive: a. HEK cells transiently transfected with ΔF or DAA were cultured at 37°C for 31 hours before shifted to 30°C and incubated for additional 16 hours. Cell lysates were immunoblotted for CFTR. b. CFTR levels in different glycoforms (bands B and C) were quantified by densitometry. Mean ± SEM were shown, n = 2.](image-url)
For further confirmation, HEK cells stably expressing ΔF508 or DAA were cultured at 37°C until the cells were approximately 80% confluent. These cells were then shifted to 30°C and harvested every three hours. Export of CFTR from the ER was checked by immuno blotting (Fig. 17a). With increasing time at 30°C, ΔF508 CFTR showed an increase in the export from the ER (indicated by C/B ratio) whereas the exit code mutant had a comparable C/B ratio from time 0 to 21 hours (Fig. 17b). This confirms that the di-acidic code mutant, DAA is not susceptible to export at reduced temperature.

Figure 17. DAA misprocessing is not temperature sensitive: a. HEK cells stably expressing ΔF or ΔF/DAA CFTR were cultured at 37°C for over 24 hours before being shifted to 30°C and incubated at 30°C for indicated time. Equal amounts of cell lysates were immunoblotted for CFTR and actin. b. CFTR levels in different glycoforms (bands B and C) were quantified by densitometry.
Increase in band B and C with increase in time were analyzed by transiently transfecting HEK cells with DAA and ΔF508 CFTR. It was demonstrated that level of band C in ΔF508 CFTR saturates at a time point earlier as compared to DAA CFTR (Elaine Chalfin, unpublished data). The post ER stability of DAA and ΔF508 CFTR were also analyzed by incubating HEK cells stably expressing WT and the mutant CFTRs with cyclohexamide (CHX) and assessing the turnover of their band C. It was suggested that post ER stability of DAA CFTR is more than that of ΔF508 CFTR (Anita Saxena, unpublished data).

1.9. DAA has lower Hsp70(s) association than ΔF508 CFTR: After observing the role of different mutants in folding and trafficking of CFTR protein we decided to see if there is a difference in the chaperone association among WT, DAA and ΔF508 CFTR prior to maturation. ΔF508 CFTR being a mutant CFTR remains extensively coupled to ER quality control proteins unlike WT CFTR. We therefore wanted to check if DAA CFTR, which is also a mutant CFTR protein, is also associated to cellular proteins similar to ΔF508 CFTR. Heat shock proteins, Hsp 70 and Hsp 90 remains bound to immature CFTR within the ER (Yang et al., 1993). Further, ΔF508 mutant CFTR is coupled to Hsp(s) more than WT CFTR (Meacham et al., 1999). As demonstrated previously exit code mutant DAA CFTR is not capable of efficiently exporting from the ER (Fig 8). In order to study the chaperone association in the ER form of WT and the mutant CFTRs, HEK cell were transiently transfected with WT, ΔF508 or DAA CFTR. Sixteen hours post transfection the cells were treated with BFA for additional twenty four hours to accumulate increased ER form of CFTR. CFTR was immunoprecipitated using NBD2 domain specific antibody (13-1). The immunoprecipitates were probed for heat shock
proteins; Hsp 70 and Hsc 70 (Fig. 18a). The signal of associated Hsps to WT, DAA or ∆F508 CFTR were quantified and normalized to their respective band B (Fig. 18b). Levels of different chaperone interaction with DAA CFTR were relatively similar to that of WT CFTR, whereas in ∆F508 CFTR the levels of bound chaperones were higher. This result suggests that DAA CFTR is capable of attaining a mature form of CFTR.

**Figure 18. DAA has lower Hsp70(s) association than ∆F508 CFTR:** a. CFTR was immunoprecipitated from HEK cells transiently transfected with WT, DAA, or ∆F CFTR followed by Brefeldin A (BFA) (10ug/ml) treatment, using anti-CFTR monoclonal 13-1 (NBD2 specific) antibody. The immunoprecipitate was run through SDS-PAGE and probed for the Hsp 70, Hsc70 and CFTR. HEK cells not expressing CFTR serve as “mock” control. The level of the associated proteins were subtracted by that of “mock” and then normalized to the level of CFTR in band B. Two independent experiments were conducted. b. The individual bands were quantified by densitometry and normalized to the respective CFTR band B.

**1.10. Impact of urea treatment on CFTR domain conformation:** We observed in Fig. 11 that DAD mutation to DAA does not generate any major conformational change in proteolytic band pattern of CFTR. According to Fig. 18 ∆F508 CFTR has increased chaperone association than DAA. Proteolytic susceptibility of proteins might get affected by their association with other cellular proteins and chaperones. To analyze if the
difference in trypsin susceptibility of ΔF508 CFTR as compared to WT and DAA CFTR is due to their association with different cellular proteins we subjected cellular extracts to urea treatment prior to trypsin digestion. Urea treatment is a commonly used scientific tool for eliminating associated cellular proteins. WT CFTR showed significant differences in proteolytic band pattern between urea treated (Fig 19a) or untreated (Fig. 10a), when immunoblotted with MM13-4 antibody. In addition increase in trypsin sensitivity was noticed with least trypsin concentration (Fig. 19a. lane1). However the bands of ~37kDa were produced in WT and DAA CFTR even with urea treatment (Fig. 19a and c lanes 7-8). This suggests that association with other cellular proteins does not attribute to for the bands of ~37kDa. Reduction in intensity of bands of ~42 and 37 kDa was also observed (Fig. 19a. lanes 7-8). ΔF508 and DAA CFTR like WT CFTR also exhibited an increase in its trypsin susceptibility (Fig. 19b and c). In ΔF508 CFTR except for the missing bands of ~25kDa, no other major difference in proteolytic band pattern was observed between urea treated (Fig. 19b) or untreated (Fig. 10b). The bands of ~42and 37 kDa in DAA CFTR were also very much reduced in intensity (Fig. 19c). Interestingly on quantification of the band intensity, the trypsin sensitivity pattern of the ~50 kDa bands (representing the NBD1 domain) was altered in WT, DAA as well as in ΔF508 CFTR (Fig. 19d and e; 10g and h). The impact of removal of associated cellular proteins to the trypsin sensitivity pattern suggests that the NBD1 domain of WT as well as the mutant CFTR interacts with various cellular proteins. Thereby from the trypsin sensitivity pattern of the ~50 kDa bands (NBD1 domain) in ΔF508 CFTR (without urea treatment) we propose that the NBD1 domain of ΔF508 CFTR has a defective conformation and therefore remains coupled to its chaperones to a greater extent than WT
or DAA CFTR. Whereas DAA mutant does not generate any major conformational defect, therefore, is not recognized as a misfolded protein and is not extensively coupled to chaperones. Other than the ~50 kDa bands trypsin sensitivity pattern the overall proteolytic band formation in WT or DAA CFTR remains relatively similar with or without associated proteins. Therefore we propose that in spite of being a trafficking mutant, DAA CFTR undergoes similar protein processing as WT CFTR.

Figure 19. Urea treatment affects NBD1 domain conformation of CFTR: *In situ* protease susceptibility of a. WT, b. ΔF and c. DAA CFTR. Microsomes of HEK cells expressing WT, ΔF and DAA were subjected to urea treatment followed by limited proteolysis with varying dilutions of trypsin for 15 min on ice. The digested samples were probed with anti-CFTR monoclonal MM13-4 (N-terminal specific) antibody. d. upper and e. lower 50 kDa bands of WT and mutant CFTRs were quantified and normalized to the respective CFTR band at trypsin amount zero.
1.11. DAA mutant shows global maturation dependent conformational change:

Unlike ΔF508, from our previous experiments we believe that DAA is capable of processing and maturing like WT CFTR but being a trafficking mutant do not exhibit efficient ER export. Processing of CFTR to its mature form can include alteration in folding pattern among WT and the CFTR mutants. In Fig. 10, BFA treatment allowed for subtracting the band C (complex glycosylation) but not arresting the WT CFTR protein in an immature form. To analyze if there is a difference in folding pattern between mature and immature form CFTR, HEK cells transiently expressing DAA CFTR were harvested after 12 hours followed by microsome preparation. This time point was chosen to generate CFTR arrested at its earlier stage of processing. The digested protein was probed with MM13-4 (Fig. 20b) and the band intensities of the ~50 kDa bands were quantified by normalizing individual band intensity to the total CFTR at zero trypsin concentration. To our surprise the bands of ~50 kDa (representing the NBD1 domain) like that of ΔF508 CFTR, showed an increase in resistance with increase in trypsin (Fig. 20g and h; 10b). However in mature DAA CFTR these bands of ~50 kDa shows increasing trypsin sensitivity with increasing trypsin concentration like that of WT CFTR (Fig. 20a,g and h). When probed with the C-terminal specific M3A7 antibody, unique bands seen in ΔF508 CFTR in region between 22 and 25 kDa emerged (Fig. 20e and 10e). These bands are not generated in mature DAA CFTR (Fig. 20b). To analyze the only matured form of DAA CFTR, stable HEK cells expressing DAA CFTR, were treated with cyclohexamide (CHX) for 12 hours followed by microsome preparation. CHX prevents protein synthesis but do not affect the export of proteins from ER. Therefore by using CHX we could eliminate any newly synthesized immature CFTR.
Figure 20. DAA mutant shows global maturation dependent conformational change: 
*In situ* protease susceptibility of DAA, DAA-12 hours and DAA-CHX CFTR. 
Microsomes after respective conditions were subjected to limited proteolysis with varying dilutions trypsin as mentioned for 15 min on ice. The digested samples were probed with anti-CFTR monoclonal MM13-4 (N-terminal specific) (a-e) and M3A7 (C-terminal specific) (d-f) antibody. 

**g.** upper and **h.** lower 50 kDa bands at different conditions were quantified and normalized to the respective CFTR band at trypsin amount zero.
The already synthesized CFTR were able to export out of the ER and accumulate as complex glycosylated band C. Microsomes from these cells were digested with trypsin and checked for digested band by immunoblotting (Fig. 20c and f). When the ~50 kDa bands were quantified they showed a much sensitive NBD1 domain (Fig. 20g and h) as compared to DAA mutant without CHX treatment (Fig 20a).

Level of chaperone interaction and progression of the NBD1 domain from trypsin resistance to trypsin susceptibility observed by quantifying the ~50 kDa bands in DAA-12 hours, DAA and DAA-CHX suggests that DAA CFTR is capable of maturing like WT CFTR. Henceforth from the above results we conclude that DAA mutation creates a trafficking mutant but not a processing mutant. Taking all these results into account, we propose that the conformation of immature DAA CFTR (DAA CFTR-12 hours) is comparable to that of ∆F508 CFTR, demonstrating that ∆F508 CFTR stay as an immature form of CFTR and thus is incapable of exporting out of the ER. DAA CFTR on the other hand can eventually attain the mature form, therefore its mature form shows a trypsin band pattern comparable to that of WT CFTR, but due to mutated di-acidic code shows inefficient ER export.

2. Sorting signals and domain conformation in ∆F508 CFTR rescue.

2.1. ∆F508 CFTR rescue by RXR mutations: It has been earlier published that simultaneous substitution of arginine residues at amino acid position 29 (R29) and 555 (R555) with lysine in BHK cells, rescued ∆F508 CFTR export at physiological temperature (Hegedus et al., 2006). As mentioned in literature review these arginine residues are the AFT sequences responsible for retrieval of misfolded protein to ER (Zerangue et al., 1999).
Figure 21. ΔF508 CFTR rescue by RXR mutations: a and b. Diagrams representing ΔF508 CFTR with both R29 and R555 mutated to K (ΔF/2RK) or only R555 mutated to K (R555K). c. The arginines at residue 29 and 555 were individually (ΔF/R29K and ΔF/R555K) or simultaneously substituted with lysine (ΔF/2RK) in ΔF508 CFTR (ΔF). The ER export was analyzed by transient transfection followed by immunoblotting. d. The band C normalized to band B were quantified by densitometry.
We substituted R29 and R555 residues individually (ΔF/R29K and ΔF/R555K) or together to lysine (ΔF/2RK) in ΔF508 CFTR backbone (Fig. 21a and b). Export from ER of these mutants at both 37°C and 30°C was analyzed by transient transfection into HEK cells, followed by immunoblotting (Fig. 21c). As expected, ΔF/2RK was capable of rescuing ΔF508 CFTR at 37°C. Interestingly in our experiments R555K mutation alone in ΔF508 CFTR was sufficient to transport ΔF508 CFTR to the plasma membrane at 37°C as indicated by presence of band C (Fig. 21c, lanes 2 and 4). The C/B ratio was calculated by quantification of B band and C band (Fig. 21d).

2.2. R555K mediated rescue of ΔF508 CFTR is dependent upon ‘DAD’: Analysis of the effect of DAA mutation in ΔF/R555K CFTR phenotype was studied.

![Diagram](image1)

**Figure 22.** R555K mediated rescue of ΔF508 CFTR is dependent upon ‘DAD’: a. Diagram representing ΔF/R555K CFTR with a DAA mutation. b. The DAD motif is replaced with DAA in ΔF/R555K (ΔF/R555K/DAA). Its export from the ER at both 37°C and 30°C was analyzed by transient transfection followed by immunoblotting. c. CFTR levels were quantified by densitometry.
DAD in ΔF/R555K CFTR was mutated to DAA (Fig. 22.a) and transfected into HEK cells. Rescue of ΔF/R555K was determined by Western analysis (Fig. 22b). The rescue of ΔF/R555K CFTR was drastically reduced once the di-acidic motif was mutated, suggesting that the di-acidic code is required at 37°C and 30°C, for rescue of ΔF508 CFTR, even when arginine at 555 is mutated to lysine (Fig. 22b and c).

2.3. R555K improves coupling of ΔF508 CFTR to Sec24: We decided to analyze if R555K mediated rescue of ΔF508 CFTR involves interaction of diacidic motif with COPII machinery. For this purpose, we performed an immunoprecipitation (IP) assay.

![Figure 23. R555K improves coupling of ΔF508 CFTR to Sec24: a. HEK cells were transiently transfected with ΔF or ΔF/R555K CFTR. 20 hours post-transfection, cells were lysed and immunoprecipitated using CFTR monoclonal antibody M3A7. The immunoprecipitates were immunoblotted for Sec24 and CFTR. Mock transfected HEK cells (no CFTR) served as the negative control. b. The level of the associated Sec24 was first subtracted by that of no CFTR, and then normalized to the level of CFTR in band B. Means and SEMs of seven independent experiments are shown in the chart. ** indicates p<0.01.

HEK cell lines transiently transfected with ΔF508 CFTR or ΔF/R555K CFTR were lysed at 4°C 20 hours post transfection. CFTR was immunoprecipitated by using antibody against NBD2 domain (M3A7). Equal amounts of eluate were run through SDS-
PAGE and probed for Sec24 (Fig. 23a). The level of Sec24 associated with ΔF508 CFTR and ΔF/R555K CFTR was calculated by normalizing the intensity of Sec 24 to the intensity of its respective CFTR band B (Fig. 23b). With the insertion of R555K mutation the amount of Sec24 coupling with ΔF508 increased significantly.

2.4. R555K induces conformational reversion in ΔF508 CFTR: The next question was if the R555K mutation rescues ΔF508 CFTR export at 37°C by promoting conformation folding correction in the mutant. Initially we prepared HEK cell lines stably expressing ΔF/R555K CFTR. Microsomes were prepared from these cells and were subjected to in situ limited proteolysis as described earlier. N and C-terminal regions were immunoblotted with their respective antibodies (MM13-4 and M3A7) (Fig. 24).

![Figure 24. R555K induce conformational reversion in ΔF508 CFTR:](image)

Band patterns of double mutant were much comparable to ΔF508 CFTR cultured at 30°C and to WT CFTR. When probed with MM13-4, the 50 kDa bands showed
decrease in band intensity with increase in trypsin concentration as in WT CFTR (Fig. 24a). Quantification of these 50 kDa bands suggested an increase in trypsin sensitivity (Fig. 26a and b). Even the bands of ~37 kDa bands were generated similar to that of WT CFTR (Fig. 24a and 8a lanes 7-8). With M3A7 antibody also, a similarity of banding pattern between ΔF/R555K and WT CFTR was observed (Fig. 24b and 8c). Further like WT CFTR, the ~30 kDa band fragment was also observed (Fig. 24b. lane 7) together with the 28 kDa fragment at highest trypsin concentration (Fig. 24b. lane 8). The signature bands of ~25 and 22 kDa of ΔF508 CFTR were also lost with the R555K mutation in it. Based on these results demonstrating that R555K mutation inΔF508 CFR alter its conformation comparable to WT CFTR. Therefore we propose that this change in conformation exposes its exit code motif thereby facilitating interaction with COPII machinery. This enhanced interaction rescues transport of double mutant to plasma membrane.

2.5. Reduced temperature (30°C) induce global conformational reversion in ΔF508 CFTR: Culturing ΔF508 CFTR at reduced temperature rescues its export to the plasma membrane (Denning et al., 1992a). Based on the above in situ proteolysis results it was worthwhile to analyze the proteolytic pattern of ΔF508 CFTR when cultured at reduced temperature. Microsomes were isolated from HEK cells stably expressing ΔF508 CFTR cultured at 30°C and subjected to trypsin digestion as mentioned earlier. When N and C-terminal regions were immunoblotted ΔF508 CFTR showed a trypsin resistance and band pattern very much comparable to that of WT CFTR (Fig. 25a, b and 9a, b).
Figure 25. R555K induce global conformational reversion in ΔF508 CFTR: Microsomes of HEK cells expressing WT and the mutants were cultured at reduced temperature and then subjected to limited proteolysis with varying dilutions trypsin as mentioned for 15 min on ice. The digested samples were probed with (a, c and e) anti-CFTR monoclonal MM13-4 (N-terminal specific) antibody and (b, d and f) anti-CFTR monoclonal M3A7 (C-terminal specific) antibody.
When probed with MM13-4 the ~50 kDa bands showed increase in intensity with increase in trypsin concentration (Fig. 25a). In addition the ~37 kDa bands were generated as was seen in WT CFTR (Fig. 25a and 9a lanes 7-8). With M3A7 antibody also, a partial correction of band pattern was observed (Fig. 25b). First and foremost the ~30 kDa band was observed (Fig. 25b. lane 7) like WT CFTR followed by appearance of the ~28 kDa band at highest trypsin concentration (Fig. 25b and 9b lane 8). However certain characteristics of ΔF508 are retained as deduced by the appearance of bands of ~25 kDa in N-terminal region (Fig. 25a. lanes 7 and 8) and the bands between ~25 and ~22 kDa in the C-terminal region (Fig. 25b. lanes 7 and 8). Microsomes of WT CFTR cultured at 30ºC were also digested with trypsin. This was done to analyze if reduced temperature also affects the conformation of WT CFTR. Western analysis showed constancy in band pattern both of N and C-terminal as compared to WT CFTR at 37ºC (Fig. 25c and d). Additionally stable HEK cells expressing ΔF/R555K CFTR were also cultured at 30ºC, microsomes were isolated and trypsinized. This was done in order to see if low temperature produces any additional folding pattern. Even in ΔF/R555K CFTR at 30ºC no supplementary bands were observed as compared to that of at 37ºC (Fig. 25e and f). Nevertheless stabilization of the protein was noticed with the increased band intensities in WT as well as ΔF/R555K CFTR.

2.6. Low temperature and R555K induces conformational correction in NBD1 domain of ΔF508 CFTR: The bands in the region of 50 kDa represent the NBD1 domain according to the available trypsin digestion sites in CFTR. The changes in the band intensities thereby represent the change in trypsin resistance with increase in trypsin amount. These bands were quantified as earlier to understand the difference in NBD1
structure in WT and different mutant CFTR (Fig. 26). Both the upper and lower set of bands of ~50 kDa in ΔF508 CFTR shows an increased resistance to trypsin than WT. Thereby symbolizing a misfolded NBD1 domain in ΔF508 CFTR. Interestingly the trypsin resistance of ΔF508 cultured at 30ºC and that of ΔF/R555K was reduced and comparable to WT CFTR. Henceforth we propose that the misfolded NBD1 domain in ΔF508 CFTR plays a major role in its incapability to export from the ER. This misfolding is partially corrected by reduced temperature or by rescue mutant R555K which further helps the trafficking mutant to export to the plasma membrane. It has been addressed that reduced temperature causes an energetic stabilization which facilitates ΔF508 CFTR export (Wang et al., 2008).

![Figure 26. Low temperature and R555K induces conformational correction in NBD1 domain of ΔF508 CFTR: a. upper and b. lower 50 kDa bands of WT and mutant CFTRs at different conditions were quantified and normalized to the respective CFTR band at trypsin amount zero.](image)

2.7. ΔF508 CFTR rescue mediated by low temperature (30ºC) and AFT mutations in BHK cells: Multiple studies have used BHK cell line to analyze export of ΔF508 CFTR
by AFT mutations and at reduced temperature. From the results of our previous experiments we believe that in HEK cells conformational correction of ΔF508 CFTR at reduced temperature or by secondary mutation facilitates its export from ER to plasma membrane. Thus, our next objective was to study the conformation patterns of WT CFTR and its mutants in BHK cells. HEK and BHK cells were transiently transfected with ΔF508 CFTR and checked for its export at 37°C and 30°C by immunoblotting (Fig. 27a. first and second panel). Surprisingly on quantification of C/B ratio we observed that at 30°C there was an increased rescue of ΔF508 CFTR in HEK cells than in BHK cells.

Figure 27. ΔF508 CFTR rescue mediated by low temperature (30°C) and AFT mutations in BHK cells a. HEK and BHK cells transiently transfected with ΔF were cultured at 37°C for 24 hours followed by additional 16 hours at 37°C or 30°C. Cell lysates were immunoblotted for CFTR (first and second panel). BHK-ΔF cells were cultured at 37°C for 24 hours followed by additional 16 hours at 37°C or 30°C (third panel) b. CFTR levels in different glycoforms (bands B and C) were quantified by densitometry. c. The AFT mutants, ΔF/R29K, ΔF/R555K or ΔF/2RK along with ΔF were transfected into BHK cells. The ER export at 37°C or 30°C was analyzed by immunoblotting.
BHK-ΔF is a distinctive cell line, stably expressing ΔF508 CFTR, which by culturing at reduced temperature does not facilitate export of ΔF508 CFTR (Seibert et al., 1995; Wang et al., 2008). When cultured at 37°C and 30°C and checked for export level, there was no appearance of band C (Fig. 27a. third panel). Also the C/B ratio at 30°C was equivalent to that of at 37°C (Fig. 27b). The rescue mutants ΔF/R29K, ΔF/R555K or ΔF/2RK was transiently transfected into BHK cell lines. Export from ER of these mutants at both 37°C and 30°C was analyzed by immunoblotting (Fig. 27c). 2RK as shown previously (Hegedus et al., 2006) rescues ΔF508. However, we show that R555K mutation alone in ΔF508 CFTR was sufficient to transport ΔF508 CFTR to the plasma membrane at 37°C. R29K alone was not capable to rescue ΔF508 CFTR export.

2.8. Conformational maturation in ΔF508 CFTR is necessary for R555K mediated rescue in BHK cells: The next obvious question was to analyze if the R555K mediated rescue of ΔF508 CFTR at 37°C in BHK cells is also due to conformational correction of the mutant. First and foremost we prepared BHK cell lines stably expressing ΔF/R555K CFTR. Microsomes prepared from these cells along with that from BHK cells stably expressing WT CFTR (BW) or ΔF508 CFTR (BHK-ΔF) were digested as described earlier and checked for digestion pattern by immunoblotting (Fig. 28). Band formation observed of both N-terminal and C-terminal halves of WT as well as ΔF508 CFTR in BHK cells was similar to HEK cells (Fig. 28a, b, e, f and 9). Thus we believe that the conformational processing of the WT and ΔF508 CFTR in both the cell lines are similar. Proteolytic bands of ΔF/R555K CFTR in BHK cells also showed general constancy in digestion pattern to that in HEK cell lines in its N-terminal region (Fig. 28c and 17a). Nonetheless bands in lanes 7 and 8 show increase in intensity in BHK cells as compared
to HEK cells. This suggests that folding pattern of ∆F/R555K in BHK cells is similar to that of WT CFTR. Immunoblotting by M3A7 antibody demonstrates a similarity in digestion pattern between ∆F/R555K CFTR and WT CFTR in both HEK cells (Fig 24b and 9b) and BHK (Fig. 28g).

![Image of Figure 28](image)

**Figure 28. Conformational maturation in ∆F508 CFTR is necessary for its rescue:** *In situ* protease susceptibility studies with MM13-4 (N-terminal Ab) of a. BW, b. BHK-∆F, c. BHK-∆F/R555K and d. BHK-∆F-30°C CFTR. Microsomes of BHK cells expressing WT and mutant CFTR were subjected to limited proteolysis with varying dilutions trypsin as mentioned for 15 min on ice. The digested samples were probed with anti-CFTR monoclonal MM13-4 (N-terminal specific) antibody.
Considering the fact that in BHK-ΔF cell line, reduced temperature export of ΔF508 CFTR is not facilitated (Seibert et al., 1995), we used BHK-ΔF cell line as a tool to verify if the rescue of ΔF508 at reduced temperature is related to its conformational correction. Microsomes prepared from BHK-ΔF cells cultured at 30°C were digested and immunoblotted as earlier. When probed for N-terminal region, it was fascinating to see

![Figure 28. Conformational maturation in ΔF508 CFTR is necessary for its rescue: *In situ* protease susceptibility studies with M3A7 (C-terminal Ab) of e. BW, f. BHK-ΔF, g. BHK-ΔF/R555K and h. BHK-ΔF-30°C CFTR. Microsomes of BHK cells expressing WT and mutant CFTR were subjected to limited proteolysis with varying dilutions trypsin as mentioned for 15 min on ice. The digested samples were probed with anti-CFTR monoclonal M3A7 (C-terminal specific) antibody.](image-url)
the band pattern very similar to that of BHK-ΔF at 37°C (Fig 28b and d). The ~50 kDa bands showed an increase in intensity with increasing trypsin concentration and the bands of ~37 kDa were not produced just as in ΔF508 CFTR in HEK cells at 37°C (Fig 9b). Taken together the N-terminal digestion pattern demonstrates that folding of BHK-ΔF does not alter at reduced temperature.

With M3A7 antibody also the digestion pattern of BHK-ΔF at 30°C (Fig 28h) and 37°C (Fig 28f) was comparable to ΔF508 at 37°C in HEK cells (Fig. 9d). However additional bands of ~42 kDa were noticed in BHK-ΔF at 30°C (Fig 28h). Since in case of BHK-ΔF cells unlike HEK cells, neither conformational correction nor export is detected, we propose that the conformational change observed in ΔF508 CFTR in HEK cells cultured at 30°C and ΔF/R555K CFTR plays a role in its export from the ER. This conformational correction probably exposes the di acid code of mutant CFTR thereby facilitating its interaction with Sec 24 the cargo selection protein of COPII machinery.

3. The impact of domain-domain interactions on CFTR misprocessing and rescue

3.1. NBD2 impacts the processing efficiency of WT and ΔF508 CFTR: It has been set forth that the inherent conformational defect in the NBD2 domain of ΔF508 CFTR prevents its proper conformational maturation necessary for export from the ER (Du and Lukacs, 2009; Haardt et al., 1999; Lukacs et al., 1994). On the other hand in Fig. 7, we observed that deletion of the NBD2 domain (ΔNBD2) did not inhibit WT CFTR export from the ER to the plasma membrane. On the basis of these results, analyzing the role of the NBD2 domain in the export of ΔF508 CFTR at 37°C as well as at 30°C seemed interesting. For this we performed an experiment wherein NBD2 deleted WT (ΔNBD2) or ΔF508 CFTR (ΔF/ΔNBD2) along with their full lengths were transiently transfected in
HEK cells and export was analyzed both at 37°C and 30°C by Western analysis (Fig. 29a).

Figure 29. NBD2 impacts the processing efficiency of WT and ΔF508 CFTR. 

a. Diagram showing CFTR with NBD2 domain deleted. 

b. The NBD2 domain was deleted in WT and ΔF (WT/ΔNBD2 and ΔF/ΔNBD2). Its export from the ER at both physiological and reduced temperature is monitored by transient transfection followed by Western blotting. 

c and d. CFTR levels were quantified. Mean ± SEM were shown, n = 2.
Level of export was calculated by quantifying C/B ratio (Fig. 29b and c). Deletion of NBD2 domain reduced the export of WT CFTR by 60% as compared to its full length counterpart. In case of ∆F508 CFTR also, deletion of NBD2 decreases the residual export at 37°C. At 30°C also a more than 50% decrease in the level of export was observed. Taken together all these results suggest that the deletion of NBD2 domain does not inhibit CFTR export but definitely plays a role in lowering the efficiency of the export of WT CFTR as well as ∆F508 CFTR both at 37°C and 30°C.

**Figure 30. NBD2 impacts the rescue efficiency of ∆F508 CFTR:** Diagram representing a. ∆F/2RK and b. ∆F/R555K CFTR with deleted NBD2 domain. c. ∆F/2RK/∆NBD2 and d. ∆F/R555K/∆NBD2 CFTR mutants were transiently transfected into HEK cells. 24 hours later cells were lysed and equal amounts of lysates immunoblotted for CFTR.
3.2. NBD2 impacts the rescue efficiency of ΔF508 CFTR: Subsequently to observe the effect of ΔNBD2 in 2RK and R555K mediated rescue of ΔF508 at 37°C, the NBD2 domains of these rescue mutants were deleted (ΔF/2RK/ΔNBD2 CFTR and ΔF/R555K/ΔNBD2 CFTR) (Fig. 30a and b). These CFTR mutants were then transiently transfected in HEK cells. Twenty four hours post transfection, cells were lysed and equal amounts of lysates were immunoblotted for CFTR. Remarkably the rescue phenotype nearly disappeared with deletion of the NBD2 domain (Fig. 30c and d). This indicates that NBD2 domain is also required for the AFT mutation mediated rescue of ΔF508 CFTR.

3.3. NBD2 is involved in the 2RK mediated conformational change in WT CFTR: To assess the rescue mutants’ effect on WT CFTR, R29 and R555 were mutated individually or together in WT CFTR backbone (WT/R29K, WT/R555K and WT/2RK). Further the NBD2 domain of WT/2RK was additionally deleted to make WT/2RK/ΔNBD2 CFTR (Fig. 31a). These CFTR mutants were transiently transfected into HEK cells and checked for CFTR phenotype by Western analysis. Relatively similar export efficiency was observed with the AFT mutants except for WT/2RK CFTR which showed a degraded protein product (Fig. 31b. lane 4). Nevertheless, when the NBD2 domain was deleted in WT/2RK CFTR (WT/2RK/ΔNBD2 CFTR) the protein managed to fold properly (Fig. 31c. lane 4). To verify the degraded product, the protein was probed with antibodies recognizing different epitopes (Fig. 31d). Deletion of NBD2 domain not only prevents degradation of WT/2RK CFTR mutant but also facilitates its export. These results demonstrate that CFTR protein undergoes a domain dependent maturation.
3.4. Conformational reversion of N-terminal module of ΔF508 CFTR by R555K mutation is not affected by ΔNBD2: Considering the above results, we thought it would be interesting to see if C-terminal truncation motivates conformational change in WT or mutant CFTR which leads to the inefficient export from the ER. We made stable HEK cell lines expressing NBD2 domain deleted WT, ΔF508 or ΔF/R555K CFTR.
Microsomes were prepared from these cell lines and subjected to trypsinization. Band pattern was analyzed by western blotting using N-terminal specific MM13-4 antibody.

The ~36 and 42 kDa bands were present in both NBD2 deleted WT (Fig 32a) and ∆F/R555K CFTR (Fig 32c) as well as in their full length forms (Fig 9a and 24a). In case

Figure 32. Conformational reversion of N-terminal module of ∆F508 CFTR by R555K mutation is not affected by ΔNBD2: In situ protease susceptibility of a. WT, b. ∆F and c. ∆F/R555K ΔNBD2 CFTR. Microsomes of HEK cells expressing deletion mutants were subjected to limited proteolysis with varying dilutions trypsin as mentioned for 15 min on ice. The digested samples were probed with anti-CFTR monoclonal MM13-4 (N-terminal specific) antibody.
of ∆F508 CFTR also (Fig 32b) bands of ~42 kDa were observed both in full length and NBD2 deleted ∆F508 CFTR (Fig. 9b). Thereby it suggests that truncation of the NBD2 domain in matured WT CFTR as well as the mutants does not lead to a major conformational change.

3.5. ∆F508 CFTR might have caused conformational defect in N-terminal region: By in situ proteolysis we have observed the presence of ~37 kDa with high trypsin concentration (with N-terminal specific, MM13-4 antibody) in WT (Fig. 9a), DAA (Fig. 10a), ∆F/R555K (Fig. 24a) and ∆F508 at 30ºC. (Fig. 25d) However, these bands were not produced in ∆F508 CFTR at 37ºC (Fig 9b).

![Figure 33. ∆F508 CFTR causes conformational defect in N-terminal region: In situ protease susceptibility of ∆2-26 CFTR. Microsomes of HEK cells transiently transfected with ∆2-26, 24 hours post transfection were subjected to limited proteolysis with varying dilutions trypsin as mentioned for 15 min on ice. The digested samples were probed with a. anti-CFTR monoclonal MM13-4 (NBD1 specific) antibody and b. anti-CFTR monoclonal M3A7 (NBD2 specific) antibody.](image)

We wanted to identify the region and trypsin sensitive sites yielding ~37 kDa bands in CFTR protein. In CFTR protein trypsin sensitive sites at residues 370, 377 and
381 in NBD1 domain produces a band of ~42 kDa. Additional trypsin sensitive site in NBD1 domain is also present at residue 352. Multiple trypsin sensitive sites are also present in first 26 residues in N-terminal. Tryptic cleavage at residue 352 or of the bands ~42 kDa at N-terminal will produce bands of ~37 kDa. To find out which of these sites generates the bands of ~37 kDa, a deletion mutation from N-terminal 2-26 residues in WT CFTR (∆2-26) was constructed. Deletion of residues 2-26 removes the trypsin sensitive site at residue 26. To identify the missing digestion site in ∆F508 CFTR we used this ∆2-26 construct. Microsomes were prepared from HEK cells transiently transfected with ∆2-26 and subjected to trypsininization. The digested product was checked by immunoblotting with N and C-terminal specific antibodies (MM13-4 and M3A7) (Fig. 33). A band shift of ~6kDa in all the bands was noticed due to the deletion of 24 (2-26) amino acids (Fig. 33). The proteolytic band pattern with both N and C-terminal antibodies was similar to that of the full length WT CFTR (Fig. 33a and b; Fig. 9a and c). Even the bands of ~37 kDa representing the bands of ~42 kDa in full length CFTR were observed. From the proteolytic patterns we show that except for the absence of the ~30 kDa bands (which represent the ~37 kDa in full length CFTR), deletion of the residues from 2-26 did not create a conformationally defective CFTR (Fig. 33a). We further suggest that the absence of ~37 kDa bands is due to the lack of the digestion site in the N-terminal of CFTR.

Taken together we conclude that ∆F508 CFTR a) was digested at sites 370, 377 and 381 therefore produced bands of ~42 kDa; b) the ~42 kDa bands were not further digested at the N-terminal therefore did not produce the bands of ~37 kDa; c) the lack of the bands of ~37 kDa was due to the defective conformation in its N-terminal region.
CFTR folding has been suggested to be co-translational (Kleizen et al., 2005). Existence of interdomain interaction has been summarized in CFTR (Du et al., 2005; Pagant et al., 2008). From the above results (Fig 29-33) we propose that after complete synthesis, CFTR also requires a post translational domain dependent conformational maturation. This step facilitates proper presentation of the di-acidic code (DAD) to the Sec 24 the cargo selection protein of COP II machinery. Interaction with the Sec24 finally helps CFTR to get recruited into the COP II vesicles followed by export from the ER to the plasma membrane. ∆F508 CFTR due to its conformationally defective N-terminal region (Fig. 33), MSD1 (Cui et al., 2007), NBD1 (Fig. 9), and NBD2 (Du et al., 2005) domain has an impaired interdomain interaction. Henceforth the mutant protein ∆F508 CFTR is incapable of attaining a mature conformation required for proper presentation of its di-acidic code to the COP II machinery. Inability to interact with Sec24 leads to ER retention of ∆F508 CFTR followed by degradation. Our study also explains the mechanism by which export of ∆F508 CFTR is rescued at low temperature and by mutation in the arginine framed tripeptide signals.
DISCUSSION

A great number of mutations present in multiple domains of CFTR compromise its processing but the mechanisms are not clear (Cheng et al., 1990; Clain et al., 2005; Cormet-Boyaka et al., 2004; Du and Lukacs, 2009; Ostedgaard et al., 1999; Seibert et al., 1997; Seibert et al., 1996; Smit et al., 1995; Vankeerberghen et al., 1998; Wang et al., 2007b). We characterized the residual processing of ∆F508, a conformational mutant, and DAA, an ER exit code mutant. ∆F508 mutation generates a CFTR trafficking mutant (Cheng et al., 1990) which can be rescued from the ER to the cell surface by culturing cells at low temperature (Denning et al., 1992) as well as by point mutations like R29K/R555K (Hegedus et al., 2006) and R553M (Teem et al., 1993).

Di-acidic code DAD was revealed to interact with Sec24, a cargo selection protein of the COP II machinery (Nishimura et al., 1999). This code was suggested to help the cargo proteins concentrate prior to recruitment into the COP II vesicles for exporting from the ER (Nishimura et al., 1999). Disruption of the DAD exit code in WT CFTR results in lower association with Sec24 than ∆F508 (Wang et al., 2004), suggesting reduced coupling to COP II machinery. In Fig. 7, we demonstrate that deleting the MSD1-NBD1-R (which includes residues 565-567) domains completely abolished CFTR export whereas, deleting NBD2 did not inhibit the export. In Fig. 8, also we observed that mutating this particular code results in significant reduction in WT CFTR export from the ER. Further, when we mutated the DAD code in ∆F508 CFTR (Fig. 12 and 13), we observed that, its residual export at 37°C was prevented. Further, even at low temperature (30°C) the double mutant ∆F/DAA CFTR was not rescued. These data support a necessary role for the DAD motif as the ER exit signal for CFTR.
Since the utilization of sorting signals depends upon domain conformation and/or protein-protein interactions, we tested the impact of DAA mutation on WT and ΔF508 CFTR conformation. DAA CFTR, by limited proteolysis, did not generate any major difference in the proteolytic pattern when compared with WT CFTR (Fig. 10 and 11). Even in ΔF508 CFTR background, mutation of DAD to DAA did not cause any additional folding defect (Fig. 15).

ΔF508 CFTR, due to a conformational defect, has prolonged association with Hsp70 (Meacham et al., 1999; Yang et al., 1993). The mis-folded mutant CFTR is believed to be recognized by the ER quality control system, withheld from ER export, and shuttled to the ERAD pathway. In contrast, DAA was found to be able to achieve full global conformational maturation (Figure 11). Consistent with this, its association with Hsp70 was much lower than ΔF508 CFTR (Fig. 18). We demonstrate that DAA processes better than ΔF508 CFTR at the steady state (Fig. 8), suggesting that defective ER exit code alone only cause mild CFTR misprocessing at the steady state whereas conformational defects can cause a far more severe misprocessing phenotype. The post ER stability of DAA CFTR was also observed to be higher than that of ΔF508 CFTR (Anita and Wang, data not shown). Further, DAA CFTR, unlike ΔF508 CFTR, did not show temperature sensitive ER export (Fig. 16 and 17). Low temperature (30°C) did not increase the export efficiency of WT CFTR either (Wang, et al., 2008). Taken together our data suggest that DAA mutation in WT CFTR produces a trafficking mutant but not a conformational mutant like ΔF508 CFTR.

Based on these specific characteristics of DAA CFTR, we used it as an important tool to study the misprocessing of ΔF508 CFTR. We studied the processing of DAA
CFTR at an early stage (DAA-12 hours) by in situ limited proteolysis, to our surprise, we observed a proteolytic pattern comparable to ΔF508 CFTR (Fig. 10 and 20).

ΔF508 CFTR on in situ limited proteolysis demonstrates a variant digestion pattern as compared to WT CFTR (Du et al., 2005) (Fig. 10). The ~50 kDa bands (when probed with MM13-4), representing the amino terminal tryptic fragment ending in NBD1 domain, show a greater increase in intensity with increasing trypsin concentration in DF508 than in wild-type CFTR (Fig. 10). The pattern of the 50-kDa bands in DAA CFTR is comparable to those in WT CFTR (Fig. 20g and h). However the pattern of the ~50 kDa bands of DAA-12 hours was comparable to that of ΔF508 CFTR (Fig. 20g and h). Further on trypsinization of the only matured DAA CFTR (DAA-CHX) these ~50 kDa bands demonstrated a wild-type-like pattern. A progressive change in trypsin resistance from the immature to mature form of CFTR is clearly depicted by cells expressing DAA CFTR at different levels of maturation. Proteolysis after urea treatment (to remove associated proteins) demonstrated that the changes in the pattern of the ~50 kDa bands in WT, DAA as well as ΔF508 CFTR are dependent upon domain-domain or protein-protein interactions (Fig. 19), suggesting that NBD1 domain is involved in interaction with various cellular proteins or other domains of CFTR.

Therefore we believe that DAA CFTR is capable of folding properly to mature form which is necessary for export from the ER. However due to the mutated exit code, DAA CFTR shows significantly reduced ER export. While ΔF508 CFTR is not capable of attaining the mature conformation and is therefore retained in the ER. Based on the similarity in ΔF508 and the immature DAA CFTR we propose that the deletion of the phenylalanine in the 508 position generates an immature CFTR results in a mis-folded
protein including its NBD1 domain; as a result of which the DAD code fails to interact with the Sec 24, followed by ER retention.

AFT motifs exist in CFTR, which when mutated were capable of rescuing ΔF508 CFTR at 37°C (Hegedus et al., 2006). We found that R29K alone does not contribute to ΔF508 CFTR rescue, however R555K does (Fig. 21). Even R555K mutation failed to rescue ΔF508 CFTR out of the ER when the DAD code was mutated (Fig. 22). In Fig. 14, we demonstrated a significant decrease in association with Sec24 in ΔF508 CFTR with a mutated di-acidic code. Although not restored to WT CFTR levels, the rescue mutant (ΔF/R555K CFTR) showed an increased association with the Sec24 (Fig. 23). An increase in association of ΔF508 CFTR and Sec24 has been demonstrated at reduced temperature (Wang et al., 2004 and 2008) suggesting the necessity of the coupling of the DAD code to COPII for ER export. In Yor1p, a yeast homologue of CFTR, recruitment of ΔF670 (an equivalent of ΔF508 CFTR) into COP II vesicles was demonstrated to be inefficient (Pagant et al., 2007). Interestingly, even in rescued ΔF508 CFTR either by 30°C or by R555K mutation, the pattern of the ~50 kDa bands becomes similar to that of WT CFTR (Fig. 26). Although not identical but partial correction of the domain conformation in rescued ΔF508 CFTR was observed. Considering the fact that DAD belongs to the NBD1 domain, conformational correction of the NBD1 domain in ΔF508 CFTR further corrects the presentation of the exit code to the COP II machinery. Based on these results we believe that like WT CFTR, the DAD code in ΔF508 CFTR is also necessary for exporting from the ER to the plasma membrane. We have not yet addressed the issue if impaired di-acidic code inhibits the recruitment of CFTR into COP II vesicles.
BHK-ΔF cell line, stably expressing ΔF508 CFTR, is a unique cell line since it is not capable of exporting ΔF508 CFTR at reduced temperature (Seibert et al., 1995; Wang et al., 2008) (Fig. 27). Interestingly the specific pattern of the ~50 kDa bands in ΔF508 CFTR in HEK cells at 37°C is comparable to BHK-ΔF cell line not only at 37°C but also at 30°C (Fig. 28). Hence we propose that the inability of the BHK-ΔF cells to export DF508 CFTR at reduced temperature is due to its inability to undergo conformational alteration. We observed R555K mediated rescue of ΔF508 CFTR in BHK cells and the results suggest that this rescue is also supported by partial conformational correction in the NBD1 domain of ΔF508 CFTR (Fig. 27 and 28).

Another mutation, R553M, in NBD1 domain, has also been demonstrated to rescue ΔF508 CFTR (Teem et al., 1993). R553M mutation has also been notified to improve folding efficiency in ΔF508 CFTR (Qu et al., 1997b). Multiple studies have shown that folding dynamics and kinetics of the NBD1 domain is affected by ΔF508 (Serohijos et al., 2008a; Serohijos et al., 2008b), prior to the formation of ATP binding sites (Qu et al., 1997b; Qu and Thomas, 1996). Taken together our data suggest that it is very likely that ΔF508 CFTR misfold in such a way that its di-acidic code, DAD, is not properly presented to the cargo selection protein Sec 24. Thus, it is possible that ΔF508 CFTR fails to be recruited in the COP II vesicles and thereby the mutant CFTR is retained in the ER and eventually degraded.

Truncation of the NBD2 domain in CFTR did not generate any major conformational change in WT, ΔF508 or ΔF/R555K CFTR (Fig. 32). Nonetheless, the deletion of NBD2 domain reduced the export efficiency of WT as well as ΔF508 CFTR (Fig. 29). Additionally NBD2 domain deletion also reduces the rescue efficiency of the
AFT mutants: ΔF/2RK and ΔF/R555K (Fig. 30). Interestingly, in Fig. 31 we observed that deletion of the NBD2 domain in WT/2RK CFTR prevented its degradation by endogenous protease(s).

In Fig. 33, proteolytic study of Δ2-26 CFTR demonstrated that removal of the N-terminal region failed to generate the band of ~37 kDa characteristic of wild-type CFTR conformation (actual molecular weight should be 34 kDa due to the lack of the amino terminal 25 residues). Proteolytic pattern of ΔF508 CFTR also fails to produce the ~37 kDa band. Therefore our data suggest that the generation of the 37kDa band characteristic of wild-type CFTR is dependent upon the N-terminal region. In other words, ΔF508 mutation also impacts the conformation of the N-terminal region. Given that ΔF508 CFTR causes a misfolding in its N-terminal region we reason that CFTR also undergoes a post translational folding which finally facilitates the maturation and export of the protein. Unlike WT CFTR, ΔF508 CFTR has a conformational defect in its N-terminal region (Fig. 33), MSD1 (Cui et al., 2007), NBD1 (Fig. 10), and NBD2 (Du et al., 2005) and therefore has impaired interdomain interaction. Due to the misfoldings in multiple domains, ΔF508 CFTR is unable to attain a mature conformation; therefore it seems likely that this prevents proper presentation of its di-acidic exit code, DAD, to the cargo selection protein Sec 24. As a result ΔF508 CFTR fails to enter the COP II vesicles followed by ER retention and degradation. Reduced temperature and R555K mutation rescues ΔF508 CFTR by partially correcting its conformational defects, and therefore enhance coupling to COPII.

The cell surface localization and functioning of ΔF508 CFTR can be greatly enhanced by improving the folding of NBD1 and its interactions with other domains.
Small molecule correctors can be generated through rational molecular design or high throughput screening (Carlile et al., 2007; Pedemonte et al., 2005; Van Goor et al., 2006) that bind directly to CFTR and alter its conformation (Wang et al., 2007b). The chaperone machinery can be modified in a way that further favors the cellular rescue of AF508 CFTR (Wang et al., 2006; Wright et al., 2004). Combining R555K with low temperature additively improves the conformational maturation of AF508 CFTR (Fig. 25). Such effect is dependent upon cellular chaperone machinery as the additive effect is much reduced in BHK cells (Fig. 27 and 28). Therefore, combinational approaches (Wang et al., 2007a) aimed at the global conformational correction of AF508 CFTR have a great potential in the development of therapeutics for CF patients.
CONCLUSIONS

1. ER exit code mutation DAA does not cause major conformational defect in CFTR.
2. DAA CFTR undergoes conformational maturation in the ER.
3. The DAD motif is necessary for ∆F508 CFTR rescue by reduced temperature or R555K mutation.
4. ∆F508 CFTR has impaired conformation in the N-terminal region and in the NBD1 domain.
5. Low temperature or R555K mutation rescues ∆F508 CFTR by inducing global conformational reversion.
6. Deletion of NBD2 domain does not inhibit but reduces the processing efficiency of WT CFTR as well as ∆F508 CFTR at 37ºC and 30ºC.
7. Deletion of NBD2 domain also affects the efficiency of rescue of ∆F508 CFTR by R555K or 2RK.
8. NBD2 impacts the 2RK-induced conformational change in WT CFTR.
9. CFTR undergoes post translational conformational maturation in a process highly depended upon interdomain interaction, which eventually facilitates its export from the ER to the plasma membrane.
SUMMARY

CFTR is a cell surface chloride channel consisting of two membrane spanning domains (MSDs), two nucleotide binding domains (NBDs) and a regulatory domain (R). A deletion of phenylalanine at residue 508 (∆F508) is present in over 90% of the CF patients. ∆F508 mutation results in ER retention and subsequent degradation of CFTR protein through ubiquitin-proteosome pathway. The precise mechanism of the defective ER-to-Golgi export of ∆F508 CFTR is not known. A di-acidic ER exit code (DAD) has been identified within NBD1 domain. Disruption of this exit code leads to the defective coupling of CFTR to the COPII machinery resulting in impaired export of CFTR from the ER.

Given that both the phenylalanine at residue 508 and the DAD code reside in the NBD1 domain we have studied the functional connection between ∆F508 and the di-acidic exit code DAD. We performed a systematic analysis of the possible ER-to-Golgi sorting signals within CFTR, and specifically examined the functional relationship between different sorting signals in the context of ∆F508 CFTR. We found that reduced temperature as well as R555K mutation mediated rescue of ∆F508 CFTR in a di-acidic code dependent manner, suggesting that the di-acidic code has a functional role in the rescue of ∆F508 CFTR. These results emphasize an important role of the di-acidic motif in CFTR processing. We believe that ∆F508 leads to an impaired presentation of the DAD code to the COPII machinery which is partially corrected by reduced temperature as well as R555K mutation.

We performed in situ limited proteolysis and demonstrated that ∆F508 CFTR causes misfolding in the NBD1 domain in addition to other domains. NBD1 domain
conformational correction was observed in ΔF508 CFTR at reduced temperature and in the presence of R555K mutation. Therefore we propose that both low temperature and R555K mutation rescue ΔF508 CFTR through conformational correction which facilitates proper presentation of the di-acidic code followed by ER export. We have also demonstrated that ΔF508 CFTR has impaired conformation in the N-terminal region. In our experiments we have observed that the NBD2 domain is required for efficient rescue of ΔF508 CFTR at reduced temperature as well as by R555K mutation. Consequently we propose that altered interdomain interactions are involved in ΔF508 CFTR rescue.

A similarity in the proteolytic pattern of immature DAA CFTR as compared to ΔF508 CFTR was also observed which confirms the early notion that ΔF508 CFTR fails to attain a mature form (Lukacs et al., 1994; Zhang et al., 1998). Our data support a cooperative conformational repair during ΔF508 CFTR rescue, which overcomes the arrest in its conformational maturation, and allow it to couple to COP II machinery and exit the ER.


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