The Cystic Fibrosis-causing Mutation ΔF508 Affects Multiple Steps in Cystic Fibrosis Transmembrane Conductance Regulator Biogenesis*

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The deletion of phenylalanine 508 in the first nucleotide binding domain of the cystic fibrosis transmembrane conductance regulator is directly associated with >90% of cystic fibrosis cases. This mutant protein fails to traffic out of the endoplasmic reticulum and is subsequently degraded by the proteasome. The effects of this mutation may be partially reversed by the application of exogenous osmolytes, expression at low temperature, and the introduction of second site suppressor mutations. However, the specific steps of folding and assembly of full-length cystic fibrosis transmembrane conductance regulator (CFTR) directly altered by the disease-causing mutation are unclear. To elucidate the effects of the ΔF508 mutation, on various steps in CFTR folding, a series of misfolding and suppressor mutations in the nucleotide binding and transmembrane domains were evaluated for effects on the folding and maturation of the protein. The results indicate that the isolated NBD1 responds to both the ΔF508 mutation and intradomain suppressors of this mutation. In addition, identification of a novel second site suppressor of the defect within the second transmembrane domain suggests that ΔF508 also effects interdomain interactions critical for later steps in the biosynthesis of CFTR.

The maturation of polytopic multidomain membrane proteins is a complex process that requires the proper folding and assembly of individual domains to form a functional complex (1). These processes may be tightly coupled and occur simultaneously or may proceed in a hierarchical fashion. In addition, these processes may proceed in either a co- or post-translational manner (2, 3). The unique nature of these proteins often requires chaperone systems to promote the proper interactions both within and across multiple protein domains. Perturbations that alter the structures of the individual domains or that alter the interactions of these multi-domain complexes are recognized by the cellular quality control (QC) machines, which ultimately target the newly synthesized protein for maturation or degradation.

Studies of the cystic fibrosis transmembrane conductance regulator (CFTR), the protein whose loss results in cystic fibrosis (CF) have provided insight into the folding of polytopic membrane proteins (4). CFTR is a member of the ABC-transporter family of proteins and is composed of five distinct domains; two transmembrane domains, TMD1 and TMD2; two nucleotide binding domains, NBD1 and NBD2; and a regulatory domain, R (4). The most common CF-causing mutation, the deletion of phenylalanine 508 (ΔF508), is located in the N-terminal cytoplasmic NBD1 (5–9). This single amino acid deletion results in a dramatic reduction of mature, plasma membrane resident CFTR. The immature protein is arrested in an intermediate conformational state that is recognized by the cellular quality control machinery and targeted for degradation by the ubiquitin-proteasome system (10–13). Previous work has shown that the ΔF508 CFTR can be “rescued” by a variety of treatments; that is, low temperature protein expression, the addition of osmolytes and chemical chaperones to cell culture medium, alterations to cellular quality control systems, and by additional mutations within NBD1 (14–17).

Although most manipulations that rescue ΔF508 CFTR are likely nonspecific, mediated through gross changes to protein-protein and/or protein-solvent interactions, the identification of suppressor mutations indicates that the specific rescue of this folding defect is possible. A single mutation, R553Q, was first identified in a patient homozygous for the ΔF508 allele but having only a mild CF phenotype (18). Subsequently, in a screen for suppressor mutations of the ΔF508 defect, the original R553Q suppressor mutation was identified as was I539T,
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G550E, R553Q, and R555K (19–21). When introduced into a ΔF508 background these mutations promoted trafficking and partially restored ΔF508 function at the plasma membrane.

As the ΔF508 and suppressor mutations are located within NBD1, they may alter the biochemical properties of the NBD (i.e. folding efficiency or stability). Alternatively, they may alter the interaction of CFTR domains while leaving the biochemical and biophysical properties of the isolated NBD unaltered. The suppressors might also have little influence on the properties of the CFTR polypeptide in cis but may alter the interaction of cellular quality control machinery with CFTR, thereby promoting CFTR trafficking in trans (22, 23). Finally, suppression of the ΔF508 defect may be the result of a combination of effects on specific intradomain, interdomain, and cellular components interactions.

High and low resolution structural information is available for the CFTR NBDs and homologous ABC -transporters, providing insight into the putative structure and association of CFTR domains. Structures of homologous bacterial transporter systems suggest that the Phe-508 position lies at the interface between the NBD and the fourth intracellular loop (ICL4) of TMD2 (7–9, 24). This interface is predicted to couple the energy of ATP-binding and hydrolysis in the NBDs to the transport or channel activity of the TMDs and provide specificity for the TMD-NBD interaction (25).

The structures of CFTR NBD1 show that the Phe-508 side chain is surface-exposed in the isolated domain. The chemical and physical character of this position contributes directly to the characteristics of the putative TMD-NBD domain-domain interaction surface (5, 6, 26). Consistent with the relatively high surface exposure of the 508 side chain, NBD1 tolerates several non-conservative missense mutations with minimal structural changes, although full-length CFTR fails to fold when charged, and bulky substitutions are made for the Phe-508 side chain (12, 26).

Structures of ΔF508 NBD1 have been solved (6, 27). Minimal changes to the protein backbone are evident, although local perturbations to the putative domain-domain interaction surface proximal to the Phe-508 position are seen (27, 28). The alterations noted in the static structures of the missense and ΔF508 NBDs and the sensitivity of full-length CFTR to charged and bulky substitutions at the 508 position underlies models wherein appropriate NBD-TMD associations are altered. The structures of ΔF508 NBD1 solved to date include additional mutations introduced to increase soluble protein production and facilitate crystallization. These include known second-site suppressors of the ΔF508 mutation and novel solubilizing mutations (6, 27).

The introduction of these additional mutations partially rescues the folding, trafficking, and function of ΔF508 CFTR (29). However, these mutations are not proximal to Phe-508 nor do they contribute directly to the TMD-NBD surface defined by the Phe-508 side chain (29). This suggests that alterations to the TMD-NBD surface of NBD1, as seen statically in the NBD1 crystal structures, are not the sole defect in ΔF508 CFTR maturation (19–21).

Previous biochemical and biophysical studies have demonstrated that the properties of NBD1 are directly altered by the introduction of the ΔF508 mutation. The soluble production of protein both in vitro and in vivo has been shown to be directly effected by the ΔF508 mutation, suggesting that the physical properties of the globular NBD1 domain are altered (6, 26, 30). In contrast, analyses of the soluble, native protein have demonstrated that the wild type and ΔF508 NBD proteins are similar with respect to their native state structures. This suggests that the primary effect of the mutation is not a dramatic alteration of native state structure (6, 26, 30).

To determine how the ΔF508 mutation interferes with CFTR folding and structure, we have probed NBD1 production in isolation and in the context of full-length CFTR. These results demonstrate that the biochemical properties of NBD1 are altered in the absence of other CFTR domains by the introduction of the ΔF508 mutation. Rescue of ΔF508 CFTR correlates well with folding and solubility measurements of NBD1 in isolation. In addition, the ability of a novel second-site suppressor, located within TMD2, to rescue the ΔF508 and F508K mutants demonstrates that proper domain-domain assembly is critical to CFTR maturation. Together, these studies suggest that the ΔF508 mutation perturbs multiple steps critical for CFTR maturation.

EXPERIMENTAL PROCEDURES

Full-length CFTR Expression and Pulse-Chase Analysis—pCMV-CFTR-No6.2 expression plasmids, a generous gift from J. Rommens, were mutagenized using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by automated DNA sequencing. Expression plasmids were transiently transfected using the FuGENE6 reagent (Roche Applied Science) and allowed to express for 48 h. Twenty-four hours post-transfection, the cells were treated with 5 mM sodium-glucamine chloride, 3 mM MgCl2, 1 mM EGTA, and 10 mM TES, pH 7.3. Glibenclamide (400 μM) was added at the end of each recording to block CFTR channels. Data shown are representative of at least four experiments with each CFTR construct.

Electrophysiological Measurement of Full-length CFTR—The pCMV-CFTR-No6.2 constructs were transiently expressed in HEK 293T cells that were cultured at 37 °C until experimentation. Excised, inside-out macropatch recordings were performed in the presence of PKA (110 units/ml) and ATP (1.5 mM) at room temperature. All patches were obtained using similar pipette sizes (ca 2 meaoths tip resistances). Recordings were made in symmetric solutions containing 140 mM NaCl, 3 mM MgCl2, 1 mM EGTA, and 10 mM TES, pH 7.3. Glibenclamide (400 μM) was added at the end of each recording to block CFTR channels.

Computational Analysis—CFTR NBD1 was used as a BLAST seed to identify ABC transporter NBD sequences. Approximately 19,000 sequences were used to generate multiple
sequence alignments with Clustal consisting of 500 sequences per alignment. The amino acid distribution at specific positions was then assessed in the alignments for both the eukaryota and prokaryota subsets.

**Bacterial Yield Assay**—Bacterial expression plasmids containing His$_6$-Smt3 tagged murine and human NBD1 proteins, containing CFTR residues 389–673, previously described, were used for the in vivo yield assays (5, 26, 31). Overnight donor cultures were diluted 1:50 into LB with 50 mg/ml kanamycin and grown to an OD$_{600}$ of 1.0 AU. The cultures were shifted to 25°C, induced with 750 µM isopropyl-1-thio-β-d-galactopyranoside, and allowed to grow for 18–20 h. The final OD$_{600}$ of each culture was determined, and lysis volumes were adjusted for differences in growth. From the 1-liter cultures, 500 ml were removed, and cells were harvested by centrifugation at 4000 x g relative centrifugal force for 15 min at 4°C. The cells were resuspended in lysis buffer (100 mM Tris, 150 mM NaCl, 5 mM MgCl$_2$, 2 mM ATP, 1 mM DTT, 12.5% w/v glycerol, pH 7.6) and sonicated on ice 3 x 1-h intervals with at least 1 h between cycles per sample (Branson Sonifer, 50% duty cycle, output level 5). After sonication, a sample of the lysate was removed for use as the whole cell lysate expression control. The remainder of the lysate was spun for 40 min at 40,000 x g relative centrifugal force at 4°C, and a sample of the supernatant was removed and used as the soluble fraction. Samples were separated by SDS-PAGE on 10% Tris-Tricine gels, transferred to nitrocellulose, and probed with either α-His (Novagen) or α-NBD1 (L12B4, Upstate Biotechnology) monoclonal antibodies and HRP-conjugated α-mouse secondary. Blots were developed with ECL-Plus chemiluminescent reagent and imaged on a GE Healthcare Storm PhosphorImager. The data shown are representative of 4–6 experiments with each NBD construct. Experiments were performed in a single blind manner to exclude contributions of processing order and/or subtle changes in handling.

**Mammalian Complementation Assay**—The His-Smt3-NBD1 sequence was cloned into a pcDNA vector containing an in-frame fusion of the HA-α sequence. HEK 293 cells were transfected with 1.5 µg of NBD-α and 1 µg of ω-plasmins using FuGENE 6 following the manufacturer’s protocols (Roche Applied Science). 24 h post-transfection, cells were treated with 5 mM sodium butyrate. 48 h post-transfection, the cells were washed twice in PBS and lysed by sonication on ice for 15 s (Branson Sonifer, 30% duty cycle, output level 3) in 1 x Reporter Lysis Buffer (Promega). The lysates were mixed 1:1 with 1 x Reporter Lysis Buffer supplemented with fluorescein-di-β-galactopyranoside (2.5 µM final concentration), aliquoted into 96-well plates, and read on a SpectraMax Gemini EM at room temperature in kinetic mode.

**Limited Proteolysis of CFTR**—Microsomes were prepared by nitrogen cavitation and differential centrifugation as described (32). The final preparations were resuspended in 10 mM HEPES, 0.25 mM sucrose, pH 7.5. Microsomes (1–1.5 mg/ml protein) were treated in PBS buffer with the indicated concentrations of trypsin or chymotrypsin for 15 min on ice. The reaction was terminated with 2 mM MgCl$_2$, 1 mM PMSF, 5 µg/ml leupeptin and pepstatin. 0.4 mg/ml soybean trypsin inhibitor was added for trypsin digestion. Digested microsomes were analyzed by SDS-PAGE and immunoblotted with the indicated anti-CFTR antibodies. Data shown are representative of at least three experiments for each construct.

**RESULTS**

**Trafficking and Functional Rescue of CFTR by Second-site Suppressor Mutations**—The introduction of a mutation or group of mutations within a multidomain protein may have multiple effects on the processing and activity of the complex of domains in the native state. Such is the case with CFTR; the deletion of Phe-508 results in ER retention and subsequent degradation of a biosynthetic intermediate. To ascertain how the ΔF508 mutation alters the conformational maturation of the nascent polypeptide, we probed the folding of CFTR utilizing a series of mutations that are predicted to either effect the folding of NBD1 and/or its association with other CFTR domains to better map the biosynthetic steps that are directly perturbed by the ΔF508 mutation.

The introduction of the single mutations, G550E, R553M or R553Q, and R555K, has previously been shown to partially rescue the ΔF508 trafficking defect in CFTR and restore channel activity at the plasma membrane (Fig. 1A) (19–21). To evaluate the combined effects of the second-site suppressors with respect to the ΔF508 mutation and identify the mechanism(s) by which they correct the ΔF508 defect, CFTR harboring the various mutations was expressed in transiently transfected HEK-293 cells and analyzed by Western blotting and pulse-chase analyses (Fig. 1, B and C). Maturation of the CFTR polypeptide includes co-translational core glycosylation (band B) in the ER followed by the posttranslational folding/assembly and subsequent complex glycosylation in the Golgi (band C). At steady state, wild type CFTR shows a mixture of both core and complex glycosylated protein in these heterologous expression systems, indicative of protein at various stages of biosynthesis and trafficking out of the ER and Golgi compartments. The ΔF508 protein appears as only the core glycosylated, band B form, consistent with its retention in the ER. Pulse-chase analyses show the transition from band B to band C in the wild type, wild type -3M, and ΔF508-3M proteins. The ΔF508 protein fails to produce detectable levels of band C CFTR under these conditions. The formation of band C by the wild type, and -3M-containing variants, is indicative of proper folding, ER-exit, and trafficking of CFTR to the Golgi (Fig. 1, B and C).

The side chain of the Phe-508 lies at a predicted domain-domain interface between the NBDs and the TMD(s), and maturation of the full-length protein is sensitive to substitutions at this position (12, 17). To determine whether the suppressor mutations rescue substitutions at the Phe-508 position, which disrupts CFTR folding without measurable impact on NBD1 folding, the -3M suppressor mutations were introduced into Phe-508 missense proteins (Fig. 1B). The inclusion of the -3M mutations failed to significantly rescue the folding of the F508D and F508K mutants, suggesting that the -3M suppressors do not directly influence the interaction between NBD1 and other domains of CFTR (Fig. 1B). Consistent with this result, the introduction of the -3M mutations onto F508A and F508C had little effect on protein maturation. Interestingly, the -3M mutations rescued the folding and maturation of the F508P protein,
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![Diagram of CFTR showing the five distinct domains and the relative locations of the ΔF508 and suppressor mutations.](image)

**FIGURE 1. Rescue of ΔF508 CFTR by -3M mutations.** The introduction of the -3M mutations (G550E, R553M, R555K) rescues the trafficking defects associated with the ΔF508 mutation and restores near wild type function. **A,** a schematic of CFTR showing the five distinct domains and the relative locations of the ΔF508 and suppressor mutations is shown. Transmembrane domains are colored blue, and the nucleotide binding domains are yellow. The locations and residue numbers approximating N- and C-terminal domain boundaries are shown for reference. The location of the Phe-508 position is shown as a red circle, and the four RXR motifs are shown as orange squares. **B,** the introduction of the -3M mutations (G550E, R553M, R555K) rescues the trafficking defects associated with mutation at position 508 and the rescue of these mutants by the inclusion of the -3M mutations. Band B, core glycosylated protein; Band C, complexly glycosylated protein. C, pulse-chase analysis of the CFTR constructs shows an increase in the production of Band C ΔF508 CFTR in the presence of the -3M suppressors. Wild type and ΔF508 CFTR, both with and without the -3M suppressors, are shown. D, functional rescue of the ΔF508 protein accompanies the rescue of CFTR ΔF508 trafficking as measured in whole cell and macropatch techniques of HEK-293T cells expressing the pCMV-CFTR constructs utilized in B. Measurements were made in the presence of PKA and ATP. Holding potential = −80 mV. Data shown are representative of at least three experiments.

which has previously been reported to be refractory to low temperature rescue (12).

To ascertain the functionality of the -3M-rescued ΔF508 protein, HEK-293T cells were transiently transfected with the pCMV-CFTR constructs, and Cl− currents were measured in excised macropatches. A summary of the functional activity of the WT, ΔF508, and -3M constructs is shown in Fig. 1D. The introduction of the -3M mutations rescued the function of the ΔF508 protein to near wild type levels, consistent with the trafficking rescue shown in Fig. 1, B and C. Consistent with prior studies on individual mutations at the 550 and 555 positions, the wild type protein showed an increase in activity when the -3M mutations were introduced (20, 22). ΔF508 channel activity for cells cultured at low temperature is shown for reference.

**Role of the RXR Motifs within NBD1**—Both the ΔF508 and the second-site suppressor mutations are located in NBD1, although the suppressors are distal to the Phe-508 position (Fig. 2A). Previous studies have demonstrated that mutation of the RXR motifs in CFTR improves the trafficking and maturation of ΔF508 CFTR, although the mechanisms behind this rescue are not fully understood (22, 33).

To evaluate the evolutionary conservation of the RXR sequences in NBD1, an alignment of more than 19,000 NBD sequences was generated, and sequence conservation at the CFTR NBD1 RXR motif sites was then assessed. The alignment is of high quality at the 553RAR555 sites as these sequences immediately follow the highly conserved LSGGQ motif. The 553RXR555 sequence is also highly conserved and has been attributed to an extended ABC signature motif sequence (LSGGQXRX). The Arg at the 555 equivalent position is conserved across >75% of the NBD sequences in alignments containing eukaryotic and prokaryotic NBD sequences. The 553-equivalent position is also conserved with respect to three amino acids: arginine, lysine, and glutamine. Interestingly, a second RXR motif, R518RXR518 is not well conserved across these divergent sequences (data not shown).

Previous studies have shown that single-site substitution of the second Arg in an RXR motif facilitates trafficking of other membrane proteins (34, 35). Disruption of the RXR by substitution of Arg-555 with lysine showed no discernible effects on wild type CFTR maturation. By contrast, substitution with alanine, glycine, or threonine in wild type CFTR resulted in the significant decrease in band C protein. The loss of a retention/retrieval signal would not be predicted to alter the trafficking of wild type CFTR, which normally traffics in the presence of such a signal.

**Effects of the -3M Mutations on NBD1**—To ascertain the effect that the ΔF508 and the -3M mutations have on the NBD itself, these mutations were evaluated using NBD1 protein expressed heterologously in *Escherichia coli* in isolation from other CFTR domains or eukaryotic quality control proteins. Previous studies have described the expression, purification, and crystallization of NBD1 utilizing a Smt3-based fusion system and have qualitatively indicated that the ΔF508 NBD1 protein is either insoluble in these systems or is significantly less soluble than wild type (5, 26).

NBD1 was expressed in *E. coli*, and the quantity of soluble protein was determined by Western blotting after controlling for differences in culture growth and separation by high speed centrifugation. The ΔF508 mutation decreased the soluble production of NBD1 protein (Fig. 3A, SOL), although total expression was unaffected by the ΔF508 mutation (whole cell lysate). Quantification of the soluble fraction of NBD1 shows a three- to 5-fold difference in the soluble quantities of wild type and ΔF508 NBD1, consistent with a direct effect of the ΔF508 mutation on NBD1 (Fig. 3B).

The -3M mutations were then introduced into both the wild type and ΔF508 NBD1 backgrounds. The introduction of these mutations caused a significant increase in the quantity of soluble protein (Fig. 3A, SOL); again, total expression levels were unchanged with the inclusion of the -3M mutations (whole cell
lysate). The -3M mutations increased the soluble quantity of NBD1 protein under identical expression conditions in both wild type and /H9004 F508 NBD1 (Fig. 3A). The magnitude of this effect was similar for both wild type and /H9004 F508 NBD1 proteins and was similar with both Arg-553 substitutions (Met and Gln). These data demonstrate that the /H9004 F508 and suppressor mutations alter the properties of the NBD1 protein in the absence of other CFTR domains, and these effects are independent of the mammalian chaperone systems.

To probe the effects of these mutations in a mammalian system, NBD1 was expressed in HEK-293 cells and monitored by a /H9252-galactosidase folding/solubility assay (36). This enzymatic assay relies on the complementation of the /H9251-fragment of /H9252-galactosidase, fused to the target protein (Smt3-NBD1), with the /H9255-fragment of β-galactosidase, expressed independently. Enzymatic activity has been shown previously to correlate with the production of soluble protein (36, 37). Transient cotransfections of the Smt3-NBD1-α constructs and the /H9255-fragment of β-galactosidase were performed, and activity was monitored utilizing a fluorogenic substrate, fluorescein-di-β-D-galactopyranoside.

When co-expressed with the /H9255-fragment, wild type Smt3-NBD1-α fusion proteins produced fluorescence significantly above controls, including HEK293 background and cells expressing only the /H9255-fragment. The inclusion of the ΔF508 mutation into this construct significantly decreased the relative fluorescence signal, p value <0.005 (Welch’s analysis of variance)(Fig. 3B). Western blot analysis of the whole cell lysates showed that both proteins were expressed, and levels of soluble expression correlated with relative enzymatic activity, consistent with differential solubility and/or turnover of the wild type- and ΔF508-α fusion proteins (data not shown).

The -3M second-site suppressors were then introduced into the Smt3-NBD1-α constructs and evaluated for their ability to influence soluble protein production in this system. Similar to the results seen in bacteria, the introduction of the second-site suppressor mutations into NBD1 in mammalian cells produced an increase in enzymatic activity for both the wild type and ΔF508 protein fusions (Fig. 3B). The fluorescence signal of the ΔF508–3M–α fusion proteins increased ~2.5-fold relative to ΔF508 to near wild type levels (p < 0.005). The -3M mutations also increased the wild type NBD1-α signal 2.3-fold (p < 0.005). Western blot analyses of the NBD1-α fusion proteins showed a strong correlation between expression levels of the fusion proteins and the corresponding enzymatic activities (data not shown).

Structural Analysis of CFTR—To evaluate the effects that the ΔF508 and -3M mutations have on the structure of full-length CFTR, limited proteolysis of CFTR stably expressed in BHK cells was performed and analyzed using antibodies specific to epitopes in NBD1 (660, L12B4) and NBD2 (M3A7). Previous studies have indicated that the ΔF508 mutation has adverse effects on the function of the protein, disrupting its interaction with other domains and altering its stability. In contrast, the -3M suppressor mutations help restore the protein’s normal function, making it more stable and easier to analyze for structural changes. The ΔF508 and -3M mutations are located within the NBD1 domain distal to the Phe-508 locus and do not directly contribute to the surface or structure altered by the deletion of the phenylalanine. Two views of NBD1 are shown rotated ~90° with respect to one another. The Phe-508 position is shown in red, and the location of the second site suppressor positions are shown in blue. A, analysis of the RR domains within NBD1 shows high conservation at the 555 position, consistent with its role in the ABC transporter signature motif but much lower conservation at the 516, 518, and 553 positions. B, substitution of the Arg-555 position alters wild type CFTR trafficking. The substitution of R555A, R555G, and R555T resulted in a marked reduction in the formation of band C CFTR, whereas the R555K, as measured by Western blotting of transiently transfected HEK-293 cells displays near wild type CFTR maturation. Data shown are representative of at least four independent experiments.
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A and B, expression of the NBD1 protein in E. coli is directly affected by the inclusion of the ΔF508 and the -3M mutations. NBD1 protein was expressed as a fusion with an N-terminal His-Smt3 and assayed by Western blotting after sonication and centrifugation. The soluble protein samples (SOL) are clarified by centrifugation at 40,000 × g relative centrifugal force, and the whole cell lysates (WCL) are shown as controls for expression and loading. Representative data are shown. B, soluble production of NBD1 in HEK-293 cells is influenced by the introduction of the ΔF508 and -3M mutations, as measured by β-galactosidase enzymatic activity. Changes in signal intensity reflect changes in soluble NBD production and enzymatic activity. Quantification of the end point β-galactosidase data is presented. Data shown are the mean and S.D. from at least 12 experiments for each mutant.

Effects on the structure of the cytoplasmic domains of full-length CFTR (12). Specifically, the inclusion of the ΔF508 mutation in NBD1 increased the proteolytic sensitivity of NBD2 while only modestly altering the proteolytic cleavage of NBD1, as measured with the M3A7 and L12B4 antibodies, respectively.

Limited digestion of CFTR with chymotrypsin was performed, and the relative proteolytic stabilities of the NBD1 and NBD2 domains were probed with the L12B4 and M3A7 antibodies, respectively. As previously described, the inclusion of the ΔF508 mutation within the NBD1 sequence dramatically altered the production of a stable NBD2 fragment containing the M3A7 epitope (Fig. 4A). The M3A7 epitope-containing NBD2 fragment (~30 kDa) decreased dramatically in the ΔF508 protein but was partially restored when the -3M mutations were included in the ΔF508 background (Fig. 4A, M3A7). Similarly, in analysis with the L12B4 (NBD1) antibody, a cluster of bands at ~35 kDa showed sensitivity to the ΔF508 mutation that was restored by the -3M mutations (Fig. 4A, L12B4).

Recent studies have suggested that the L12B4 epitope may report on the proteolytic susceptibility of both NBD1 and TMD1 (38). The L12B4 epitope (residues 385–410) is located in the extreme N terminus of NBD1 and is separated from the core of NBD1 by a large disordered loop (residues 410–430) that is likely susceptible to early cleavage. Thus, to further assess the proteolytic sensitivity of NBD1 in full-length CFTR, limited trypsinolysis was performed, and blots were probed with both L12B4 and a second antibody, Ab660, whose epitope lies between residues 484–589 in the core of NBD1 (Fig. 4B).

Analysis with the 660 antibody clearly demonstrated the formation of a stable, NBD1 core epitope-containing band of ~30 kDa (Fig. 4B, boxed). The degradation of this product was significantly increased by the ΔF508 mutation and stabilized by the inclusion of the -3M mutations in the ΔF508 background. Consistent with the chymotrypsin results and previous studies, analysis of the trypsin digestion with the L12B4 antibody yields a predominant band of ~40 kDa in the wild type, ΔF508, and -3M proteins (Fig. 4B, boxed). The intensity of this band was decreased, and the band appeared to be more susceptible to proteolytic degradation in the ΔF508 as a function of the total amount of CFTR present in the undigested lane. Furthermore, a band of ~20 kDa appeared in the ΔF508-containing samples and was decreased with the inclusion of the -3M suppressor mutations. The Ab660 antibody failed to identify the ~40-kDa products associated with the L12B4 antibody, suggesting that the core sequence of the NBD was not included in this digested fragment.

NBD-NBD Interactions Involved in CFTR Maturation—The proteolytic data indicate that NBD2 conformation is sensitive to the ΔF508 mutation in NBD1 and that the suppression of the ΔF508 defects by the -3M mutations partially restores the native, proteolytically resistant conformation to NBD2. It is not known how this occurs nor is it known what role NBD2 plays in the recognition of the ΔF508 mutation by the quality control machinery. To assess the potential role that NBD dimerization plays in CFTR maturation, mutations within the ATP-binding sites were introduced into pCMV-CFTR plasmid and expressed in HEK293 cells.

ABC-transporter NBDs have been shown to dimerize during their ATP binding and hydrolysis cycles (39, 40). The canonical ATP binding sequences associated with ABC transporters include the Walker A and B sequences and the signature motif (Fig. 5A). Mutations of the Walker A lysine (K464A and K1250A in NBD1 and NBD2, respectively) have been shown to dramatically decrease ATP affinity (40). Conversely, mutation of the catalytic glutamate to glutamine in NBD2, E1371Q, has previously been shown to stabilize NBD dimers by trapping ATP at the NBD-NBD interface (39). Both sets of mutations were evaluated in the wild type and ΔF508 backgrounds after transient transfection and expression in HEK 293 cells to assess the potential role(s) of NBD heterodimerization in CFTR trafficking (Fig. 5, A and B).

Mutations made in the NBD2 composite ATP-binding site, the functionally active ATP site, had no dramatic effect on CFTR trafficking (Fig. 5B). Stabilization of the putative NBD1-NBD2 dimer via the E1371Q mutation did not facilitate the trafficking of the ΔF508 protein and had no discernible effect on the maturation of the wild type protein. Similarly, the introduction of the K1250A mutation had minimal effects on the maturation of wild type CFTR and failed to rescue the ΔF508 CFTR protein. The NBD1 K464A mutation also failed to rescue ΔF508 trafficking. However, when introduced into the wild type background, the K464A reduced CFTR maturation, as evidenced by a decrease in band C.

Although stabilization of the NBD1-NBD2 interaction had little effect on CFTR trafficking, it was possible that NBD2 plays a key role in the suppression of the ΔF508 defects by the -3M mutations (12). To test this hypothesis, a ΔNBD2 CFTR protein previously employed to demonstrate that NBD2 is not required for the maturation of full-length wild type CFTR (41), was utilized. Other studies have demonstrated that maturation of similar ΔNBD2 CFTR proteins are sensitive to the inclusion of the ΔF508 mutation, although the deletion of NBD2 interferes with low temperature rescue (38, 42, 43).
Consistent with those findings, the deletion of NBD2 did not inhibit the trafficking of wild type CFTR, as evidenced by the presence of band C/H9004 NBD2 CFTR (Fig. 5C), and the ΔF508 mutation caused the loss of band C, consistent with its retention in the ER. The -3M mutations promoted the folding and trafficking of the ΔF508-ΔNBD2 CFTR protein, as evidenced by the presence of band C by Western blotting, indicating that their effect does not require NBD2.

TMD-NBD Interactions in CFTR Maturation—Recent studies have suggested that the core CFTR structure, capable of exiting the ER, is formed by the protein sequences including the N terminus, TMD1, NBD1, R, and TMD2 but does not require NBD2 (38, 41–43). To evaluate the possibility the interdomain interactions in the core CFTR structure could be effected by ΔF508, models of the NBD-TMD interactions in CFTR were produced using the extant Sav1866 crystal structures. These CFTR structural models indicate that the first and fourth intracellular loops (ICL1 and ICL4) are predicted to interact with NBD1, with ICL4 in close proximity to the Phe-508 residue side chain. Based on sequence alignments and homology modeling, sites within ICL4 were chosen to probe the NBD-TMD interactions in wild type and mutant CFTR (Fig. 6A). A single tryptophan residue was introduced at positions within ICL4 and evaluated for its ability to rescue the ΔF508 trafficking defect (Fig. 6B). A tryptophan residue was chosen with the hypothesis that the aromatic side chain would physically fill the void created by the ΔF508 mutation, increasing the affinity of the TMD-NBD interaction. The substitution of R1070W had little effect on the maturation of wild type CFTR but measurably promoted trafficking of ΔF508 CFTR (Fig. 6B). Substitution of either Lys or Ala at the 1070 position did not facilitate maturation of ΔF508 CFTR. To evaluate the potential mechanisms by which the R1070W mutation rescued ΔF508, this mutation was also introduced into the ΔF508-3M and F508K backgrounds (Fig. 6, C and D). F508K is expected to disrupt the interdomain interaction, as it interferes with maturation but does not affect the isolated NBD1 (26). The combination of the -3M mutations with the R1070W mutation increased ΔF508 Band C production, as compared with ΔF508-3M and ΔF508/R1070W alone. The increases in Band C production suggest distinct or independent mechanisms of action. In this regard, the R1070W mutation induced the formation of Band C in the F508K mutant predicted to disrupt the interdomain interaction, an effect not seen for low temperature or with the -3M mutations (Fig. 6D).

DISCUSSION

CFTR biosynthesis is a complex process that requires the folding and subsequent assembly of multiple independent domains. Utilizing a series of experiments aimed at identifying the defects associated with the ΔF508 mutation and its suppression by second-site mutations, a more detailed model of the folding pathway of CFTR has been generated (Fig. 7). In this model the primary manifestation of the ΔF508 defect lies within the NBD1 domain itself, which has previously been shown to fold co-translationally and autonomously of other CFTR domains. In the model, failure of this domain to fold and
are of differing severities. This is consistent with the structural models based on bacterial ABC transporters wherein the Phe-508 residue packs against an ICL from TMD2 (ICL4). The loss of Phe-508 is predicted to alter the geometry of the surface without significant alteration to its hydrophobicity. Alterations in chemical character within this interface are predicted to alter this association. Consistent with this, structures of NBD1 F508S and F508R and trafficking of F508S and F508R full-length CFTR demonstrate that the severity of physicochemical alterations at the 508 position correlate with protein trafficking (12, 26). These data suggest that this putative domain-domain interface is critical to the maturation of the CFTR protein.

Second, the loss of the Phe-508 side chain can be accommodated by full-length CFTR when complemented by the NBD1 suppressor mutations. This rescue of ΔF508 NBD1 and CFTR without the full restoration of the NBD-ICL interface (i.e. physical replacement of the missing Phe side chain) suggests that NBD properties are altered more generally. Restoring these physical properties (i.e. solubility, dynamics, stability), NBD-TMD interactions facilitates maturation even though the surface of NBD1 is not.

Finally, rescue of the ΔF508 defect can also be accomplished via alterations within multiple CFTR domains. Domain-domain association events are critical to forming the core CFTR structure. Facilitating these interactions from either side of the interaction interface putatively restores the requisite domain-domain association, thereby promoting CFTR maturation. Furthermore, either improving the folding of one or both of the domains forming the surface or stabilizing the interaction itself would be reflected as increased maturation.

The refined model of CFTR folding and maturation highlights these individual events (Fig. 7). NBD1 is capable of associating with the core structure of CFTR when folded and with cellular chaperone systems when misfolded or partially folded in dynamic equilibria. The inclusion of the ΔF508 mutation alters these equilibria, resulting in prolonged NBD1-chaperone interactions. Failure of the NBD to associate with the core structure of CFTR may also be recognized by cellular quality control systems as alterations in TMD structure. NBD2 seems to be particularly sensitive to a disruption of these interactions. The loss of the appropriate folding and association results in the lower branch of the folding model wherein NBD1 fails to complement other domains in the core CFTR structure. These events ultimately lead to ERAD targeting of CFTR to the proteasome.

Rescue of ΔF508 may be accomplished by facilitating NBD1 folding directly or by stabilizing its interactions with the core CFTR structure. The -3M suppressor mutations directly alter the biochemical properties of the NBD in isolation (Fig. 3). The increased solubility and reported stability potentially shift the equilibria toward a native NBD state, capable of interacting with the core CFTR structure. This association is putatively critical to completing the CFTR core structure (Fig. 7, upper arm). In the model, by stabilizing and/or improving the yield of the native NBD fold, the -3M mutations also facilitate appropriate domain-domain interactions. This may occur indirectly via decreasing NBD1-chaperone interactions, thereby allowing a physical interaction between NBD1 and the TMDs. More
likely, the -3M suppressors act directly via stabilizing the native NBD fold, thereby increasing its availability and indirectly promoting domain-domain interactions.

To further probe the role of the -3M mutations in NBD1, a bioinformatic analysis was used to evaluate the Arg-555 and Arg-555 positions across a large alignment of sequence alignments. Analysis of the RXR sequences suggests an evolutionarily conserved role for the di-arginine residues at these positions. It is possible that these residues were also utilized to serve as retention/retrieval sequences in eukaryotes, as has been previously suggested. However, bioinformatic analyses demonstrate conservation across both prokaryotic and eukaryotic sequences, consistent with a basic structural role. Specifically, the conservation at the Arg-555 position (>75% in both eukaryotes and prokaryotes) suggests strong evolutionary pressure for Arg at this site. As the conservation at this site is preserved in both eukaryote and prokaryotes, the evolutionary pressure exerted on this position is independent of cellular trafficking machinery.

The decrease in wild type CFTR trafficking seen with the R555A/G/T demonstrates that the basic side chain at the 555 locus is required for proper trafficking. The loss of CFTR trafficking with the R555A/G/T mutations suggests this site defines more than a simple signal motif. Consistent with these data, the R555G mutation has previously been identified in a heterozygous CF patient (R555G/Y1307X). Furthermore, structural and functional roles of the Arg-555 residue have been identified in a homologous system wherein intradomain movements are coordinated by interactions between the Arg side chain and a neighboring Asp residue. Together, these data suggest that the conserved Arg-555 residue plays a critical role in NBD structure and function.

Proteolytic analyses also demonstrate that the -3M mutations alter the structure of the wild type and ΔF508 CFTR proteins (Fig. 4, A and B). Changes in NBD1 proteolysis, as a result of the ΔF508 mutant are partially reverted by the -3M mutations, as evidenced by stabilization of both NBD1 and NBD2 bands in trypsin and chymotrypsin digests. Furthermore, these changes in proteolytic protection and CFTR maturation correlate with the changes in NBD1 solubility (Figs. 2, 3, and 4), providing a structural correlate for the alterations in solubility and stability within NBD1. Reversion of the NBD2 proteolytic sensitivity suggests that appropriate domain-domain interactions (and global structure) are partially restored by the -3M mutants. This is consistent with a model wherein proper NBD1 folding in turn facilitates appropriate domain-domain interactions and global CFTR structure.

To evaluate the possibility that domain-domain association and stabilization could be facilitated by stabilization of the NBD1-NBD2 heterodimer, mutations that are known to alter NBD oligomerization and channel gating were introduced into wild type and ΔF508 CFTR (Fig. 5A). Stabilization of the NBD heterodimer by the E1371Q mutation had no discernible effect on wild type or ΔF508 maturation (Fig. 5B). The failure of the E1371Q mutant to rescue ΔF508 CFTR is consistent with ΔF508 influence on the early step of NBD1 folding and that
stabilization of the NBD-NBD dimer may not facilitate ΔF508 maturation.

As well, disruption of the composite ATP-binding site in NBD2 by the K1250A mutant had no discernible effect on CFTR maturation. In contrast, the K464A NBD1 ATP-binding mutant decreased wild type CFTR maturation. These data are consistent with previous reports of effects of Lys-464 mutants on maturation and support a model wherein ATP serves as a structural co-factor for NBD1 but not NBD2.

Analysis of ΔNBD2 CFTR suggests that CFTR trafficking is generally refractory to the presence of NBD2. Both the ΔF508 and -3M mutants behaved similarly when expressed in full-length or ΔNBD2 CFTR. The ΔF508 ΔNBD2 protein failed to produce significant quantities of band C but was partially rescued when the -3M mutations were introduced. However, the observation of less band C in ΔF508 ΔNBD2–3M protein relative to ΔF508 –3M suggests that the NBD2 has minimal effects on CFTR maturation and/or stability, although it is itself profoundly impacted by the folding status of the rest of the molecule.

To evaluate the possibility that ΔF508 CFTR could be rescued by stabilization of other domain-domain interactions within the minimal core structure of CFTR, structural models were produced to identify positions critical to the NBD1-TMD2 interface. Sites within ICL4 were chosen based on their proximity to the Phe-508 residue. The identification of a single site, Arg-1070, within ICL4 that promotes ΔF508 maturation suggests that the NBD1-TMD2 interaction is critical for CFTR biosynthesis.

Although both the Arg-1070 and -3M suppressors rescue ΔF508, suppression by the R1070W mutation is likely accomplished by independent mechanisms. The properties of NBD1 are not directly altered by the Arg-1070 locus. Thus, the R1070W mutation putatively promotes appropriate domain-domain associations by increasing hydrophobic interactions (affinity) at the NBD1-ICL4 domain-domain interaction surface. The relatively hydrophobic surface proximal to the Phe-508 position could potentially accommodate the hydrophobicity and volume of the R1070W substitution. Decreases in side-chain volume (Ala/Gly/Thr substitution) and the presence of charge (Arg-1070 and R1070K) fail to facilitate ΔF508 trafficking. Maturation of the F508K CFTR molecule was potentially facilitated by interactions between the indole side chain from R1070W and the NBD1 surface. An increase in domain-domain affinity as a result of increased hydrophobic interactions may overcome the repulsive forces introduced by the Lys side chain.

Intriguingly, the R1070W mutation, which rescues ΔF508 CFTR, has been identified in patients with mild disease (congenital bilateral absence of the vas deferens, pancreatic sufficient CF). Previous studies suggest that in the wild type background the R1070W mutation alters protein expression, localization, and function (44). It is possible that changes in interdomain dynamics contribute to ΔF508 maturation and altered wild type CFTR properties, although further study is needed to fully evaluate this possibility.

Taken together, these data suggest that the formation of a core CFTR molecule, including TMD1-NBD1-R-TMD2, is critical for CFTR maturation. The assembly of this core structure requires the proper folding of individual domains and the proper assembly of these domains. Alterations to the processes of domain folding and assembly may both contribute to the misfolding and rescue of ΔF508 CFTR. These data provide evidence for the direct biochemical and biophysical alterations of NBD1 with the ΔF508 and -3M suppressor mutations, demonstrating a critical role of NBD1 folding in CFTR maturation. Modulation of NBD1 folding, therefore, represents an attractive therapeutic target for ΔF508 CFTR. Additionally, the identification of a novel second site suppressor within TMD2 provides evidence that stabilization of the global fold may facilitate NBD1 folding via coupled folding and assembly events, providing additional regions of CFTR that could be targeted for the rescue of ΔF508 CFTR folding and biosynthesis.

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