Mutations in the NBD1 signature motif region rescue processing and functional defects of CFTR ΔF508

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Running Title: Mutations in the ABC signature motif region rescue CFTR ΔF508
Summary

The gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), an ATP-binding cassette (ABC) transporter that functions as a phosphorylation-and nucleotide-regulated chloride channel, is mutated in cystic fibrosis (CF) patients. Deletion of a phenylalanine at amino acid position 508 (∆F508) in the first nucleotide binding domain (NBD1) is the most prevalent CF-causing mutation and results in defective protein processing and reduced CFTR function, leading to chloride impermeability in CF-epithelia and heterologous systems. Using a STE6/CFTRΔF508 chimera system in yeast, we isolated two novel ΔF508 revertant mutations, I539T and G550E, proximal to and within the conserved ABC signature motif of NBD1, respectively. Western blot and functional analysis in mammalian cells indicate that mutations I539T and G550E each partially rescue the CFTR ΔF508 defect. Furthermore, a combination of both revertant mutations resulted in a 38-fold increase in CFTRΔF508 mediated chloride current, representing 29 % of wildtype channel activity. The G550E mutation increased the sensitivity of CFTRΔF508 and wildtype CFTR to activation by cAMP agonists and blocked the enhancement of CFTRΔF508 channel activity by 2 mM IBMX. The data show that the ΔF508 defect can be significantly rescued by second-site mutations in the NBD1 region that includes the LSGGQ consensus motif.
Introduction

Cystic fibrosis (CF) is the most frequent lethal genetic disease associated with a single gene in Caucasians (1). CF results from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes an ATP-binding cassette (ABC) transporter that functions as a phosphorylation and nucleotide-regulated chloride channel located in the apical membrane of epithelial cells (2,3). The ABC transporters constitute a large family of ubiquitously expressed proteins, mostly involved in ATP-driven translocation of diverse substrates across biological membranes (4,5). It has been proposed that a functional ABC transporter has a minimal structural requirement of two membrane-spanning domains (MSDs) and two nucleotide binding domains (NBDs) (5). The NBDs, or ABC cassettes, share 30-50% sequence identity (6) and are characterized by the presence of three conserved motifs; Walker A and Walker B motifs are present in several nucleotide binding and hydrolyzing proteins (7), and the ABC-signature motif, located just upstream of the Walker B, is diagnostic of ABC cassettes (5,6).

The deletion of the Phe 508 (ΔF508) in the first nucleotide binding domain (NBD1) of CFTR is the most frequent CF-causing mutation, present in 90% of CF chromosomes. ΔF508 impairs normal protein maturation and trafficking to the plasma membrane (8,9), presumably through a localized effect on the folding of the NBD1 domain (10-12). This misfolding results in retention of CFTRΔF508 by the ER-associated quality control and in subsequent degradation with the participation of the cytoplasmic proteasome (13). The CFTRΔF508 biosynthetic processing defect can be partially rescued by low temperature (14), high concentrations of glycerol (15) and other low molecular weight compounds that affect the cellular folding environment (16).
CFTR channel is regulated by phosphorylation by cAMP-dependent protein kinase (PKA) at multiple sites in the regulatory (R) domain, and by ATP binding and hydrolysis at the NBDs (3,17). Maximal phosphorylation of PKA sites in the R domain control the channel bursting rate and open probability ($P_o$) of CFTR wt channels, by increasing the apparent affinity of NBDs for ATP (18,19). CFTR is modified in vivo by different levels of phosphorylation, resulting in channels with corresponding different biophysical characteristics (3,17,20). The ΔF508 mutation alters CFTR function, by decreasing the channel open probability ($P_o$) (21,22). The defective activity of CFTRΔF508 channel can be ameliorated pharmacologically (23-25).

To identify regions in the NBD1 that are affected by ΔF508, we isolated point mutations that rescued the functional and processing defects of CFTRΔF508. Making use of the sequence homology of NBDs of CFTR and the S. cerevisiae mating peptide pheromone transporter, Ste6p (26,27), a STE6/CFTR chimera was previously developed to study the ΔF508 mutation in yeast (28,29). Mutations analogous to ΔF508 disrupt function of other ABC transporters (30-34). The mutation equivalent to ΔF508 in the STE6 gene, ΔL455, did not result in defective phenotype (35), but in the context of the CFTR sequences ΔF508 disrupts the α-factor transport function of STE6/CFTR chimera (28,36), providing a yeast system for identification of ΔF508 revertant mutations within CFTR sequences. Here we used the STE/CFTRΔF508 chimera system to identify novel amino acid substitutions just upstream (I539T) and within (G550E) the ABC signature motif of CFTR NBD1, that partially suppressed the CFTRΔF508 defect in HeLa cells and in Fischer rat thyroid (FRT) cells. The G550E mutation introduces a negatively charged amino acid in the highly conserved LSGGQ core signature motif of CFTR NBD1. Interestingly, the site of this mutation is flanked by two residues where CF-causing mutations have been identified that impair folding/trafficking of CFTR (S549R) or ATP-
dependent channel gating (G551D) (37-41). We assessed the effect of the G550E mutation on the PKA-dependent activation of wildtype and mutant CFTR chloride channel. We also investigated the effect of two compounds known to optimize PKA-dependent CFTRΔF508 activity, genistein and 3-isobutyl-1-methylxanthine (IBMX) (23-25), on the ΔF508 revertant channels.

**Experimental Procedures**

**Mutagenesis and Screen for ΔF508 Revertants in Yeast**

The construction of the plasmids for expression of the STE6/CFTR and STE6/CFTRΔF508 chimeras in yeast has been previously described (28). Derived from JTS6 plasmid carrying the STE6 gene, JTS6-H5 is a single copy CEN plasmid that carries the selectable marker URA3 and the STE6/CFTR hybrid gene H5, where sequences corresponding to Ste6p amino acid residues R441-I516 were replaced by the corresponding region from CFTR (F494-L558). Similarly, CFTRΔF508 sequence was used to make plasmid H5-ΔF508. A fragment containing the 193 bp of CFTRΔF508 DNA flanked by STE6 sequences, 101 bp on the amino terminus and 100 bp on the carboxy terminus, was generated by polymerase chain reaction (PCR) amplification of plasmid H5-ΔF508 using the following primers: forward (5’-GTTCTACGATAGCTATAATGGAT-3’), and reverse (5’-GCCTAATTGCCTCATCAAACAG-3’). In order to generate random point mutations within this fragment, PCR reactions were performed under mutagenic conditions (42,43), using Taq DNA polymerase (Promega Co.). For site-directed mutagenesis at the position corresponding to CFTR G550, degenerate DNA oligos were used in the PCR reactions to generate multiple codons. Yeast strain JPY201 (MATa, STE6Δ::HIS3, gal2, ura5-52, lys2-801, trp1, leu2-3,112, his3Δ200) (26) was co-transformed with the linearized H5-ΔF508 plasmid and the mutagenized 394 bp DNA fragments, using the
lithium acetate method. Mutations were inserted into H5-ΔF508 as a result of homologous recombination (44). The transformed JPY201 cells were plated in Sc-Ura medium, selective for transformants expressing the URA3 gene and then mated with the yeast strain 22-2D4 (Mat α, ura3-52, leu2-3,112, trp1). The yeast mating assays were performed as previously described (28,29). Briefly, a lawn of 22-2D4 cells and transformed JPY201 colonies were replica-printed to a non-selective medium (YPD) and incubated at 30°C, to allow mating. After 8 hours, cells were replica-printed to a medium selective for diploids (SD + Leu). After a retest, plasmid DNA was isolated from single haploid JPY201 colonies that gave rise to diploid colonies and sequenced. For quantitative mating assay, JPY201 cells transformed with each H5 variant were grown to log phase in 0.1% glucose Sc-URA medium. From each culture, 3 x 10^6 cells were mixed with an equal number of 22-2D4 cells and collected by filtration onto a filter, that was then placed on a YPD plate for 4 h at 30°C. Cells were resuspended, sonicated briefly and plated from serial dilutions onto SD-Leu plates. Plates were incubated for 3 days at 30°C and diploid colonies were counted (45).

Vector construction and CFTR Expression in Mammalian Cells

For the expression of CFTR variants in HeLa cells, pTM CFTR (28) digested at unique Smal and XhoI sites flanking the F508 region was used to clone the sequences from the H5-ΔF508 variants amplified by PCR. For expression in FRT cells, the pSwick (pMT3-Swick) vector (46) was similarly constructed. The CFTR region of the H5-ΔF508 hybrid gene containing second site mutations was amplified by PCR using the oligos Sma-L (5’-GGATTATGCCCCGGACCATTAAAG-3’), forward, and Xho-R (5’- GATGAATGCTCGAGCTAAAGAAA-3’), reverse. The PCR product was digested with Smal and XhoI, resulting in a 178 bp fragment, corresponding to CFTR cDNA
nucleotide residues 1630 to 1808, and introduced in-frame into pTM-CFTR and pSwick CFTR. Constructs were verified by DNA sequencing.

*Transient Expression of CFTR in HeLa Cells.* HeLa cells were maintained at 37°C in a humidified, 5% carbon dioxide atmosphere. Growth medium was EMEM (Sigma) supplemented with 10% Fetal Bovine Serum (Summit Biotechnology) and 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate and 0.25 µg/ml amphotericin B (GibcoBRL). The vaccinia virus/bacteriophage T7 hybrid expression system (47) was used for transient expression of the CFTR variants in HeLa cells, as described previously (28). Briefly, sub-confluent 100 mm plates of HeLa cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase (10 MOI) and transfected with pTM plasmids carrying each CFTR variant cDNA under the control of the T7 promoter. Cells were incubated for 18 hours and lysed.

*Transient Expression of CFTR in FRT Cells.* Fischer rat thyroid (FRT) epitheloid cells and FRT cell lines stably expressing CFTR wt and CFTR ΔF508 (48) were a gift from Michael Welsh, University of Iowa. FRT cells were maintained at 37°C in a humidified, 5% carbon dioxide atmosphere. Growth medium was Coon’s modification of Ham’s F-12 (Sigma) supplemented with 5% Fetal Bovine Serum (Summit Biotechnology) and 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate and 0.25 µg/ml amphotericin B (GibcoBRL). Dividing FRT cells were trypsinized in a solution containing 0.25% trypsin and 0.1% EDTA, pelleted and resuspended in serum free F-12 Coon’s medium. Aliquots of cell suspension equivalent to 5 x 10^5 cells were transferred to microfuge tubes, pelleted and resuspended in F-12 Coon’s medium containing DMRIE-C reagent (GibcoBRL) complexed with pSwick plasmid, carrying each CFTR variant cDNA. Cells were transfected at 37°C for 2 hours, with slow rotation and plated on Millicell –HA
permeable cell culture inserts (pore size 0.45 µm, Millipore Co.). Transepithelial chloride currents were recorded 4 days following transfection.

**Generation of FRT Stable Cell Lines.** FRT cells were co-transfected with pSwick CFTR and pcDNA plasmid (Invitrogen Co.), using the DMRIE-C reagent. Clonal cell lines resistant to zeocin (Invitrogen Co.) were expanded and screened for CFTR expression, using short-circuit chloride current measurements in Ussing chambers.

**Western Blot Analysis of CFTR Protein**

**Cell Lysates.** For analysis of CFTR processing, transiently transfected HeLa cells were washed three times with phosphate buffer saline (PBS) and lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH 8.0), containing a protease inhibitor cocktail (1 mM benzamidine, 5 µg/ml pepstatin, 5 µg/ml leupeptin, 1 µg/ml aprotinin and 17.4 µg/ml PMSF). After unbroken cells and nuclei were discarded by low speed centrifugation, proteins in the lysates were denatured in SDS-gel loading buffer and stored at –20°C.

**Protein Enriched Fraction.** Cells growing on 100 mm plates were washed three times with PBS, collected in 2 ml of PBS, pelleted and resuspended in ice cold 1 ml TEAA buffer (20 mM Tris/HCl pH 8.0, 1 mM EDTA, 3 mM EGTA), supplemented with protease inhibitor cocktail (1 mM benzamidine, 5 µg/ml pepstatin, 5 µg/ml leupeptin, 1 µg/ml aprotinin and 17.4 µg/ml PMSF). After 5 min incubation in TEAA, the cell suspension was passed ten times through a 27-gauge needle. Nuclei and cell debris were pelleted (5 min/ 4,000 g) and discarded, and the cleared supernatant was submitted to high speed centrifugation (100,000 g/ 30 min). The pelleted membranes were resuspended in SDS-gel loading buffer and stored at –20°C.

**Immunoblot analysis.** Fifty micrograms of total protein were separated by SDS-PAGE on 6% gels. Proteins were electrophoretically transferred to nitrocellulose membranes.
CFTR variants were probed with the monoclonal antibody M3A7, which recognizes an epitope within the CFTR NBD2 (9). A secondary anti-mouse IgG antibody peroxidase conjugate (Jackson ImmunoResearch Laboratories Inc.) was added and protein bands were visualized using ECL (Amersham Pharmacia Biotech Limited).

Electrophysiology

FRT cell lines stably expressing CFTR variants or transiently transfected were plated at 2.5 x 10^5 cells/cm² on Millicell-HA cell culture inserts. Transepithelial resistance was monitored daily (Millicell Electrical Resistance System, Millipore Co.) and was typically greater than 3000 Ω/cm² after 4 days. FRT monolayers were mounted in modified Ussing chambers (Jim’s Instruments, Iowa City, IA), and continually gassed with O₂. Temperature was maintained at 37°C. Transepithelial chloride gradient was imposed by bathing the basolateral surface with a recording solution containing (in mM): 135 NaCl, 1.2 CaCl₂, 1.2 MgCl₂, 2.4 K₂HPO₄, 0.6 KH₂PO₄, 10 HEPES and 10 dextrose, pH 7.4, and the apical surface with a similar recording solution, except that 135 mM sodium gluconate replaced the 135 mM NaCl, bringing the chloride concentration to 4.8 mM. The potential difference between the potential sensing electrodes was compensated. The transepithelial voltage was clamped to zero (Voltage Clamp Channel Module, model 558C-5, Dept. of Bioengineering, University of Iowa), transepithelial resistance was monitored by recording of current deflections in response to 2s pulse of 1 or 5 mV every 50 seconds. The short circuit currents were recorded continuously on a charter recorder (Model SR6335, Western Graphtec, Inc.). After a stable baseline current was observed (usually within less than 10 minutes), IBMX, forskolin and genistein (Sigma) were added to the apical chamber, as described for each experiment, and the current reflecting the flow of chloride promoted by its concentration gradient (Isc) was recorded as a downward deflection. The Isc was calculated as the difference
between the baseline and the sustained phase of the response (plateau), or the peak current. Values were normalized by the area of the insert (0.6 cm²) and results were then expressed in µA/cm².

Statistical Analysis

Data is expressed as mean ± s.e.m. Current values were compared before normalization, by the Student's t-test, or by one-way ANOVA with a Student-Newman-Keuls' (SNK) follow up test, at a 95% (p<0.05) or greater confidence level.

Results

Isolation of ΔF508 suppressor mutations using the STE/CFTR chimera system in yeast

A STE6/CFTR hybrid gene (H5-wt), in which a region coding for 74 amino acid residues within NBD1 of STE6 (R441 to I516) was replaced by the corresponding region of the CFTR NBD1 (F494 to L558), has been previously described (28). H5-wt complements the yeast ste6Δ mutation (JPY201 strain), restoring a-factor transport and consequent mating. However, when the ΔF508 mutation was introduced in the CFTR portion of H5 (H5-ΔF508), the mating efficiency, assessed by a quantitative mating assay, was reduced to 0.28% of H5-wt (Table 1) (28). Because the ΔF508 mutation can be modeled in yeast, this system can be used for the identification of second site mutations within the CFTR NBD1 region that restore a-factor transport function to the H5-ΔF508 chimera. To generate random point mutations, the entire CFTR portion of H5-ΔF508 was subjected to in vitro PCR mutagenesis and introduced into JPY201 yeast. Transformants were screened using a qualitative mating assay to identify colonies with an increased mating efficiency relative to H5-ΔF508. Plasmids yielding a revertant phenotype were rescued and reintroduced into JPY201 to confirm the phenotype by a quantitative mating assay. Plasmids associated with improved mating efficiencies were
sequenced. Two novel point mutations were isolated in the CFTR sequence that substantially rescued the H5ΔF508 mating defect (Table 1), resulting in change of Ile 539 of CFTR sequence to a Thr residue (I539T) and of Gly to Glu at the 550 position (G550E).

Mutations I539T and G550E partially rescue CFTR ΔF508

The two novel ΔF508 revertant mutations isolated in yeast were located either just upstream (I539T) or within (G550E) the CFTR NBD1 signature motif (Figure 1). Interestingly, the three ΔF508 revertant mutations previously isolated using the STE6/CFTR system, R553Q, R553M and R555K, are also located within the NBD1 signature motif (28,29). In order to evaluate the effect of the novel revertant mutations on CFTRΔF508 processing, I539T and G550E mutations were introduced into the full length CFTRΔF508 cDNA (ΔF/I539T and ΔF/G550E) for expression in mammalian cells. To test whether the combination of the I539T and G550E mutations would result in an additive or synergistic effect in correcting the ΔF508 phenotype, we also constructed a CFTRΔF508 allele containing both revertant mutations (ΔF/DB). The CFTR variants were expressed in HeLa cells using a vaccinia virus hybrid expression system, and the steady-state level of CFTR protein in the cell lysates was analyzed by SDS-PAGE followed by Western analysis (Figure 2A). Only the core-glycosylated ER form of CFTR, referred to as “band B”, was observed for CFTRΔF508. CFTR wt was present as both band B and “band C” forms. The latter represents mature CFTR that trafficked to the Golgi where complex oligosaccharide processing takes place. We observed that I539T, and to a lesser extent G550E, partially rescued the CFTRΔF508 processing defect. The steady-state level of mature CFTRΔF/DB protein was higher compared to each revertant alone (Figure 2A).
Next, we assessed the phosphorylation-activated chloride channel function of the fraction of CFTR\(\Delta F508\) revertants present in the mature form at the plasma membrane. Mutant and wildtype CFTR alleles were transiently expressed in Fischer rat thyroid (FRT) epithelial cells, which are well suited for this purpose, since they polarize to form monolayers with high transepithelial resistance and do not express a cAMP-activated chloride channel (48,49). CFTR channels are routinely activated by a cAMP-agonist cocktail containing 100 \(\mu M\) IBMX and 10 \(\mu M\) forskolin (48). Four days after transfection, FRT monolayers were mounted in modified Ussing chambers and the transepithelial short circuit chloride current \(I_{sc}\) was recorded following activation with 10 \(\mu M\) forskolin and 100 \(\mu M\) IBMX. In the absence of CFTR expression, FRT monolayers remain impermeable to chloride in response to cAMP agonists (48). In contrast, FRT cells transfected with CFTR wt respond to cAMP agonists with a rapid increase in \(I_{sc}\) reflecting increased chloride permeability (48). Transepithelial chloride permeability was markedly decreased in FRT monolayers expressing CFTR\(\Delta F508\), to 0.76 % of CFTR wt \(I_{sc}\) (Figure 2B). The \(\Delta F508\) revertants CFTR\(\Delta F/I539T\) and CFTR\(\Delta F/G550E\) exhibited 6-fold and 12-fold increase in chloride current relative to CFTR\(\Delta F508\), respectively (Figure 2B). The combination of the two revertant mutations (CFTR\(\Delta F/DB\)) resulted in 29 % of CFTR wt chloride currents (Figure 2B), representing a substantial rescue (38-fold) of the chloride impermeability characteristic of epithelia expressing CFTR\(\Delta F508\).

To assess the effect of the \(\Delta F508\) revertant mutations on CFTR wt chloride channel function, CFTRG550E, CFTRI539T and a CFTR allele containing both I539T and G550E mutations (CFTRDB), were transiently expressed in FRT cells and chloride current was measured after activation with 10 \(\mu M\) forskolin and 100 \(\mu M\) IBMX. Results in Figure 2C indicate that chloride currents mediated by CFTRI539T and CFTRG550E
were not significantly different from CFTR wt. However, CFTRDB produced currents 50% higher than CFTR wt.

The G550E Mutation Increases the Sensitivity of CFTR and CFTR ∆F508 to Activation by cAMP-Agonists in FRT Cells Transiently Expressing CFTR

As shown in Figure 2B, the G550E mutation was more effective than I539T in restoring the chloride channel function of CFTR∆F508 (12-fold vs. 6-fold increase), yet CFTR∆F/G550E cell lysates contained lower levels of mature protein relative to CFTR∆F/I539T (Figure 2A). Interestingly, the G550E mutation occurs in a conserved residue, changing the core consensus ABC signature motif from LSGGQ to LSEGQ. The functional importance of the NBD1 signature motif is evident from the characterization of the CF causing mutation G551D, which does not affect processing, but results in decreased CFTR chloride channel function (41,50) (Table 2).

To better understand the mechanism by which the G550E mutation improves function of CFTR∆F508, we tested its effect on activation of CFTR∆F508 and CFTR wt by suboptimal concentrations of forskolin. CFTR expressed in FRT cells is maximally stimulated with 10 µM forskolin (48), with lower concentrations resulting in decreased channel activity (49). Accordingly, CFTR wt, CFTR∆F508, CFTRG550E and CFTR∆F/G550E were transiently expressed in FRT cells, and the transfected cell monolayers were assayed for transepithelial chloride current in response to activation by sub-optimal concentration of forskolin (0.5 µM), in the absence of IBMX. The results for each variant were expressed as the percentage of maximum chloride current, with the maximum defined by activation of the channels with 10 µM forskolin and 100 µM IBMX (Figure 3). The G550E mutation substantially increases the sensitivity of CFTR∆F508 to PKA activation, increasing the level of chloride current activated by the sub-optimal concentration of forskolin (0.5 µM) from 4.66 % of maximum activation for CFTR∆F508
to 29.25 % for CFTR ΔF508/G550E (Figure 3A). Although the G550E mutation did not increase the chloride channel activity of CFTR wt when the channels were activated with the optimal concentration of cAMP agonists (Figure 2C), we observed that G550E did increase the sensitivity of CFTR wt to activation by the sub-optimal concentration of forskolin (compare 49.54% vs. 77.2% of maximum $I_{sc}$ for CFTR wt and CFTR G550E, respectively) (Figure 3A).

**Characterization of FRT cell lines stably expressing the CFTRΔF508 revertants**

We obtained FRT cell lines stably expressing CFTRΔF/I539T, CFTRΔF/G550E, and CFTRΔF/DB, to further characterize the effect of the revertant mutations on CFTRΔF508 processing and function. FRT-CFTRΔF508 and FRT-CFTR wt stable cell lines have been previously described (48). Results from CFTR immunoblotting analysis (Figure 4A) and functional studies (Figure 4B) confirmed the suppression of the CFTR ΔF508 processing and chloride impermeability defects by I539T and G550E mutations, as observed for the transient expression experiments (Figures 2A and 2B). Functional assays showed a 13-fold increase in transepithelial chloride current for FRT-CFTRΔF/I539T in relation to FRT-CFTRΔF508, which is in agreement with the substantial amount of mature protein observed for this cell line (Figures 4A and 4B). While the $I_{sc}$ was not significantly different between FRT-CFTRΔF/DB and FRT-CFTRΔF/I539T, we observed decreased levels of both mature (band C) and immature (band B) protein for FRT-CFTRΔF/DB. Functional studies showed a 6.3-fold increase in $I_{sc}$ for FRT-CFTRΔF/G550E relative to FRT-CFTR ΔF508 (Figure 4B), although the mature band C was barely detectable and the steady state levels of band B were decreased for the revertant (Figure 4A). These results suggest that the G550E mutation increases the channel activity of CFTR variants containing the ΔF508 mutation.

**Temperature sensitivity of the ΔF508 revertants**
The CFTRΔF508 processing defect can be partially reversed by incubating mammalian cells at 25-30°C (14), or by expressing the mutant in cells that are usually incubated at lower temperature, such as insect cells (51) and *Xenopus* oocytes (52). We also investigated the effect of the revertant mutations I539T and G550E on the temperature-sensitivity of CFTR ΔF508. To detect the rescue of CFTRΔF508 by low temperature treatment and study the effect of the revertant mutations on the temperature sensitivity of CFTRΔF508, FRT monolayers stably expressing each CFTR variant were incubated at 37°C for five days, followed by a 48 hour incubation at 30°C prior to I_{sc} measurements, which were performed at physiological temperature (37°C) (Figure 4B). Under these conditions, low temperature treatment of FRT-CFTR ΔF508 resulted in 4.8-fold increase in I_{sc} (Figure 4B). Also consistent with the results by others (14,53), we did not observe an increase in cAMP activated chloride current of FRT monolayers expressing CFTR wt after low temperature treatment (not shown). The G550E mutation attenuated the temperature sensitivity of CFTRΔF508, as the low temperature treatment resulted in a two-fold increase in chloride current for CFTRΔF/G550E. The I539T mutation rendered CFTRΔF508 and CFTRΔF/G550E insensitive to incubation at 30°C (Figure 4B). The I_{sc} measured after low temperature treatment of FRT-CFTRΔF508 (12.27 μA/cm²) was comparable to the I_{sc} of FRT-CFTRΔF/G550E incubated at physiological temperature (15.57 μA/cm²) (Figure 4B).

**Sensitivity of the CFTR ΔF508 revertants to cAMP activation**

The increase in sensitivity to forskolin activation observed for the CFTRΔF/G550E mutant in transient expression (Figure 3) we hypothesize to be an intrinsic property of the mutant channel. Alternatively, it is possible that the enhanced sensitivity to activation is secondary to a cooperative interaction among the increased number of mutant channels at the plasma membrane (54). To assess the effect of
G550E and I539T on the modulation of sensitivity of CFTRΔF508 to activation, while minimizing the potential effect of different channel levels at the plasma membrane, we compared the sensitivity to forskolin activation of CFTR ΔF508 rescued by incubation for 48h at 30°C, with CFTRΔF/G550E, CFTRΔF/I539T and CFTRΔF/DB incubated at 37°C. FRT monolayers stably expressing each CFTR variant were mounted in Ussing chambers and the cAMP-activated chloride current was measured in response to decreasing concentrations of forskolin. The results are expressed as percentage of maximum I_{sc} (obtained by activation with 10 µM forskolin and 100 µM IBMX) for each variant. The results demonstrate that CFTR variants containing the G550E mutation have increased sensitivity to forskolin activation (Figure 4C). When CFTRΔF/G550E, incubated at physiological temperature, was compared with CFTRΔF508 we observed a significant increase in sensitivity to activation by 0.5 µM forskolin, confirming the results from transient expression (Figure 3). FRT-CFTRΔF/DB (containing both I539T and G550E) also exhibited a significant increase in sensitivity to activation relative to FRT-CFTRΔF508 over the entire range of suboptimal forskolin concentrations tested. However, in contrast to the variants containing G550E, increased sensitivity to activation by suboptimal concentrations of cAMP agonist was not observed for the variant containing I539T alone.

**The G550E Mutation Increases the Sensitivity of CFTR wt to Activation by cAMP-Agonists in FRT Cells Stably Expressing CFTR**

Next, we investigated the dose-response for forskolin activation of CFTR wt and CFTRG550E stably expressed in FRT cells. Polarized FRT monolayers expressing CFTR wt and CFTRG550E (Figure 5a) were mounted in Ussing chambers and activated with decreasing amounts of forskolin. The resulting I_{sc} values were expressed as percentage of maximum I_{sc}, achieved by activation with 10 µM forskolin and 100 µM IBMX.
IBMX. The dose-response curve for activation by forskolin shows a remarkable increase in sensitivity for FRT-CFTRG550E, relative to CFTR wt, over the entire sub-optimal concentration range tested (0.1 to 1µM) (Figure 5B), confirming the results obtained with the transient expression experiments (Figure 3).

Additional ΔF508 Revertant Mutations at Position G550

The significant rescue of the ΔF508 defect by the G550E mutation prompted us to screen for other ΔF508 revertant mutations at this codon, using site-directed mutagenesis. Additional revertant of the ΔF508 defect, G550H, was identified by the yeast mating assay (not shown). Because the G550E mutation replaces a Gly residue with the negatively charged Glu, we also constructed a CFTRΔF/G550D variant to test whether the negative charge resulting from an aspartate substitution would have a similar effect on CFTR channel activation. The effect of mutations at position G550 on chloride channel function of CFTRΔF508 was investigated by I_sc measurements of FRT monolayers transiently expressing each variant. Results were expressed as percentage of cAMP-activated I_sc measured for monolayers expressing CFTR wt (Table 2). CFTR variants bearing CF-causing mutations within the NBD1 signature motif, G551D and S549R, were included in the experiment and produced low I_sc, as expected. A significant increase in I_sc for monolayers expressing each novel ΔF508 revertant at position G550, relative to CFTRΔF508 was observed. CFTRΔF/G550H and CFTRΔF/G550D displayed 50% and 68% of CFTRΔF/G550E I_sc, respectively, demonstrating that these additional revertants were not as effective as G550E in suppressing the CFTR ΔF508 defect. We tested the CFTRΔF/G550D response to activation by low concentration of forskolin (0.5 µM), as described in Figure 3. However, unlike the results observed for CFTRΔF/G550E, suboptimal forskolin concentration failed to activate CFTRΔF/G550D (not shown).
The G550E Mutation Modulates the CFTR ΔF508 Response to Activation by Genistein and Millimolar Concentration of IBMX

Several compounds have been isolated that optimize channel activity of phosphorylated CFTR, by mechanisms that are independent of increase in cAMP (55,56). The effect of the G550E mutation to increase sensitivity to cAMP-mediated activation of mutant and wt CFTR led us to ask if this mutation would modulate the response of CFTRΔF508 channels to optimization by two such compounds. We tested the effect of 2 mM IBMX and 50 μM genistein on the PKA-dependent activity of CFTRΔF508, CFTRΔF/I539T, CFTRΔF/G550E, CFTRΔF/DB and CFTR wt. FRT stable cell lines expressing the CFTR variants, including CFTRΔF508, were incubated at physiological temperatures for these experiments, since genistein and high concentrations of IBMX are reported to enhance the channel activity of even small amounts of mutant CFTR present at the plasma membrane (25). Genistein, a natural isoflavone compound, enhances PKA-dependent activity of wild type and mutant CFTR in several cell types (22,24,25,55,57). Optimal concentrations of genistein have been reported to be around 50 μM (58,59). Fifty micromolar genistein was added to the apical side of FRT monolayers mounted in Ussing chambers, and 5 minutes later the channels were activated with a cAMP-agonist cocktail (10 μM forskolin and 100 μM IBMX). Under these conditions, we did not observe channel activation by genistein alone for any of the FRT cell lines. Genistein significantly increased the PKA-activated I_{sc} for all the cell lines containing the ΔF508 mutation, although it produced a smaller increase for cell lines containing the G550E mutation: compare 58% and 70% increase for CFTRΔF508 and CFTRΔF/I539T, respectively, with 45% and 25% for CFTRΔF/G550E and CFTRΔF/DB (Figure 6). We did not observe enhancement of PKA-activated chloride currents for FRT-CFTR wt by genistein under the experimental conditions employed (Figure 6).
When present at millimolar concentrations, the xanthine derivative IBMX has been shown to have an effect on CFTRΔF508 activity that is independent from its well-established activity as a phosphodiesterase (PDE) inhibitor (23,24,55,60). FRT monolayers stably expressing the CFTR variants were mounted in Ussing chambers and activated with 10 µM forskolin and 2 mM IBMX. Two millimolar IBMX resulted in approximately 80% increase in I_sc for FRT-CFTRΔF508 and FRT-CFTRΔF/I539T (Figure 6). Interestingly, 2 mM IBMX did not affect the PKA-activated I_sc of the FRT-CFTR wt, FRT-CFTRΔF/G550E or FRT-CFTRΔF/DB under the experimental conditions employed.

Discussion

Two novel ΔF508 revertant mutations were identified just upstream (I539T) and within (G550E) the core consensus ABC signature motif LSGGQ in the NBD1 of CFTR. Each mutation partially restored processing of mutant CFTRΔF508 expressed in HeLa cells, with I539T being the most effective. Increased cAMP-activated chloride permeability was also observed in FRT monolayers expressing CFTRΔF/I539T and CFTRΔF/G550E; to levels 6-fold and 12-fold higher than CFTRΔF508, respectively. The larger fraction of processed CFTRΔF/I539T and CFTRΔF/G550E observed relative to CFTRΔF508 thus represents functional channels localized at the plasma membrane. Furthermore, functional studies using a double revertant allele (CFTRΔF/DB) showed that I539T and G550E mutations act synergistically to increase CFTRΔF508 chloride currents to approximately 29% of CFTR wt, representing a 38-fold increase over the CF-mutant. Processing of CFTRΔF/DB, as indicated by the fully glycosylated form of CFTR, was correspondingly increased. Therefore, the processing defect of CFTRΔF508 was substantially rescued by second-site mutations in the region of the LSGGQ motif of
NBD1, leading to increased functional activity of mutant channels at the plasma membrane.

The I539T and G550E mutations were identified as revertants of the CF-causing mutation ΔF508. It might thus be expected that these revertant mutations, identified by virtue of their effects to reverse the ΔF508 defect, would be specific for suppression of ΔF508. However, results by others suggest that G550E can partially rescue another processing-defective CF mutant, A561E (61). Possibly, the ΔF508 and A561E mutations cause misfolding in a similar manner, allowing each to be partially compensated by G550E. Alternatively, the suppressors may have a general effect to increase CFTR processing. In support of the latter possibility, it was observed that the combination of I539T and G550E within wildtype CFTR increased functional activity. Further experiments will be necessary to determine the extent to which ΔF508 revertant mutations I539T and G550E suppress other CF-mutations within the NBD1 that are associated with defective protein processing.

To further assess the effects of the revertant mutations on CFTRΔF508, we compared the functional activity of CFTRΔF508, CFTRΔF/G550E, and CFTRΔF/I539T under various experimental conditions. Because ΔF508 is a temperature sensitive mutation (14), we determined whether revertant mutations alter the temperature-sensitivity of CFTRΔF508. The I539T mutation, when introduced in either CFTRΔF508 or CFTRΔF/G550E, rendered these variants insensitive to low temperature treatment. It is possible that the I539T revertant mutation and the low temperature treatment stabilize the same step in the mutant protein folding pathway; thus their effects are not additive. The temperature insensitivity of CFTRΔF508 rendered by suppressor mutations has also been observed by others (62). In contrast to I539T, G550E had little effect on
CFTRΔF508 temperature-sensitivity, suggesting that it affects the protein folding pathway differently.

In addition to the processing defect, the ΔF508 mutation also impairs the chloride channel function of CFTR. The channel $P_o$ of CFTRΔF508 is decreased relative to CFTR wt (21,23), due to prolonged closed times, which are also observed for CFTR wt activated by suboptimal concentrations of forskolin (25). Results from patch-clamp studies using NIH 3T3 cells stably expressing CFTR and CFTR ΔF508 indicate that the ΔF508 mutation affects CFTR activation by attenuating PKA-dependent phosphorylation (22). Our results show that the G550E mutation decreased the concentration of forskolin required for half-maximal stimulation of all the CFTR variants tested: CFTR wt, CFTRΔF508, CFTRΔF/I539T. Furthermore, G550E mutation improved PKA-dependent activity of CFTR variants bearing the ΔF508 mutation under optimal and suboptimal forskolin concentration, and improved the wildtype channel activity only under suboptimal concentrations of forskolin, but not when maximal PKA activity was promoted, suggesting that this mutation could specifically increase function of underphosphorylated CFTR. Alternatively, G550E could facilitate CFTR phosphorylation under suboptimal PKA activity. Although the role of G550E in CFTR phosphorylation remains to be determined, the effect of G550E to increase the PKA-dependent activity of CFTRΔF508 is consistent with the higher levels of function associated with CFTRΔF/G550E relative to the low levels of processed protein observed.

Because IBMX and genistein are known to enhance the functional activity of CFTRΔF508 (23-25), we assessed effect of these molecules on CFTRΔF/G550E, CFTRΔF/I539T and CFTRΔF/DB. Our results show that 2 mM IBMX caused an increase (1.8-fold) in PKA stimulated chloride current for CFTR ΔF508. In other studies addressing the effect of IBMX on CFTRΔF508, it has similarly been shown that 2 mM
IBMX increases activity of the CFTR ΔF508 channel activated with 10 μM forskolin, without inducing further increase in cAMP (55). We further observed no detectable effect of IBMX on CFTR wt, which is consistent with functional studies in Xenopus oocytes (52) and with the observation that IBMX has a higher affinity for NBD1 ΔF508 relative to NBD1 wt (63). The effect of 2 mM IBMX on the activation of CFTRΔF/I539T was similar to the effect observed for CFTRΔF508. However, 2 mM IBMX did not increase the PKA-dependent currents of FRT-CFTRΔF/G550E or FRT-CFTRΔF/DB. We speculate that G550E could directly alter the binding of IBMX to CFTRΔF508, impairing increase in P₀ or further contributing to the decrease of current amplitude (24). Genistein significantly enhanced PKA-activated chloride currents of CFTRΔF508, CFTRΔF/G550E, CFTRΔF/I539T, and CFTRΔF/DB, although the currents mediated by CFTR variants containing the G550E mutation were stimulated to a lesser extent. It has been shown that genistein increased the iodide efflux from NIH 3T3 cells expressing CFTRΔF508 following activation with a range of forskolin concentrations (0.01 to 100 μM), but increased CFTR wt mediated efflux only when the channels were activated by suboptimal concentrations of forskolin (25). Genistein has been shown to activate specifically underphosphorylated CFTR (64), and to cause substantial increase in CFTRΔF508 channel P₀ (22). The revertant mutations I539T and G550E did not preclude genistein enhancement of the PKA-dependent activity of CFTRΔF508, implying that, similarly to the CF mutant, the revertants could be underphosphorylated at maximal PKA activity.

I539T significantly enhanced the processing of CFTRΔF508. Notably, Thr is the most conserved amino acid residue at position 539 for CFTR of most non-primate species, as well as for other ABC transporters, including members of the MRP/CFTR subgroup (ABC C) (Figure 7). A significant level of CFTRΔF508 chloride channel activity
and detectable level of mature protein have been observed for the homozygous ΔF508 CF mouse (65-68). Our results suggest that the T539 could contribute to the attenuated defect caused by the ΔF508 mutation in the murine CFTR. G550 is a conserved residue within the ABC signature motif of several ABC transporters, including those with high homology to human CFTR (Figure 7). The G550E mutation represents a non-conservative introduction of a negatively charged Glu residue, changing the LSGGQ core consensus signature sequence of NBD1 to LSEGQ. Interestingly, LSEGQ is the core signature motif found in few members of the human ABC A subgroup: NBD of ABCA6 (69) and NBD2 of ABCA8 and ABCA9 (Accession Numbers NP009099 and XP085646, respectively).

The highly conserved signature motif fulfills an essential role in the functioning of ABC transporters. Functions assigned to this motif include coupling of energy to translocation (6,70), activation of ATP hydrolysis following substrate binding to other components of the transporter (71), and mediation of intra-domain interactions (72,73). Direct participation of the ABC signature motif in the ATP-dependent dimerization of NBDs has also been suggested (74-76). The CF-causing mutation of the invariant Gly residue in the NBD1 signature motif of CFTR (LSGGQ), G551D, has been shown to reduce ATP binding (41,77) and hydrolysis (18). Crystallographic structures determined for several ABC cassettes (74,78) reveal a similar subdomain organization: an ATP binding pocket formed by the Walker A and Walker B motifs, and a α-helical subdomain containing the ABC signature motif and upstream sequences. Thus, sequences corresponding to the CFTR NBD1 region that includes the F508 residue and the revertant mutations (I539-G550) are localized to the same α-helical subdomain. The position of LSGGQ in the ABC cassette structure is in agreement with the proposed function in coupling the activities of the catalytic domains with those involved in
regulation or transport (79). We speculate that G550E and I539T mutations could restore the LSGGQ mediated interactions disrupted by the ΔF508 mutation.

The functional importance of the LSGGQ region of CFTR NBD1 has been supported by previous mutational analysis, describing processing and functional defects associated with amino acid substitutions in the conserved motif (37-41). Our results indicate that mutations in this region also enhance CFTRΔF508 processing and function, and highlight the importance of the LSGGQ motif as a focus for understanding the defect associated with ΔF508. Elucidation of the role of the LSGGQ motif to mediate the defects associated with ΔF508 may be instrumental in the design of new therapies for CF.

Acknowledgements

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References


Figure Legends

**Figure 1. Schematic representation of CFTR.** The positions of F508, I539 and G550 amino acid residues within CFTR NBD1 are indicated. The shadowed boxes show the location of Walker A and Walker B motifs and the hatched box shows the location of the C motif, also referred to as ABC signature motif.

**Figure 2. The I539T and G550E mutations partially rescue CFTR ΔF508 processing and functional defects.** A) The steady-state level of CFTR protein resulting from transient expression in HeLa cells. HeLa cells were infected with vTF7-3 and transfected with pTM plasmid carrying each CFTR variant cDNA. Cell lysates were obtained 18 hours after transfection and 50 µg protein samples were separated by SDS-PAGE. Western blots were probed with anti-CFTR monoclonal antibody M3A7. HeLa cells infected with vTF7-3 served as control. Positions of the core glycosylated (band B) and complex glycosylated CFTR (band C) are indicated by the arrows. B) FRT monolayers transiently expressing the CFTRΔF508 variants were incubated in permeable supports for 4 days at 37°C. Monolayers were mounted in Ussing chambers and transepithelial chloride currents were recorded after activation with 10 µM forskolin and 100 µM IBMX. The results are expressed as percentage of chloride current mediated by CFTR wt (26.40 ± 2.79 µA/cm², n=20), after subtracting the background current observed for non-transfected FRT monolayers (0.25 ± 0.02 µA/cm²), and represent mean ± s.e.m. for the number of experiments shown (n). Non-normalized current values were compared by one-way ANOVA and the asterisks indicate the CFTR variants presenting a significant increase in chloride current over CFTRΔF508 (α=0.05), by SNK follow-up test. C) Chloride currents mediated by CFTR variants were assayed...
as in (B). Non-normalized current values were compared by one-way ANOVA and the asterisks indicate the CFTR variants presenting a significant increase in chloride current over CFTR wt (α=0.05), by SNK follow-up test.

Figure 3. The G550E mutation increases sensitivity of CFTR wt and ∆F508 to activation by forskolin following transient expression in FRT cells. A) FRT monolayers transiently expressing the CFTR variants were incubated in permeable supports for 4 days at 37°C. Monolayers were mounted in Ussing chambers and transepithelial chloride currents were recorded after activation with a sub-optimal forskolin concentration (0.5 µM). Results are expressed as percentage of the maximum current for each variant, achieved with activation by 10 µM forskolin and 100 µM IBMX, and represent mean ± s.e.m. for n = 6 experiments. Current ratios were compared by one-way ANOVA and the asterisks indicate significant difference between the two CFTR variants (α=0.05), by SNK follow-up test. B) Representative tracings for no CFTR control, CFTR∆F508 and CFTR∆F/G550E. Currents were recorded continuously and the arrows indicate when forskolin and IBMX were added. The dashed lines represent the baselines. C) Representative tracings for CFTR wt and CFTR G550E, as described in (B).

Figure 4. Effect of I539T and G550E mutations on CFTR ∆F508 temperature sensitivity and dose-response to forskolin activation in FRT stable cell lines. A) Western Blot analysis of the steady-state level of CFTR ∆F508, CFTR∆F/I539T, CFTR∆F/G550E and CFTR∆F/DB stably expressed in FRT cells. A membrane enriched fraction for each cell line was obtained and 50 µg protein samples were separated by SDS-PAGE. Western blots were probed with anti-CFTR antibody M3A7, as described in
Figure 2A. B) Effect of low temperature on transepithelial chloride current for the cell lines in (A). FRT cells stably expressing each CFTR variant were grown on permeable supports for seven days at 37°C (solid bar) or for five days at 37°C followed by 48 h at 30°C (stripped bar). The polarized monolayers were mounted on Ussing chambers and the transepithelial chloride current was measured after activation with 10 µM forskolin and 100 µM IBMX. Results represent mean ± s.e.m. for n = 6 experiments. The asterisks indicate the variants for which the low temperature treatment resulted on a significant increase in current over the control monolayers (37°C) (p<0.05, t-test). C) Dose-response curve for forskolin activation. Monolayers of FRT stable cell lines described in (A) and (B) were mounted on Ussing chambers and activated with 10 µM IBMX and decreasing concentrations of forskolin. FRT-CFTR∆F508 monolayers were incubated for five days at 37°C followed by 48 h at 30°C; the other FRT cell lines were incubated for seven days at 37°C. Results are expressed as percentage of maximum activation, as in Figure 3A. Results represent mean ± s.e.m. for n = 6 measurements.

Figure 5. Effect of G550E mutation on CFTR dose-response to forskolin activation in FRT stable cell lines. A) Western Blot analysis of the steady-state level of protein for CFTR wt and CFTR G550E stably expressed in FRT cells, as described in Figure 2A. Transepithelial chloride currents for each CFTR variant were recorded after activation with 10 µM forskolin and 100 µM IBMX (I_{sc}), results are shown as mean ± s.e.m for n = 6 experiments. B) Dose-response curve for forskolin activation. Monolayers of FRT stable cell lines described in (A) were incubated for seven days at 37°C and mounted on Ussing chambers. CFTR channels were activated with decreasing concentrations of forskolin. Results are expressed as percentage of maximum activation. Results represent mean ± s.e.m. for n = 6 experiments.
Figure 6. Effect of the I539T and G550E mutations on CFTR ΔF508 activation by 2 mM IBMX and 50 μM genistein. FRT monolayers stably expressing each CFTR variant were mounted in Ussing chambers and activated with 10 μM forskolin and 2 mM IBMX (solid bar), or 50 μM genistein, followed by 10 μM forskolin and 100 μM IBMX (stripped bar). Results are shown as mean ± s.e.m for n = 6 experiments and are expressed as percentage of control currents for each cell line, achieved by activation with 10 μM forskolin and 100 μM IBMX. The asterisks indicate that the I sc values were significantly different from control for each CFTR variant (t-test, p<0.05).

Figure 7. Alignment of peptide sequences from various ABC transporters to the LSGGQ region of human CFTR NBD1. A multiple alignment sequence of NBD1 of CFTR from several species and other highly homologous ABC transporters was generated by the Pileup program (Wisconsin Package Version 10.2. Genetics Computer Group). The positions of the ΔF508 revertant mutations are indicated. CFTR sequences are from Mus musculus (mouse), Ovis aries (sheep), Bos taurus (bovin), Oryctolagus cuniculus (rabbit), Xenopus laevis (xenla), and Squalus acanthias (squac); sulfonylurea receptor 1 (acc8), from Ratus norvergicus (rat) and Homo sapiens (human); multidrug resistance-associated protein 6 (MRP6) from Ratus norvergicus (rat) and Homo sapiens (human).
Tables

Table 1. Yeast mating efficiency mediated by the STE6/CFTR chimeras.

Results of quantitative mating are expressed as percentage of H5 wt, and represent the mean ± s.e.m. for n = 3 experiments.

<table>
<thead>
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<th>STE6/CFTR (H5) variant</th>
<th>Mating efficiency (% of H5)</th>
</tr>
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<tbody>
<tr>
<td>∆F508</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>∆F508/I539T</td>
<td>42.70 ± 0.40</td>
</tr>
<tr>
<td>∆F508/G550E</td>
<td>79.90 ± 4.50</td>
</tr>
</tbody>
</table>
Table 2. Effect on CFTR-mediated transepithelial chloride currents of CF-causing mutations and ∆F508 revertant mutations within the LSGGQ motif.

FRT cells monolayers transiently expressing the CFTR variants were mounted in Ussing chambers and chloride current values were measured, as described in Figure 2B. Results are expressed as percentage of CFTR wt chloride and represent mean + s.e.m. for the number of experiments indicated (n). The asterisks indicate the variants presenting a significant increase in chloride current over CFTR∆F508 (ANOVA, α=0.05), by SNK follow-up test.

<table>
<thead>
<tr>
<th>CFTR variant</th>
<th>I sc (% of CFTR wt)</th>
<th>n</th>
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<tbody>
<tr>
<td>CFTR S549R</td>
<td>1.70 ± 0.11</td>
<td>4</td>
</tr>
<tr>
<td>CFTR G551D</td>
<td>1.17 ± 0.12</td>
<td>4</td>
</tr>
<tr>
<td>CFTR ∆F508</td>
<td>0.76 ± 0.07</td>
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<td>14  (*)</td>
</tr>
<tr>
<td>CFTR ∆F/G550D</td>
<td>6.06 ± 0.63</td>
<td>12  (*)</td>
</tr>
<tr>
<td>CFTR ∆F/G550H</td>
<td>4.17 ± 0.40</td>
<td>8   (*)</td>
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</table>
Fig. 5

A

- band C
- band B

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>G550E</th>
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<tr>
<td>Isc (µA/cm²)</td>
<td>51.44 ± 4.62</td>
<td>177.75 ± 3.04</td>
</tr>
</tbody>
</table>

B

[Graph showing forskolin concentration (µM) vs. I_sc/I_max (%)]
Fig. 7
Mutations in the NBD1 signature motif region rescue processing and functional defects of CFTR deltaF508
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