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Original Article

Ivacaftor potentiation of multiple CFTR channels with gating mutations

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Abstract

Background: The investigational CFTR potentiator ivacaftor (VX-770) increased CFTR channel activity and improved lung function in subjects with CF who have the G551D *CFTR* gating mutation. The aim of this in vitro study was to determine whether ivacaftor potentiates mutant CFTR with gating defects caused by other *CFTR* gating mutations.
Methods: The effects of ivacaftor on CFTR channel open probability and chloride transport were tested in electrophysiological studies using Fischer rat thyroid (FRT) cells expressing different *CFTR* gating mutations.
Results: Ivacaftor potentiated multiple mutant CFTR forms with defects in CFTR channel gating. These included the G551D, G178R, S549N, S549R, G551S, G970R, G1244E, S1251N, S1255P and G1349D *CFTR* gating mutations.
Conclusion: These in vitro data suggest that ivacaftor has a similar effect on all CFTR forms with gating defects and support investigation of the potential clinical benefit of ivacaftor in CF patients who have *CFTR* gating mutations beyond G551D.

1. Introduction

The underlying cause of cystic fibrosis (CF) is the loss of epithelial chloride transport due to mutations in the CF *transmembrane conductance regulator* gene (*CFTR*) that encodes the CFTR protein (CFTR) [1]. CFTR is an epithelial chloride channel that is composed of two membrane spanning domains that form the chloride channel pore, two nucleotide-binding domains (NBD) that bind and hydrolyze ATP, and a regulatory domain with several protein kinase A (PKA) phosphorylation sites [1]. The opening and closing of the CFTR channel pore, or channel gating, is a tightly regulated process, requiring phosphorylation of the regulatory domain by PKA and subsequent ATP binding and hydrolysis by the NBDs [2]. Normally, CFTR is present at the epithelial cell surface where it allows chloride transport across the epithelial cell membrane to maintain salt, fluid, and pH balance in multiple organs [3]. In CF, *CFTR* mutations cause a loss of chloride transport through CFTR that results in the accumulation of thick, sticky mucus in the bronchi of the lungs, loss of exocrine pancreatic function, impaired intestinal absorption, reproductive dysfunction and elevated sweat chloride concentrations [3–4].

There are over 1800 *CFTR* mutations, resulting in a wide spectrum in the severity of the loss in chloride transport and disease phenotype [4]. Evaluation of the molecular defect caused by some of the more common *CFTR* mutations has shown that the loss of chloride transport can be due to a decrease in the quantity and/or function of CFTR channels at the cell surface [4–5]. For example, the most common *CFTR* mutation, *F508del-CFTR* which accounts for two-thirds of all *CFTR* alleles in patients with CF [4], impairs CFTR processing in the endoplasmic reticulum (ER), greatly reducing the quantity of F508del-CFTR protein at the cell surface [5]. In contrast, approximately 4–5% of patients with CF carry a missense *CFTR* mutation that results in CFTR protein which is present at the cell surface but does not open and close properly (defective channel gating), resulting in minimal chloride transport

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as measured in vitro or in vivo [6–8]. Such *CFTR* mutations are called *CFTR* gating mutations (also known as Class III mutations) [5]. The most common *CFTR* gating mutation in patients with CF is G551D [6–7]. Other known *CFTR* gating mutations include G178R, G551S, G970R, G1244E, S1255P, and G1349D [9–11].

One potential strategy to treat CF is to increase chloride transport by restoring or enhancing CFTR function with small molecule drugs known as CFTR modulators. A CFTR potentiator is a type of CFTR modulator that acts by increasing CFTR channel gating to enhance chloride transport. For a CFTR potentiator to act, CFTR at the cell surface must first be activated by PKA-dependent phosphorylation [12]. Experimentally, the effect of a CFTR potentiator on channel gating can be quantified using patch-clamp electrophysiology to directly measure the fraction of time the channel is open, which is called the channel open probability. Ivacaftor (VX-770), an investigational CFTR potentiator [12], increased the channel open probability of G551D-CFTR to enhance chloride transport in vitro [12]. In patients with CF who have the G551D mutation, ivacaftor increased clinical measures of chloride transport through CFTR, and improved clinical measures of lung function [13]. Ivacaftor has also been shown to potentiate normal CFTR and F508del-CFTR, suggesting that the compound is not specific for G551D-CFTR. We therefore hypothesized that ivacaftor might potentiate multiple mutant CFTR forms with *CFTR* gating mutations.

In this set of in vitro studies, the ability of ivacaftor to potentiate mutant CFTR was examined in a panel of Fischer rat thyroid (FRT) cells engineered to express previously reported *CFTR* gating mutations or *CFTR* mutations believed to be located in the ATP binding sites required for normal channel gating [2]. The results presented here indicated that ivacaftor potentiated CFTR channel gating and enhanced chloride transport in all examples of *CFTR* gating mutations tested. These in vitro data support the investigation of the clinical benefit of CFTR potentiators such as ivacaftor, in patients with CF who have *CFTR* gating mutations beyond G551D.

2. Results

2.1. Generation and characterization of cell lines expressing CFTR gating mutations

To systematically compare the effects of ivacaftor on mutant CFTR with defective channel gating, a panel of stable cell lines was generated using FRT cells. Each cell line was engineered to express a mutant CFTR form with a specific *CFTR* gating mutation or *CFTR* mutation believed to be located in the ATP binding sites required for normal channel gating [2]. These included G551D-, G178R-, S549N-, S549R-, G551S-, G970R-, G1244E-, S1251N-, S1255P-, and G1349D-CFTR [4,7,9–11]. No significant difference ($P > 0.05$; ANOVA followed Tukey's multiple comparisons test; $n = 3–6$) in the level of CFTR mRNA was observed between normal CFTR and the individual mutant CFTR forms expressed in FRT cells (Fig. 1A), suggesting similar CFTR protein levels.

In order to further characterize these cell lines, the delivery of mutant CFTR protein to the cell surface was indirectly assessed in immunoblot studies that measure an increase in the molecular mass of CFTR (from a 135–140 kDa band to a 170–180 kDa band) due to glycosylation (Fig. 1B–D), an indicator of CFTR exit from the endoplasmic reticulum and passage through the Golgi. After CFTR is processed by the Golgi, the glycosylated CFTR (mature CFTR) is delivered to the cell surface [5]. The ratio of mature to total CFTR (mature + immature CFTR) was used to estimate the fraction of CFTR synthesized that was processed normally and delivered to the cell surface (Fig. 1C). In addition, the steady-state level of mature mutant CFTR at the cell surface was expressed as a percentage of that measured in four separate FRT cell lines expressing normal CFTR (% normal CFTR; Fig. 1D). F508del-CFTR was used as a reference to calibrate severe defects in CFTR processing and delivery to the cell surface [5]. This analysis showed that, as expected for known *CFTR* gating mutations (G551D, G178R, G551S, G970R, G1244E, S1255P, and G1349D) [5,9–11], the amount of CFTR delivered to the cell surface was generally similar between CFTR with gating defects and normal CFTR. In contrast, only a small amount of F508del-CFTR was delivered to the cell surface (Fig. 1B–D). Interestingly, there was significantly more G551S-CFTR at the cell surface than normal CFTR, although the total amount of G551S-CFTR synthesized and the ratio of mature to total CFTR were similar to normal CFTR.

2.2. Ivacaftor increased the channel gating of mutant CFTR with defective channel gating

The effect of ivacaftor on CFTR channel gating was monitored by quantifying the channel open probability by patch-clamp electrophysiology using membrane patches excised from FRT cells expressing the known *CFTR* gating mutations, G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, or G1349D-CFTR. To activate CFTR prior to ivacaftor addition, PKA (75 nM) and ATP (1 mM) were added to the cytoplasmic surface (bath solution) of the membrane patch. Under these conditions, the baseline CFTR channel open probability of G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR was $\leq 5\%$ of normal CFTR (Fig. 2, B; Table 1). For most mutant CFTR forms, the single channel current amplitude, a measure of channel conductance, was similar to normal CFTR (between 77% and 122% of normal CFTR), although a small but statistically significant difference in single channel current amplitude was observed for S1255P-CFTR (Table 1). Acute (5 min) addition of 10 μM ivacaftor increased the channel open probability of all *CFTR* gating mutations tested, reaching levels equivalent to 30% to 118% of normal CFTR (Fig. 2B; Table 1). No change in the single channel current amplitude was observed following ivacaftor addition (Table 1). These data indicated that ivacaftor increased the channel open probability of all mutant CFTR forms with defective channel gating tested to a similar or greater extent than G551D-CFTR.

2.3. Ivacaftor enhanced chloride transport through mutant CFTR with defective channel gating

The impact of the increase in CFTR channel gating by ivacaftor on total chloride transport was assessed in Ussing chamber studies using FRT cells expressing the known *CFTR* gating mutations, G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR. To activate CFTR prior to ivacaftor addition, 10 μM forskolin was added to the bath to increase the intracellular levels of cAMP and activate PKA. The level of chloride transport for each mutant CFTR form was expressed as a percentage of that measured in four separate FRT cell lines expressing normal CFTR (% normal-CFTR; 204.5±29.8 μA/cm^2). Under these conditions, the baseline level of chloride transport in FRT cells expressing G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR was <10% of normal CFTR (Fig. 3; Table 2), which was consistent with the low CFTR channel open probability of these mutant CFTR forms (Table 1). Acute (5-min) ivacaftor addition caused a >10-fold increase over baseline chloride transport (Fig. 3; Table 2). Compared to all *CFTR* gating mutations tested, the ivacaftor response in FRT-cells expressing F508del-CFTR was minimal (Fig. 3). The EC_{50} of ivacaftor for all mutant CFTR forms tested was similar to G551D-CFTR (range: 161 to 594 nM) (Fig. 3C; Table 2).

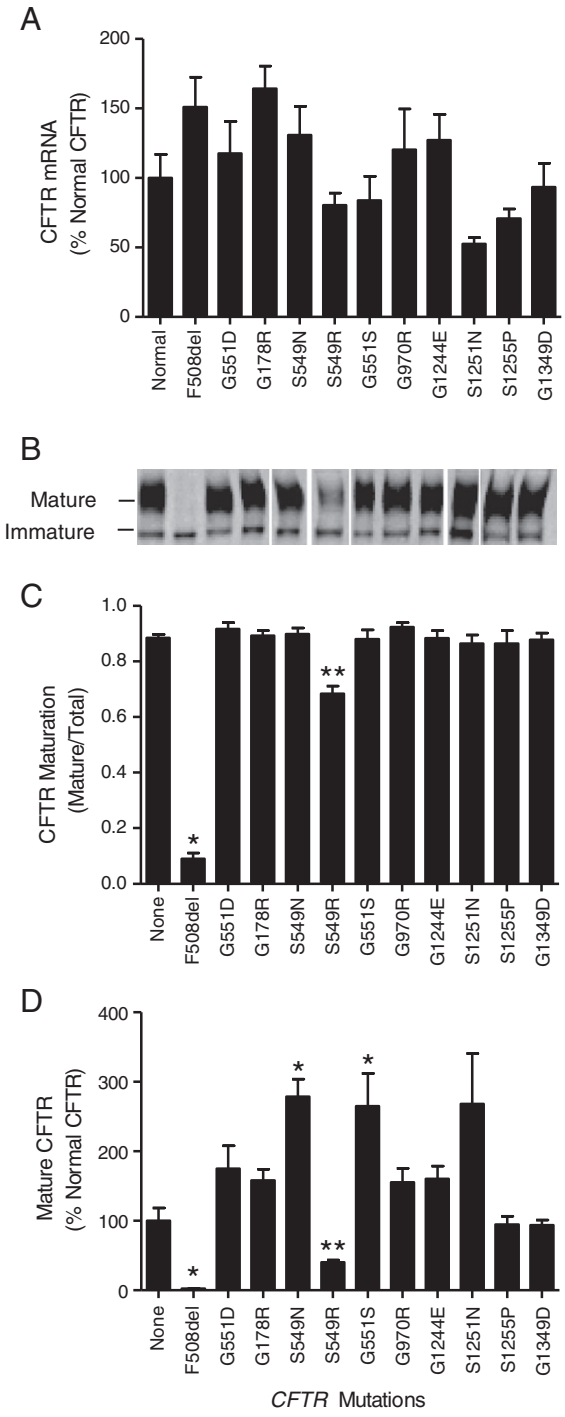


Fig. 1. CFTR mRNA expression and maturation in FRT cells. A) Mean (±SEM; n=3–5) levels of CFTR mRNA expression for each *CFTR* gating mutation expressed in FRT cells containing the Flp Recombination Target site (pFRT/lacZeo). For each mutant CFTR protein, the level of CFTR mRNA expression was normalized to the level of expression for normal CFTR prepared from four separate FRT cell lines. No significant differences compared to normal CFTR were observed (P>0.05: ANOVA followed by Tukey's multiple comparison test). B) Representative immunoblot of the glycosylation pattern of FRT cells expressing normal and mutant CFTR proteins. The bands associated with immature and mature CFTR are indicated. C) Quantification of the steady-state CFTR maturation expressed as the mean (±SEM; n=5–9) ratio of mature CFTR to total CFTR (immature plus mature) in FRT cells individually expressing the *CFTR* gating mutations indicated. F508del-CFTR was included as a reference of a severe processing defect that results in minimal CFTR at the cell surface (n=17). D) Quantification of the level of mature CFTR (±SEM; n=5–9) expressed as a percentage of normal CFTR. Single asterisk indicates significant difference (p<0.05; unpaired t-test) compared to normal CFTR and double asterisk indicates significant difference (p<0.05; unpaired t-test) compared to normal and F508del-CFTR.

2.4. Identification of additional CFTR gating mutations and effects of ivacaftor

Several of the known *CFTR* gating mutations cause defects in one of the two ATP binding pockets formed by the two NBDs known to regulate CFTR channel gating [2]. The CF-associated *CFTR* mutations, S549N, S549R, and S1251N, are located in the ABC signature sequence (Fig. 4; LSGGQ; S549N,R in NBD1) and Walker A motif (Fig. 4; S1251N in NBD2), that form the ATP binding sites [14]. Immunoblot and Ussing chamber studies indicated that, although the maturation of S549N- and S1251N-CFTR was similar to normal, the baseline level of chloride transport was <10% of normal CFTR (Figs. 1 and 3; Table 2). S549R-CFTR showed maturation comparable to 26±2% (n=5) of normal CFTR (Fig. 1D), which did not account for the <1% of normal CFTR function (Fig. 3; Table 2). These results indicated that the function of S549N-, S549R-, and S1251N-CFTR at the cell surface was minimal. Patch-clamp studies confirmed that the channel open probability of S549N-, S549R-, and S1251N-CFTR was <5% of normal CFTR, whereas the single channel current amplitude

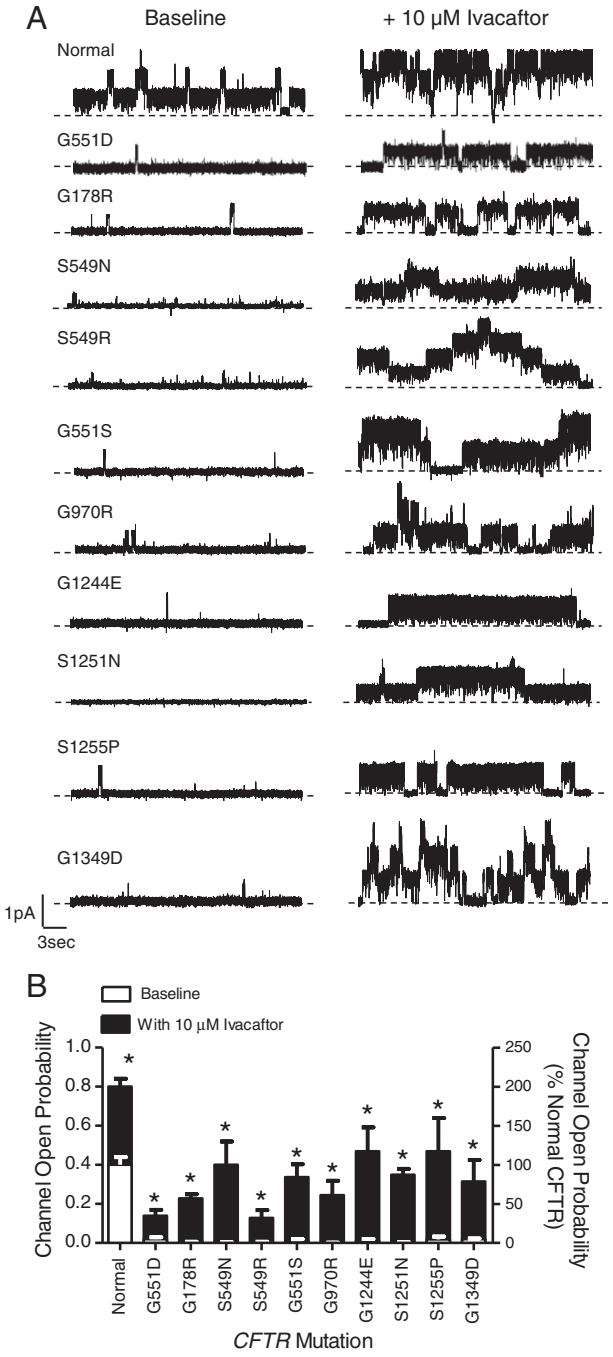


Fig. 2. Effect of ivacaftor on the channel gating activity of *CFTR* gating mutations associated with CF. A) Representative patch-clamp tracings (n=3–5 for each *CFTR* mutation) in an excised plasma membrane patch from FRT cells expressing the *CFTR* gating mutations indicated or normal CFTR. To activate CFTR, the cytoplasmic surface (bath solution) was exposed to 75 nM PKA and 1 mM ATP (baseline) prior to application of 10 μM ivacaftor. B) Mean ±SE of the CFTR channel open probability in the absence (baseline; open bars) or in the presence (filled bars) of 10 μM ivacaftor. Asterisks indicate significant difference compared to baseline (asterisks indicates $p<0.05$; paired t-test; n=3–5).

was 79 to 97% of normal CFTR (Fig. 2; Table 1). Taken together, these data suggested that defective channel gating was the predominant defect in S549N-, S549R- and S1251N-CFTR.

In FRT cells expressing S549N-, S549R-, and S1251N-CFTR, acute addition of 10 μM ivacaftor increased the channel open probability and total chloride transport by >10-fold over baseline levels (Figs. 2 and 3; Tables 1 and 2). The EC_{50} for ivacaftor in FRT cells expressing S549N-, S549R-, and S1251N-CFTR were similar to G551D-CFTR (Fig. 3C; Table 2). These results indicated that S549N-, S549R-, and S1251N-CFTR were potentiated by ivacaftor in vitro.

3. Discussion

CFTR gating mutations result in CFTR which is present at the cell surface but does not open or close normally, resulting in a low CFTR channel open probability and the loss of epithelial chloride transport as determined in electrophysiological studies. In this study we focused on CFTR gating mutations resulting in minimal channel function and generally associated with severe CF. The most common CF-causing *CFTR* gating mutation is G551D [6] but several other *CFTR* gating mutations have been previously reported [9–11]. In a panel of FRT cells expressing G551D-, G178R-, G551S-, G970R-, G1244E-, S1255P-, and G1349D-CFTR, we confirmed that all these mutant CFTR forms shared similar in vitro functional characteristics that were consistent with a defect in channel gating. In addition, we showed that the 3 additional mutations, S549N, S549R, and S1251N also have characteristics consistent with gating defects. These characteristics included the delivery of CFTR to the cell surface, a low (<5%) CFTR channel open probability, and minimal (<10% normal CFTR) baseline levels of chloride transport.

Ivacaftor addition caused a >10-fold increase in CFTR-mediated chloride transport in FRT cells expressing G551D-, G178R-, S549N-, S549R-, G551S-, G970R-, G1244E-, S1251N-, S1255P-, and G1349D-CFTR. For all *CFTR* gating mutations tested, the level of chloride transport achieved in the presence of ivacaftor was >10% of normal CFTR, which exceeds the level of chloride transport associated with lower sweat chloride levels and a mild CF phenotype [15]. Moreover, in patients with CF who have the G551D *CFTR* gating mutation, administration of ivacaftor was associated with improvements in CFTR function, as determined by a reduction in sweat chloride concentrations, and improvements in lung function, as determined by an increase in the FEV_1 [13]. Taken together, these in vitro results provide a rationale for testing the potential benefit of ivacaftor in individuals with CF who have a *CFTR* gating mutation other than G551D, including the G178R-, S549N-, S549R-, G551S-, G970R-, G1244E-, S1251N-, S1255P, and G1349D *CFTR* gating mutations.

Evaluation of CF-associated *CFTR* mutations that were expected to cause protein alterations in the ATP-binding sites formed by the NBDs indicated that S549N- and S1251N-CFTR also shared similar in vitro functional characteristics with G551D-CFTR and could be classified as *CFTR* gating mutations. Unlike S549N-CFTR and S1251N-CFTR, S549R-CFTR exhibited a partial reduction in the level of CFTR maturation compared to normal CFTR (see also Ref. [16]). The partial reduction in S549R-CFTR maturation was ~27% of

Table 1
Effect of ivacaftor on the channel gating activity of CFTR with gating mutations.

	Single channel current amplitude at 80 mV				CFTR channel open probability			
	Baseline		With 10 μM ivacaftor		Baseline		With 10 μM ivacaftor	
Mutation	pA	% Normal	pA	% Normal	P_o	% Normal	P_o	% Normal
Normal	0.57±0.03	100	0.63±0.02	111	0.400±0.04	100	0.800±0.04 [a]	200
G551D	0.46±0.06	81	0.46±0.03	81	0.019±0.01 [b]	5	0.121±0.035 [a]	30
G178R	0.59±0.11	103	0.66±0.08	116	0.005±0.001 [b]	1	0.228±0.022 [a]	57
S549N	0.55±0.02	97	0.61±0.02	108	0.003±0.010 [b]	1	0.396±0.119 [a]	99
S549R	0.45±0.01 [b]	79	0.55±0.02 [a]	96	0.004±0.010 [b]	1	0.143±0.031 [a]	36
G551S	0.57±0.13	100	0.64±0.02	113	0.010±0.001 [b]	3	0.337±0.110 [a]	84
G970R	0.55±0.03	96	0.55±0.03	97	0.001±0.001 [b]	0	0.245±0.042 [a]	61
G1244E	0.44±0.11	77	0.54±0.08	94	0.011±0.010 [b]	3	0.470±0.122 [a]	118
S1251N	0.54±0.07	95	0.63±0.04	111	0.003±0.010 [b]	1	0.350±0.03 [a]	88
S1255P	0.70±0.03 [b]	122	0.71±0.02	125	0.018±0.016 [b]	5	0.468±0.168 [a]	117
G1349D	0.49±0.08	85	0.63±0.06	111	0.019±0.015 [b]	5	0.315±0.110 [a]	79

[a] Significantly different ($P<0.05$; paired t-test, n=3–5) compared to baseline levels for each CFTR mutation.
[b] Significantly different ($P<0.05$; unpaired t-test) compared to normal CFTR.

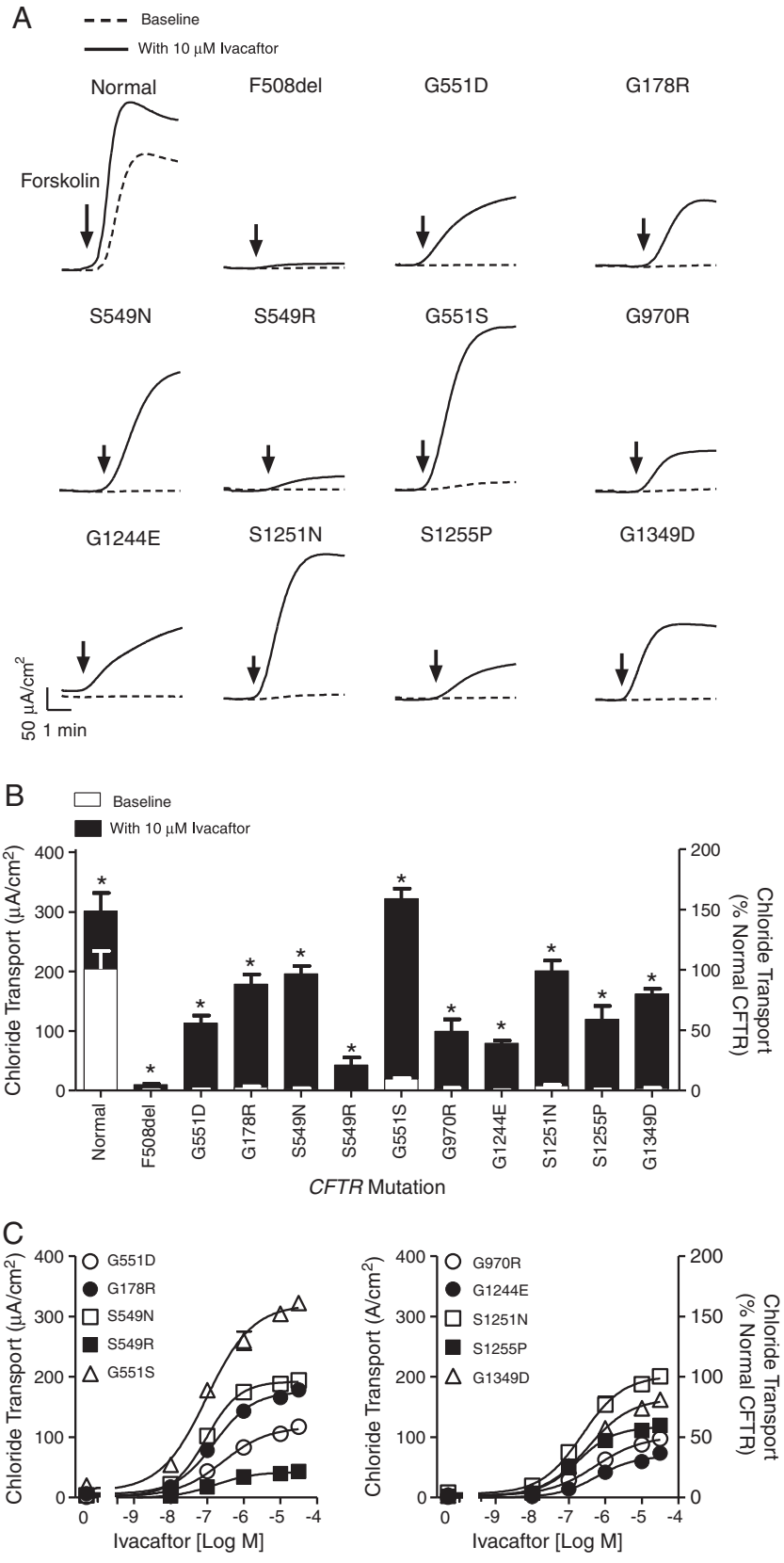
normal CFTR and did not appear to be sufficient to account for the <1% normal CFTR chloride transport in FRT cells expressing this *CFTR* mutation. Patch-clamp studies showed that the channel open probability of S549R-CFTR was less than 1% of normal CFTR. These results indicated that although the predominant defect caused by the S549R *CFTR* mutation was defective CFTR channel gating, a mild processing defect was also observed. The apparent mild processing defect in S549R-CFTR may account for the lower ivacaftor response in FRT cells expressing S549R-CFTR compared to the other mutant CFTR forms with gating defects and normal CFTR maturation.

The low baseline levels of chloride transport in FRT cells expressing mutant CFTR forms encoded by the *CFTR* gating mutations studied here were consistent with the clinical characteristics of patients with CF who carry one of these *CFTR* gating mutations. Patients with CF who carry the G551D *CFTR* gating mutation have minimal amounts of CFTR function, as determined by high (~100 mmol/L) sweat chloride concentrations, and a severe CF phenotype [8,17]. Although there are limited case studies on patients with CF who have *CFTR* gating mutations beyond G551D, they also appear to have high sweat chloride concentrations and severe CF [18–20]. This was consistent with the low CFTR channel open probability and minimal (<10% normal CFTR) baseline levels of chloride transport measured in FRT cells expressing these *CFTR* gating mutations. A milder CF clinical phenotype has been associated with the G551S *CFTR* gating mutation, as demonstrated by a lower sweat chloride concentration (75–94 mmol/L) and lower incidence of pancreatic insufficiency compared to patients with CF who carry the G551D *CFTR* gating mutation [21]. In the present study, although the expression and channel open probability of G551S-CFTR were similar to G551D-CFTR, the baseline chloride transport was higher (~9% normal CFTR). This may be due to the increased level of mature G551S-CFTR delivered to the cell surface compared to normal CFTR, as determined by immunoblot studies in FRT cells.

The potency (EC_{50}) of ivacaftor for the *CFTR* gating mutations tested was similar to G551D in vitro, suggesting that a similar dose of ivacaftor as that used in clinical trials of patients with CF who carry the G551D *CFTR* gating mutation may be appropriate for most other *CFTR* gating mutations. Previous in vitro studies using recombinant cells and primary human airway cultures have shown that higher concentrations of ivacaftor were required to potentiate G551D-CFTR compared to normal- and F508del-CFTR [12]. The potency of other CFTR potentiators has also been shown to be lower for G551D-CFTR compared to normal or F508del-CFTR [12,22–25]. This suggests that some *CFTR* mutations may alter interaction of CFTR potentiators with CFTR to affect channel gating. Further studies will be needed to identify the ivacaftor binding site on CFTR to test this hypothesis.

CFTR potentiators from structural classes distinct from ivacaftor have also been shown to potentiate mutant CFTR with defective channel gating. These included derivatives of 1,4-dihydropyridine and phenylglycine which potentiated G551D-, G970R-, and G1349D-CFTR to a similar extent as ivacaftor [23,25–26]. In contrast, sulfamoyl-4-oxoquinoline-3-carboxamides were weakly effective on G551D-, G970R-, and G1349D-CFTR [26] and phloxine B strongly potentiated G551D-CFTR, but not G1349D-CFTR [22]. Previous studies have speculated that these differences may be due to distinct binding sites which may be differentially altered by *CFTR* mutations or which may modulate CFTR channel gating through different mechanisms [22–23,26]. These in vitro data have suggested that broad-acting CFTR potentiators, like ivacaftor and derivatives of 1,4-dihydropyridine and phenylglycine, may have a wider clinical utility among patients with CF who have different *CFTR* gating mutations compared to CFTR potentiators that appear to have an effect on specific *CFTR* mutations.

The gating of the CFTR channel pore is a tightly regulated process, requiring phosphorylation of the regulatory domain by PKA and subsequent ATP binding and hydrolysis by the NBDs [2]. Like G551D, the G551S, G1244E, S1255P, and G1349D *CFTR* gating mutations, as well as the S549N, S549R, and S1251N *CFTR* gating mutations identified in the


A
Baseline
With 10 μM Ivacaftor
Normal
F508del
G551D
G178R
Forskolin
S549N
S549R
G551S
G970R
G1244E
S1251N
S1255P
G1349D
50 μA/cm2
1 min
B
Baseline
With 10 μM Ivacaftor
Chloride Transport (μA/cm2)
Chloride Transport (% Normal CFTR)
Normal
F508del
G551D
G178R
S549N
S549R
G551S
G970R
G1244E
S1251N
S1255P
G1349D
CFTR Mutation
C
G551D
G178R
S549N
S549R
G551S
Chloride Transport (μA/cm2)
Ivacaftor [Log M]
G970R
G1244E
S1251N
S1255P
G1349D
Chloride Transport (A/cm2)
Chloride Transport (% Normal CFTR)
Ivacaftor [Log M]

present study, cause protein alterations in the ATP binding pockets formed by the two NBDs required for normal CFTR channel gating (Fig. 4) [2]. The G178R and G970R *CFTR* gating mutations alter the intracellular cytoplasmic loops that are believed to link the ATP-driven conformational changes in the NBDs to the opening of the CFTR channel pore formed by the membrane spanning domains [27]. Given the importance of these regions in driving CFTR channel opening and closing, it was not surprising that *CFTR* mutations associated with these regions caused severe defects in CFTR channel gating. In vitro, ivacaftor potentiated mutant CFTR forms associated with alterations in the ATP binding pockets and the cytoplasmic loops linking the CFTR channel pore to the movement of the NBDs, suggesting that ivacaftor may bypass or augment ATP-dependent channel gating of mutant CFTR to potentiate the function of PKA-activated CFTR. Additional studies are needed to determine the ATP dependence and molecular mechanism of CFTR potentiation by ivacaftor.

In previous in vitro studies ivacaftor potentiated chloride transport by normal and F508del-CFTR [12]. Along with the data presented here on multiple mutant CFTR forms with defective channel gating, ivacaftor appeared to be a broad-acting CFTR potentiator. This suggests that ivacaftor may act on other mutant CFTR forms that are delivered to the cell surface in sufficient amounts. For example, R117H-CFTR is delivered to the cell surface in normal amounts, but exhibits a ~20% reduction in CFTR channel conductance and a ~75% reduction in the channel open probability, resulting in residual CFTR function both in vitro and clinically [28]. Other *CFTR* gene mutations associated with residual CFTR function include A445E, R347H, D1152H, and certain splice mutations (3849 +10kbC→T) [4,29–30]. Further in vitro studies and clinical measurements are needed to evaluate the ivacaftor response and potential clinical benefit for *CFTR* mutations beyond *CFTR* gating mutations.

In conclusion, ivacaftor increased the channel gating activity of all mutant CFTR forms associated with *CFTR* gating mutations tested in this study, resulting in enhanced chloride transport. This suggested that ivacaftor is not a mutation-specific CFTR potentiator, as it overcomes the underlying molecular defect caused by G551D and a number of other *CFTR* gating mutations. As a group these *CFTR* gating mutations shared the following characteristics; 1) defective CFTR channel gating as the predominant molecular defect, 2) residual or normal amounts of CFTR present at the cell surface, 3) minimal baseline chloride transport in vitro (<10% normal), and 4) a pronounced (>10-fold) increase over baseline chloride transport in response to ivacaftor. The in vitro data presented here suggest that ivacaftor has a similar effect on all CFTR forms with gating defects and support the investigation of ivacaftor in patients with CF who have *CFTR* gating mutations beyond G551D, including G178R, S549N, S549R, G551S, G970R, G1244E, S1251N, S1255P, and G1349D.

4. Methods

4.1. Cell line generation

A panel of FRT cell lines expressing different *CFTR* mutations was generated using a host cell line with a single integration site as described in the supplementary material.

4.2. RNA analysis

Total RNA was isolated and real-time PCR assays were performed as described in the Supplementary material.

4.3. Electrophysiology

Ussing chamber techniques using FRT cells were used to record the I_T due to CFTR-mediated chloride transport. The single-channel activity of CFTR was measured using excised inside-out membrane patch recordings (see Supplementary material).

4.4. CFTR immunoblot analysis

Immunoblot techniques using the monoclonal CFTR antibody 769 (J. Riordan, University of North Carolina) were used to measure CFTR maturation in FRT, HEK-293, or HBE cells expressing wild-type- or F508del-CFTR (see Supplementary material).

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Fig. 3. Effect of ivacaftor on chloride transport in FRT cells expressing CFTR gating mutations associated with CF. A) Representative recordings of the forskolin-stimulated chloride transport in the absence (baseline; dotted line) and presence (solid line) of 10 μM ivacaftor in FRT cell expressing normal *CFTR* or the *CFTR* gating mutation indicated. F508del-CFTR was included as a reference. Forskolin (10 μM; arrows) was added to the bath to activate CFTR by increasing the intracellular concentrations of cAMP. B) The mean (±SEM; n=4–6) CFTR-mediated chloride transport (left y-axis) with 10 μM forskolin in the absence (baseline; open bars) or in the presence (filled bars) of 10 μM ivacaftor in FRT cells expressing normal CFTR and the mutant CFTR proteins indicated. To compare to normal CFTR function, the forskolin-stimulated chloride transport in FRT cells expressing mutant CFTR was normalized to the forskolin-stimulated chloride transport in 4 separate FRT cell lines expressing normal CFTR (204.5±29.8 μA/cm^2) and expressed as % normal CFTR (right y-axis). Asterisks indicate significant difference compared to baseline ($p<0.05$; paired t-test; n=4–6). C) Concentration response relationship for ivacaftor in FRT cells expressing the *CFTR* gating mutations indicated (mean±SEM; n=4–6).

Table 2
Baseline chloride transport and pharmacological effect of ivacaftor in cultured FRT cells expressing *CFTR* gating mutations.

Mutation	Chloride transport (μA/cm^2)		Chloride transport (% normal)		Fold change [a]	EC_{50} [b]
	Baseline	With ivacaftor	Baseline	With ivacaftor		
G551D	1.5±0.7	113.2±13.0 [c]	1.0±0.5	55.3±6.3 [c]	55	312±73
G178R	6.0±1.1	178.4±16.8 [c]	2.9±0.5	87.2±8.2 [c]	30	178±43
S549N	3.3±0.8	195.8±13.4 [c]	1.6±0.4	95.7±6.5 [c]	59	124±43
S549R	0.1±0.1	43.0±12.5 [c]	0.0±0.0	21.0±6.1 [c]	>20	182±23
G551S	19.8±1.5	322.3±18.8 [c]	9.7±0.7	157.6±9.2 [c]	16	161±51
G970R	3.3±1.2	99.8±20.0 [c]	1.6±0.6	48.8±9.8 [c]	31	514±91
G1244E	0.6±0.2	79.6±4.6 [c]	0.3±0.1	38.9±2.2 [c]	130	594±105
S1251N	8.1±1.5	200.9±17.6 [c]	3.9±0.7	98.2±8.6 [c]	25	245±69
S1255P	1.6±0.5	119.6±22.8 [c]	0.8±0.2	58.5±11.1 [c]	73	192±45
G1349D	3.6±1.0	162.1±8.4 [c]	1.7±0.5	79.3±4.1 [c]	47	315±13

[a] The fold change in chloride transport was determined by dividing the level of chloride transport (% normal) in the presence of ivacaftor by the baseline chloride transport.
[b] No significant differences ($P>0.05$; unpaired t-test, n=4–6) compared to G551D-CFTR was observed for any of the mutant CFTR forms tested.
[c] Significant difference compared to baseline ($p<0.05$; paired *t*-test; n=4–6).

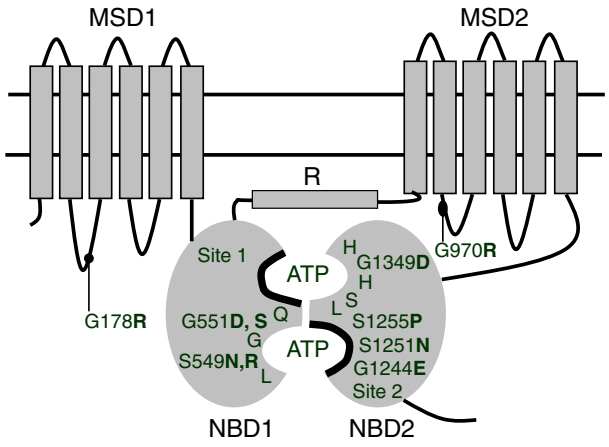

Fig. 4. Location of the amino acid alterations associated with *CFTR* gating mutations tested in this study. CFTR is composed of two membrane spanning domains (MSDs) that form an anion-selective pore, two NBDs that contain the LSGGQ and Walker A and B (bold solid lines) motifs that form the two ATP binding pockets (sites 1 and 2), and a regulatory domain (R) that contain the PKA phosphorylation sites. Intracellular cytoplasmic loops connecting the membranes spanning domains are believed to act as molecular linkers to transfer the ATP-dependent conformational changes in the NBDs to the MSD, thereby opening the CFTR channel pore formed by the membranes spanning domains. The *CFTR* gating mutations tested here are believed to cause protein alterations in the ATP binding sites and cytoplasm loops.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jcf.2011.12.005.

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