Optimization of the Degenerated Interfacial ATP Binding Site Improves the Function of Disease-related Mutant Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Channels*

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The cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, an ATP binding cassette (ABC) protein whose defects cause the deadly genetic disease cystic fibrosis (CF), encompasses two nucleotide binding domains (NBD1 and NBD2). Recent studies indicate that in the presence of ATP, the two NBDs coalesce into a dimer, trapping an ATP molecule in each of the two interfacial composite ATP binding sites (site 1 and site 2). Experimental evidence also suggests that CFTR gating is mainly controlled by ATP binding and hydrolysis in site 2, whereas site 1, which harbors several non-canonical substitutions in ATP-interacting motifs, is considered degenerated. The CF-associated mutation G551D, by introducing a bulky and negatively charged side chain into site 2, completely abolishes ATP-induced openings of CFTR. Here, we report a strategy to optimize site 1 for ATP binding by converting two amino acid residues in other ABC proteins (i.e. H1348G) or more commonly seen residues in other ABC proteins (i.e. W401Y, W401F). Introducing either one or both of these mutations into G551D-CFTR confers ATP responsiveness for this disease-associated mutant channel. We further showed that the same maneuver also improved the function of WT-CFTR and the most common CF-associated ΔF508 channels, both of which rely on site 2 for gating control. Thus, our results demonstrated that the degenerated site 1 can be rebuilt to complement or support site 2 for CFTR function. Possible approaches for developing CFTR potentiators targeting site 1 will be discussed.

The CFTR chloride channel belongs to the ATP binding cassette (ABC) proteins superfamily, whose members all share a basic architecture comprising two transmembrane domains and two cytoplasmic nucleotide binding domains (NBD1 and NBD2). Crystallographic studies have revealed that each NBD can be divided into a larger core (head) subdomain and a smaller helical (tail) subdomain; the former contains the conserved Walker motifs for binding and hydrolyzing ATP, whereas the latter is characterized by the signature motif (LSGGQ) unique to ABC proteins (1, 2). The two monomeric NBDs are so arranged that the head subdomain from one NBD faces the tail subdomain from the other NBD. Upon ATP binding, the NBDs assemble into a head-to-tail dimer connected by two ATP molecules at interfacial composite sites (site 1 and site 2, depicted in supplemental Fig. S1). This NBD dimer is subsequently destabilized when the enclosed ATP molecules are hydrolyzed.

CFTR is classified as an asymmetric ABC protein (3, 4) as the constituents of its site 2 (i.e. NBD2 Walker motifs and NBD1 LSGGQ motif) retain all conserved residues, whereas those of its site 1 (i.e. NBD1 Walker motifs and NBD2 signature motif: LSHGH) present several non-consensus substitutions. Such a structural asymmetry is accompanied by the functional asymmetry that opening and closing of the CFTR pore located in transmembrane domains are controlled by ATP binding and hydrolysis, respectively, in the catalysis–competent site 2 (5, 6). This “site 2-controlled gating” of WT-CFTR can be impaired by CF-related mutations. For example, the G551D mutation at the NBD1 signature motif (site 2) completely eliminates ATP-catalyzed openings of CFTR, presumably by perturbing normal ATP-site 2 interactions (7). ΔF508, the most common CF-associated mutation, although not located in site 2, also leads to defective gating (8–10). In this case, the exact mechanism underlying the abnormal channel function remains unsettled (8–11).

It has been shown that for some ABC proteins with two canonical composite sites, mutating conserved residues in one site does not abolish protein functions entirely (12). Thus, we reasoned that rebuilding the degenerated site 1 of CFTR might compensate for the defective site 2 and thereby improve the function of G551D channels. One of our previous studies provided some insight into how this goal could be accomplished. We found that a hydrolyzable ATP analogue PATP potentiates G551D channels by binding to the NBD1 head subdomain (13), a component of site 1. As PATP may bind to CFTR NBDs more tightly than ATP (14), this observation implies that site 1 might
not be optimized for ATP binding, and thus, only high affinity ATP analogues can support G551D-CFTR gating.

Here, guided by accumulated structural and functional understanding of NBDs, we first identified W401Y, W401F mutations that strengthened ligand binding in the NBD1 head subdomain. We demonstrated that these mutations conferred ATP responsiveness for G551D-CFTR and thus significantly improved the function of this sick channel. The presence of the Asp-551 residue, which disfavors NBD dimer formation, however, raised the question of whether ATP molecule potentiates W401Y, W401F/G551D channels by binding at the NBD interface (i.e. site 1) or interacting only with the monomeric NBD1. Our data suggested that the former is likely the case as the effects of ATP on W401F/G551D channels can be further enhanced by the H1348G mutation in the NBD2 tail subdomain. These results support the idea that optimization of site 1 for ATP binding can compensate for the disabled site 2 for G551D channels. Finally, we demonstrated that the same strategy (i.e. optimizing site 1) to improve G551D-CFTR function could be applied to amend at least partially the functional defects of ΔF508 channels and increase the open probability ($P_o$) for WT channels. Structural and pharmacological implications of our results will be discussed.

**EXPERIMENTAL PROCEDURES**

Expression System and Electrophysiological Recordings—The experimental protocol has been described in detail in a previous report (15). In brief, inside-out patches were obtained from Chinese hamster ovary (CHO) cells that were transfected with WT or mutant CFTR channels and grown at a 27 °C incubator. The pipette solution contained (in mM) 140 N-methyl-D-glucamine chloride, 2 MgCl₂, 5 CaCl₂, and 10 HEPES (pH 7.4). The bath solution contained (in mM) 150 N-methyl-D-glucamine chloride, 2 MgCl₂, 10 EGTA, and 8 Tris (pH 7.4). The bath solution contained (in mM) 150 N-methyl-D-glucamine chloride, 2 MgCl₂, 10 EGTA, and 8 Tris (pH 7.4). CFTR channels were first activated by 2.75 mM MgATP and 50 units/ml PKA to maintain a high level of phosphorylation. The concentrations for ATP, PATP, and pyrophosphate (PPi) were 2.75 mM, 50 μM, and 2 mM, respectively, for all experiments. Membrane potential was held at ~60 mV, and the inward current was inverted for clearer data presentation. Perfusion solutions were exchanged using a fast perfusion change device SF-77B (Warner Instrument Cop.) with a dead time of 30 ms.

Analysis of Macroscopic Current Recordings—Measurements of steady-state macroscopic current amplitudes and variances as well as fittings of current relaxations were done using the IGOR Pro program (version 4.07, WaveMetrics). To quantify the stability of the lock-open state induced by PPi, we measured the time constant of the current relaxation upon removal of PPi-containing solutions (see Fig. 1 and supplemental Figs. S6 and S8), as previously described (15). Because WT-CFTR channels seldom open in the absence of ligands, the time constant derived by single exponential fitting of the macroscopic current decay reflects the mean lock-open time.

For the W401F/H1348G/G551D channel, the steady-state open probability ($P_o$) in the presence of ATP was estimated by stationary noise analysis of macroscopic currents using the equation

$$\sigma^2 / i = (1 - P_o)$$  \hspace{1cm} (Eq. 1)

where $\sigma^2$ is the variance, $i$ is the unitary current amplitude, and $l$ is the amplitude of the steady-state currents. A graph (supplemental Fig. S7) of $\sigma^2 / i$ as a function of $l$ yielded a straight line with a slope of 1 − $P_o$. All data points were from stationary segments of current records of at least 2 min. It is noted that noise analysis is inappropriate for those G551D-containing channels with even lower $P_o$, as the analysis will be more susceptible to the error of measurements (see supplemental text).

Analysis of Single-channel Recordings—Recordings with less than four simultaneous opening steps were selected for single-channel kinetic analysis. These data were filtered digitally at 50 Hz and analyzed using software kindly provided by Dr. Csanády (16). A three-state kinetic model, C$\leftrightarrow$O$\leftrightarrow$B, was adopted to extract kinetic parameters, including $P_o$, mean open time, and mean opening rate. To determine the $P_o$ and opening rate, it is necessary to be certain of the number of channels in a membrane patch. This can be readily done for WT and W401F/H1348G/ΔF508 channels (as seen in Fig. 5). However, for ΔF508 and all G551D-containing channels tested in the current study, the low $P_o$ inevitably results in an uncertainty of the channel number in a patch, and thus, only the mean open time can be measured accurately for these mutants.

The method we used to estimate the $P_o$ and opening rate for ΔF508 channels was described in Miki et al. (8). The increase of the opening rate upon the application of ATP to G551D-containing channels (see Fig. 4F) was estimated indirectly. First, as the mean closed time ($\tau_c$, the reciprocal of the opening rate, $R_{co}$) is much longer than the mean open time ($\tau_o$) for most G551D-containing channels, the $P_o$ for these mutants can be approximated as

$$P_o = \tau_o / \tau_c = \tau_o \times R_{co}$$  \hspace{1cm} (Eq. 2)

From here, the -fold increase of the opening rate upon exposing G551D-containing channels to ATP can be estimated as

$$\frac{R_{co - ATP}}{R_{co - Basal}} = \frac{P_o - ATP}{P_o - Basal} \times \frac{\tau_o - Basal}{\tau_o - ATP}$$  \hspace{1cm} (Eq. 3)

By calculating the ratio of macroscopic currents in the presence or in the absence of ATP (see Figs. 2B and 3D), we can obtain the average -fold increase of the single-channel $P_o$ induced by ATP (i.e. $P_o - ATP / P_o - Basal$). Meanwhile, the ratio of $\tau_o - Basal$ over $\tau_o - ATP$ can be calculated from single-channel analysis (see Fig. 4E).

Statistics—All results were presented as means ± S.E.; $n$ represents the number of independent experiments (marked above the bars in all figures). Student’s $t$ test was performed with SigmaPlot (version 8.0, SPSS Science) with $p < 0.01$ considered significant.

**RESULTS**

Fig. 1A shows a representative current recording of G551D channels in an inside-out patch. It can be seen that the channels failed to respond to ATP and exhibited only spontaneous open-
This "ATP-independent gating" is also observed with WT-CFTR in the absence of ATP and has a Po only /H11011 /H11011 0.004 (i.e. /H11011 Po) of the maximal Po induced by ATP (7, 17). Fig. 1A also shows that PATP increased the basal activity of G551D-CFTR by /H11011 6-fold. Because this effect was diminished when the conserved aromatic residue that stacks against the adenine ring of ATP in NBD1 (Trp-401) but not in NBD2 (Tyr-1219) was mutated (18, 19), we reported previously that PATP potentiates G551D channels by interacting with NBD1 (13). (Note: The ATP binding site of a monomeric NBD is in its head subdomain.) These findings led to an intriguing question. What is the factor that determines whether or not a nucleotide ligand can activate G551D-CFTR upon binding to NBD1?

High Affinity Binding of Nucleotides in NBD1 Improves the Function of G551D Channels—As PATP was reported to assume a higher affinity than ATP in CFTR NBDs (14, 15), we hypothesized that high affinity binding in NBD1 is essential for a nucleotide ligand to activate G551D channels. If this hypothesis is correct, one would predict that mutations that enhance nucleotide binding in NBD1 will confer ATP-dependent activation as well as a stronger response to PATP for G551D channels. Identifying such gain-of-function mutations, however, presents a challenge as an altered ATP affinity in NBD1 is not well reflected by the ATP dose-response relationship of CFTR (19, 20) due to the fact that gating of WT-CFTR is mainly controlled by ATP binding/hydrolysis in site 2 (5, 6).

An opportunity to overcome this difficulty emerges from our recent findings that WT channels can be locked open in a configuration where the non-hydrolytic ligand PPi occupies NBD2 (site 2), whereas ATP or its analogues bind in NBD1 (site 1), and that the stability of this lock-open state is correlated with the strength of nucleotide-NBD1 interactions (15). This idea is recapitulated in Fig. 1B, which shows that the lock-open duration (i.e. current decay constant calculated from traces in Fig. 1C; see also “Experimental Procedures”) of WT channels elicited by ATP and PPi (left) was shortened by the W401G mutation in NBD1 (middle) but was prolonged when PATP, instead of ATP, was used (right). Thus, by identifying mutations that stabilize the lock-open state, we could design strategies to tighten nucleotide binding in NBD1.

Trp-401 was the first target for mutagenesis as it directly contacts ATP through a ring-ring stacking interaction (18). That the equivalent residue at this position in most other ABC proteins is a tyrosine (21) raised the possibility that a tyrosine substitution may improve this stacking interaction. Fig. 1D summarizes the mutational effects of Trp-401 on the mean lock-open duration induced by PPi with ATP or PATP. (Some of the original traces are shown in supplemental Fig. S2, and the results of data analysis were summarized in a supplemental table.) As expected, non-conservative substitutions of Trp-401 with Ile or Gly (W401I and W401G), which are unable to stack ATP, was used (right). Thus, by identifying mutations that stabilize the lock-open state, we could design strategies to tighten nucleotide binding in NBD1.
with ATP, facilitated channel closure from the lock-open state. Not surprisingly either, when applied with PPi, the high affinity ligand PATP was more effective than ATP in maintaining Trp-401 mutants in the lock-open state. Interestingly, we found that phenylalanine (W401F) appeared better than tyrosine (W401Y), which was in turn superior to tryptophan, in stabilizing the lock-open state, suggesting that W401Y and W401F mutations might enhance nucleotide-NBD1 interactions. (Note: As the electrophysiological recording used in the current study does not measure ATP binding directly, our functional data only gauge ATP affinity in NBDs indirectly. See under “Discussion” for more details.)

We then incorporated W401Y or W401F mutation into G551D channels, intending to help G551D-NBD1 to bind nucleotide more tightly. The resulting double mutant channels indeed became ATP-responsive and were potentiated to an even greater extent by PATP (Fig. 2A). The increase of the basal current, as high as ~30-fold, is presented in Fig. 2B. It can be seen that W401F, which yielded a more stable lock-open state in the WT background (Fig. 1D), was also more effective than W401Y in conferring ATP-dependent activation of G551D channels. Furthermore, in both double mutants, PATP induced more robust potentiation than ATP did. Plotting the lock-open duration of WT-CFTR shown in Fig. 1D with the nucleotide response for G551D-CFTR in Fig. 2B yielded a positive correlation (supplemental Fig. S3), consistent with our hypothesis that high affinity binding in NBD1 is critical for a nucleotide to activate G551D channels.

Two concerns over this proposition are addressed here. First, the response to ATP seen in W401Y, W401F/G551D channels could be due to an altered ligand-NBD1 interaction as proposed, or alternatively, is a result of a restored ability for ATP to gate CFTR through binding to site 2. We argue that the former is more likely the case as the coexistence of ATP and the negatively charged Asp-551 side chain in site 2 should still be energetically and sterically unfavorable. Moreover, we also found that mutating the Asp-401 equivalent residue in Trp-401 (Y1219G), which greatly decreases the NBD2 ATP affinity (19), had little effect on ATP-mediated activation of W401Y, W401F/G551D channels (supplemental Fig. S4).

Second, as PPi fails to lock open G551D-containing channels (data not shown), presumably due to the negatively charged Asp-551 side chain in site 2, it is desirable to have another parameter in support of the conjecture that W401Y, W401F mutations do tighten nucleotide binding in G551D-NBD1. One possible approach is to fit the current relaxation traces of W401Y, W401F/G551D channels upon removal of the nucleotide (Fig. 2A, inset) as the resulting time constants could potentially reflect the mean nucleotide dwell time in NBD1 of these channels (see also under “Discussion”). Indeed, the data summarized in Fig. 2C suggest that the two Trp-401 mutations do help G551D-NBD1 to bind nucleotides for a longer period. A comparison of Fig. 2, B and C, reveals that a stronger response of G551D-containing channels to nucleotides is correlated with a slower current decay upon removal of nucleotides (supplemental Fig. S3), thus again corroborating our proposition that the function of G551D channels can be improved when a nucleotide can bind tightly in NBD1.

Nucleotide-dependent Activity of G551D-containing Channels Requires Closure of the Interface between NBD1 Head and NBD2 Tail—We then sought to answer the following question. Does the ATP or PATP molecule that potentiates W401Y, W401F/G551D channels bind in the head subdomain of the monomeric NBD1 or NBD1-NBD2 dimer interface (i.e. site 1)? These two scenarios can be differentiated by altering the signature motif in the NBD2 tail subdomain, the other part of site 1, as in several crystal structures of NBD dimers (22–24), residues in this motif (LSHGH from positions 1346–1350 in CFTR) mediate multiple interactions with ATP bound in site 1. Interestingly, we found that non-conservative mutations L1346Q and S1347G (Fig. 3A) and G1349I greatly reduced the nucleotide-dependent activation of W401F/G551D channels. For example, ATP and PATP, which increased the basal activity of W401F/G551D-CFTR by ~12-fold (Fig. 3D, blue dashed line) and ~30-fold (green dashed line), respectively, led to only ~2.5- and ~6-fold current increases when the S1347G mutation was present (Fig. 3D). These results thus suggest that ATP or PATP resides in site 1 and interacts with both NBD1 head and NBD2 tail subdomains to support gating of W401Y, W401F/G551D channels.

The idea that both the head of NBD1 and the tail of NBD2 are involved in ATP-dependent activation of W401Y, W401F/G551D channels implicates that optimizing the interactions of ATP with the NBD2 tail subdomain may also improve the func-
tion of G551D-CFTR. His-1348, the third amino acid in the NBD2 signature motif, is of particular interest as its equivalent residue in most ABC proteins is a glycine. Because this glycine is so close to the phosphate groups of ATP in crystal structures of NBD dimers (22–24), a bulky His-1348 side chain may cause a steric clash (25) upon closing of the NBD interface. We thus converted the His-1348 residue to Gly (H1348G) in the G551D background. The resulting mutant channel indeed became responsive to ATP, and the current in the presence of ATP was 4-fold (3.9 ± 0.3, n = 9) higher than the basal activity (Fig. 3, B).

Moreover, the H1348G mutation further improved the function of W401F/G551D channels so that the application of ATP and PATP increased the basal activity by 25- and 75-fold, respectively (Fig. 3, C and D, and supplemental Fig. S5), further supporting the notion that optimizing ATP binding in site 1 enhances the function of G551D channels.

Optimized G551D Channels Enter into Longer Open Bursts—We next examined the channel kinetics for those compound G551D-containing mutants. Current traces in Fig. 4, A–D, show discernible openings and closings of G551D channels with Trp-401 or His-1348 mutation, recorded in the presence of 2.75 mM ATP. E, mean open time for G551D channels and those mutant channels in A–D. Asterisk, p < 0.01, F, estimated increase of the opening rate (Rco) upon the application of ATP for G551D-CFTR and mutant channels in A–D. There is no error bar for this panel as the ratio Rco/ATP/Rco/Basal was estimated by using data from two sets of different experiments. It is noted that some short openings could be observed for optimized G551D channels, implicating that ATP-independent openings may still be present. Therefore, it is possible that our kinetic analysis, by lumping all opening events into one single population, could underestimate the effect of ATP. Nevertheless, this analytic imprecision does not affect our conclusion that ATP increases the Po of optimized G551D channels mainly by prolonging the channel open time. The number above each bar represents the number of patches.
CFTR Function Improved by Optimized Site 1

A WT 3 sec

B ΔF508 3 sec

C Mean open time (s)

D Open probability

E Opening rate (s⁻¹)

FIGURE 5. Effects of W401F/H1348G mutations on WT and ΔF508 channels. A, 30-s single-channel recordings of WT and W401F/H1348G channels exposed to 2.75 mM ATP. B, current recordings of ΔF508 and ΔF508/W401F/H1348G channels in the presence of 2.75 mM ATP. C–E, mean open time (C), open probability (D), and opening rate (E) extracted from single-channel experiments as shown in A and B. Asterisk, p < 0.01. The number above each bar represents the number of patches.

mutants with more robust nucleotide response tend to have a longer mean open time. On the other hand, all tested mutant channels appeared to have a similarly low opening rate that is only ~1.5-fold higher than that in the absence of ATP (Fig. 4F). Therefore, mutations that optimize nucleotide-site 1 interactions only exert slight effects on the opening rate but greatly stabilize the open state once the channel passes through the opening transition process. Possible mechanisms and implications for these observations will be discussed.

W401F and H1348G Mutations Improve the Function of WT and ΔF508 Channels—To this point, we have demonstrated that optimizing the interactions of ATP with site 1 components, NBD1 head (W401Y and W401F) and NBD2 tail (H1348G), ameliorates the gating defects of G551D channels, which hold a non-functional site 2. However, it is unclear whether the same maneuver is also effective for other CFTR channels, such as WT- or ΔF508-CFTR, whose opening and closing are mainly controlled by ATP binding and hydrolysis in site 2. It is noted that our previous report suggests an allosteric communication between site 1 and site 2; closure of WT-CFTR timed by ATP hydrolysis in site 2 can be delayed by the high affinity ligand PATP bound in site 1 (14). We therefore speculate that optimizing site 1 for ATP binding may slow down closure of WT- and ΔF508-CFTR channels and leads to an increased overall $P_o$. Indeed, when W401F and H1348G mutations were engineered into WT channels (Fig. 5A), the mean open time of WT-CFTR was more than quadrupled (Fig. 5C) with the already high $P_o$ (~0.4) nearly doubled (~0.78, Fig. 5D). Furthermore, these two mutations similarly prolonged the mean open time of ΔF508 channels (Fig. 5, B and C) and increased its $P_o$ from ~0.03 to ~0.22 (Fig. 5D), reaching ~50% of WT activity. In either case, W401F/H1348G mutations did not significantly alter the opening rate (Fig. 5E). Thus, the strategy to restore the function of G551D-CFTR by strengthening nucleotide-site 1 interactions also works to augment the activity of WT or ΔF508 channels.

DISCUSSION

Two main findings were presented in the current study. First, ATP-site 1 interactions of the CFTR channel can be strengthened by introducing mutations in both the head domain of NBD1 (i.e. W401Y, W401F) and the tail domain of NBD2 (i.e. H1348G). Second, enhancing ligand-site 1 interactions improves not only the function of G551D-CFTR with a disabled site 2 but also WT and ΔF508 channels that rely mostly on site 2 for gating control. These findings provide structural insights into CFTR function (see below) and serve as a pharmacological proof of principle that site 1 could be a target for developing CFTR potentiators.

Optimization of CFTR Site 1 for ATP Binding—CFTR site 1 contains many non-canonical substitutions scattered in several ATP-interacting motifs (26). There are three in the NBD1 core subdomain: a His to Ser mutation (position 605) in the H-loop, a Ser to Ala mutation (position 576) in the D-loop, and a Glu to Ser mutation (position 573) right after the Walker B motif. The signature sequence of NBD2 contains two His substitutions at positions 1348 and 1350. A careful examination of CFTR NBD1 sequence also reveals some residues that conform to ABC-consensus but appear only infrequently in other ABC proteins. For example, the corresponding residue of Trp-401 in CFTR is usually a tyrosine, and the last two residues in the Walker A motif (ST at positions 465 and 466 for CFTR) are usually TT, TS, or SS in other human ABC proteins.

It has been known that the H-loop histidine and the glutamate next to the Walker B motif are essential for the ATPase activity of ABC proteins (1, 2). It is thus not surprising that site 1 was shown to have a very low nucleotide turnover rate (15, 27–29). However, except for this low ATPase activity, it is unclear whether some other properties of a canonical composite site have been affected by the aforementioned non-consensus substitutions or uncommon residues in CFTR site 1. Here, we reported that ATP binding in CFTR site 1 is not optimized and can be improved by converting Trp-401 to Tyr/Phe and His-1348 back to Gly, the ABC protein consensus. This finding seems surprising in light of the prevailing view that site 1 has already mediated extremely tight nucleotide binding (15, 27–29). In the future, it will be of great interest to explore whether site 1 might be further optimized not only for ATP binding but probably also for restoring catalytic ability. In either case, the research focus may be drawn not only to those non-conserved substitutions but also to those amino acids whose small differences
from their corresponding residues in other ABC proteins may also pose significant impacts on the functional properties of CFTR.

Some cautions regarding our data interpretations need to be borne in mind. First of all, it must be noted that one limitation of our functional study is that it did not allow us to measure ligand binding strength in site 1 directly. Instead, what we measured and used to infer the tightness of ligand binding is the PPi-induced lock-open time (Fig. 1, B–D). We noticed that incorporating mutations expected to destabilize ATP binding in site 1 into WT-CFTR shortens the lock-open duration. These mutations include W401G, W401I (Fig. 1, B–D), which eliminate a ring-ring stacking interaction, S1347G (supplemental Fig. S6), which may break a hydrogen bond between ATP and the NBD2 signature motif, and G1349I (supplemental Fig. S6), whose side chain likely protrudes into site 1 and causes a steric clash with ATP (22–24). It is also noticed that the shortened lock-open time observed with all mutant channels can be at least partially restored by applying PPi together with the high affinity ATP analog, PATP (Fig. 1, B–D and supplemental Fig. S6). It is this correlation between the chemical nature of mutations and the stability of the lock-open state that grants us the confidence that W401Y, W401F and H1348G mutations, which prolonged the lock-open duration of WT-CFTR, indeed tighten ATP binding in site 1. This argument gains further support when one considers the fact that the tryptophan-mediated (Trp-401) stacking interaction with the ATP molecule is observed less frequently than that mediated by Phe/Tyr in ABC proteins (21) and that a histidine residue (His-1348) in the NBD2 signature sequence is likely to cause steric hindrance for ATP binding in site 1 (25).

Because G551D-containing channels cannot be locked open by PPi, presumably because PPi cannot bind stably in site 2 with an Asp-551 side chain, we cannot use the same strategy of measuring lock-open duration to gauge the strength of ligand binding in site 1 for G551D-containing channels. Instead, we measured the time constant for the current decay of optimized G551D channels upon removal of nucleotide-containing solutions (Fig. 2A) under the assumption that the current decay reflects dissociation of the nucleotide from site 1. The results shown in Fig. 2C did suggest that W401Y, W401F mutations enhance nucleotide binding in G551D site 1, like in the case of WT site 1. Nonetheless, we would like to emphasize here that we cannot rule out the possibility that the current decay reflects other molecular events, such as the relaxation of nucleotide-induced conformational changes, and thus has little to do with nucleotide dissociation.

Possible Mechanism by Which Optimized Site 1 Improves CFTR Function—The most interesting finding in the current study is that the optimized site 1 not only compensates the defective site 2 in supporting G551D channel function but also enhances site 2-controlled gating of WT- and ΔF508-CFTR. Before we discuss possible structural mechanisms underlying these results, we first cover some technical issues about quantification of channel activity for optimized G551D-CFTR.

Unlike WT and ΔF508 channels, whose $P_o$ in the presence or absence of W401F/H1348G mutations (Fig. 5) can be measured with reasonable accuracy, the G551D-containing channels exhibit a $P_o$ too low to be derived from single-channel analysis. We therefore had to resort to alternative methods and accept certain uncertainties. One simple method is to normalize the $P_o$ of optimized G551D mutants in the presence of ATP to the $P_o$ of the ATP-independent activity by calculating the ratio $I_{ATP}/I_{Basal}$ (Figs. 2B and 3D), where $I$ is the macroscopic current amplitude for optimized G551D channels. Theoretically, if these G551D-containing mutants have a $P_o$ for basal activity similar to that of G551D- and WT-CFTR (i.e. ~0.004, Refs. 7 and 13), their $P_o$ in the presence of ATP could be approximated by multiplying the ratio $I_{ATP}/I_{Basal}$ with 0.004. For instance, that the W401F/H1348G/G551D channel has an $I_{ATP}/I_{Basal}$ of ~25 indicates that the $P_o$ for this mutant is ~0.1. To verify this value, we performed stationary noise analysis for the W401F/H1348G/G551D mutant, and the resulting $P_o$ of 0.09 ± 0.01 (supplemental Fig. S7) did provide some reassurance. Unfortunately, we cannot use this approach to estimate the $P_o$ for other compound mutants, such as W401Y/G551D, as the $P_o$ values for these channels are likely too small for noise analysis to be accurate (see supplemental text).

For WT-CFTR, it is known that NBD dimerization induced by ATP binding to the NBD2 core subdomain is coupled to the conformational changes in transmembrane domains that open the CFTR pore (5, 6). Hydrolysis of the same ATP molecule, now buried in site 2, reverses this process by separating the NBD2 core and the NBD1 helical subdomains into a partially opened NBD dimer (5, 6, 15, 29). We have observed that W401F/H1348G mutations in site 1 prolonged the mean open time of WT-CFTR (Fig. 5C). Because this compound mutation stabilizes the open state to a similar extent no matter channels close via the hydrolytic (Fig. 5C) or non-hydrolytic pathway (supplemental Fig. S8), we conclude that an elevated energy barrier for partial NBD separation, rather than an altered ATP hydrolysis rate in site 2, accounts for the stabilized open state. Thus, it appears that a stronger ATP binding in site 1, due to the presence of W401F/H1348G mutations, can allosterically tighten the connection between two NBDs around site 2, thereby slowing down channel closure. Further experiments are required to test whether optimized site 1 increases ΔF508-CFTR channel activity by a similar mechanism.

For optimized G551D channels, kinetic analysis showed that in the presence of ATP, these channels exhibited a slow opening rate (Fig. 4F) similar to that of ATP-independent gating but a prolonged open time (Fig. 4E) comparable with that of site 1-optimized WT-CFTR. Thus, the restored ATP-dependent activity of these G551D-containing channels is mainly due to an increased stability of the open state upon application of ATP. As site 2, which mediates rapid channel opening in WT-CFTR, is disabled by the G551D mutation, the slow opening rate is an expected observation. However, it is puzzling how an enhanced ATP-site 1 interaction can lead to prolonged open bursts. Three tentative conclusions drawn from the current results may provide a rough picture for the underlying structural mechanism.

First, as the ATP-induced mean channel open time can be modulated by mutations at both the head of NBD1 and the tail of NBD2 (Fig. 4E), it is likely that both of these two
subdomains interact with ATP in the open state (i.e. the two subdomains are connected with ATP buried in their interface). Second, because ATP may bind in site 1 (Fig. 2C) longer than the mean channel open time (Fig. 4E), we reasoned that the channel can close without the disengagement of the two NBD constituents of site 1 (see supplemental Fig. S9 for details). Here, if we take one step further in hypothesizing that the open state for G551D-containing channels, like WT-CFTR, also represents an NBD dimer, we can explain the ATP-elicited long open bursts with the same mechanism used for site 1-optimized WT-CFTR; that is, the tight ATP-site 1 interaction allosterically delays partial separation of NBDs. However, this NBD dimer state, if it exists, may have an unoccupied site 2 to avoid a possible steric clash between ATP and the Asp-551 residue, an idea resonant with our third conclusion that NBD2 could remain vacant during ATP-dependent gating of optimized G551D channels as the Y1219G mutation, which disrupts ATP binding in NBD2, posed no functional impact on these channels (supplemental Fig. S4). It should be noted here that more experiments will be needed to examine this provisional mechanism, and the crucial postulate that even for G551D-CFTR, NBD dimerization constitutes the fundamental mechanism for coupling NBDs and gating of the pore, particularly requires experimental verification.

Strategies for Developing CFTR Potentiators Targeting Site 1 —
A main challenge for CFTR investigators in recent years has been to identify chemical compounds called correctors that improve membrane expression of CFTR and potentiators that restore defective CFTR function. In the current study, we have demonstrated that modulating site 1 for optimal ATP binding can improve the function of WT and CF-related mutant CFTR channels. This observation implies that a high efficacy potentiator of CFTR may be developed by designing a chemical with a high affinity for site 1. Because site 1 differs from its catalysis-competent homologues of other ABC proteins by numerous non-canonical substitutions, potentiators designed for tight binding in site 1 could have a distinct advantage of possessing high specificity.

For rational design of CFTR potentiators targeting site 1, one may take full advantage of recent efforts in high throughput screenings that led to the discovery of numerous CFTR potentiators (30). We envisage that those compounds that can identify chemical compounds called correctors that improve membrane expression of CFTR and potentiators that restore defective CFTR function. In the current study, we have demonstrated that modulating site 1 for optimal ATP binding can improve the function of WT and CF-related mutant CFTR channels. This observation implies that a high efficacy potentiator of CFTR may be developed by designing a chemical with a high affinity for site 1. Because site 1 differs from its catalysis-competent homologues of other ABC proteins by numerous non-canonical substitutions, potentiators designed for tight binding in site 1 could have a distinct advantage of possessing high specificity.

For rational design of CFTR potentiators targeting site 1, one may take full advantage of recent efforts in high throughput screenings that led to the discovery of numerous CFTR potentiators (30). We envisage that those compounds that interact with site 1 to potentiate CFTR mutants can be identified by examining whether ATP serves as a competitive inhibitor for their actions on G551D channels. Iterative modifications of these existing potentiators and testing the effects of the resulting products on mutant CFTR may provide information about necessary structural properties that a molecule must possess to act on site 1. In this regard, VX-770, a potentiator currently in phase III clinical trials in CF patients carrying G551D-CFTR, is of particular interest in that the prolonged open time of G551D-CFTR channels in the presence of VX-770 appears to be reminiscent of the ATP-dependent gating of site 1-optimized G551D channels described in the current study (31).

Finally, a unique advantage of choosing site 1 for drug design is the availability of high resolution crystal structures of human CFTR NBD1 (18) and NBD2 (Protein Data Bank (PDB) code: 3GD7). Establishing a structural model of NBD1-NBD2 dimer by computational methods could provide guidance for improving pharmacological properties of existing small-molecule compounds acting on site 1 as well as initiating in silico screening for candidate CFTR potentiators.

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