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In this study, we present data indicating a robust and specific domain interaction between the cystic fibrosis transmembrane conductance regulator (CFTR) first cytosolic loop (CL1) and nucleotide binding domain 1 (NBD1) that allows ion transport to proceed in a regulated fashion. We used co-precipitation and ELISA to establish the molecular contact and showed that CL1 binding was not altered by the common clinical mutation F508del. Both intrinsic ATPase activity and CFTR channel gating were inhibited severely by CL1 peptide, suggesting that NBD1/CL1 binding is a crucial requirement for ATP hydrolysis and channel function. In addition to cystic fibrosis, CFTR dysfunction has been implicated in the pathogenesis of prevalent diseases such as chronic obstructive pulmonary disease, acquired rhinosinusitis, pancreatitis, and lethal secretory diarrhea (e.g. cholera). On the basis of clinical relevance of the CFTR as a therapeutic target, a cell-free drug screen was established to identify modulators of NBD1/CL1 channel activity independent of F508del CFTR and pharmacologic rescue. Our findings support a targetable mechanism of CFTR regulation in which conformational changes in the NBDs cause reorientation of transmembrane domains via interactions with CL1 and result in channel gating.

The cystic fibrosis transmembrane conductance regulator (CFTR)2 is a member of the ATP-binding cassette (ABC) family of proteins. Like its relatives, CFTR is composed of two nucleotide binding domains (NBDs) and two membrane-spanning domains (MSDs) that, together, form a transmembrane conduit. Unlike transporters of larger solutes that constitute the majority of the ABC protein family, CFTR mediates the conduction of chloride and bicarbonate across the plasma membrane. CFTR also contains a unique regulatory (R) domain whose phosphorylation by PKA controls channel function (reviewed in Ref. 1). CFTR subdomains, such as the regulatory insertion (RI), are also CFTR-restricted.

Mutations in CFTR cause cystic fibrosis by disrupting epithelial ion transport. This can be indirect, by interfering with proper folding and maturation of the channel in epithelial cells, or more direct, by disrupting the mechanochemistry of activation, gating, or conduction processes. The lack of a phenylalanine residue at position 508 in the first nucleotide-binding domain (NBD1) of CFTR is observed in a majority of patients with cystic fibrosis worldwide (2). This mutation results in biosynthetic arrest of CFTR in the endoplasmic reticulum, where the protein is ubiquitinated and subsequently degraded by the proteasome rather than being transported through the Golgi to the plasma membrane. Defects in both protein stability and channel gating are also well established for F508del (3–6).

An area of particular interest is the interface between the NBDs and the MSDs. The crystal structures of several whole...
bacterial ABC transporters, including BtuCD, Sav1866, HI1470, ModB2,C2, MalFGK2, MsbA, and the mammalian P-glycoprotein, have been solved (7–13). All structures indicate that the two NBDs contact the two MSDs through loops between transmembrane helices, the "cytosolic loops" (CLs). In most cases, a particular CL of one MSD connects with the cis NBD. In the case of Sav1866, CL2 connects with the opposite NBD, but CL1 contacts both NBDs (so-called "domain swapping" (7)). In P-glycoprotein, CLs 1 and 4 represent the contact points with NBD1 (13). Changes in conformation induced by ATP binding and/or hydrolysis by the NBDs can be transmitted to the MSDs through the cytoplasmic loops, allowing for reorientation of the two MSDs with respect to each other. This conformational change is believed to govern opening of ABC transporters and movement of substrate. It has been predicted that a similar mechanism also regulates CFTR channel gating. Cysteine cross-linking studies suggest interactions between NBD1 and CL1 and/or CL4 (14, 15) but revealed that other connections are possible as well (15, 16). Earlier findings also indicate that Phe-508 is required for proper interaction with CL4 (17, 18).

In this study, we used an unbiased, peptide-based mapping strategy to probe CL connections within the CF gene product. Approaches such as these have been used in the past to delineate protein binding partners, intramolecular interactions, and peptide mimetics and form the basis for successful two-hybrid screening strategies and peptide ELISAs (19–25), including studies of CFTR (26). In our experiments, we used several independent protocols (immunoprecipitation of recombinant NBD1, CL1 binding to isolated and properly folded NBD1, and cell-free ELISA) to demonstrate a strong and specific affinity between CL1 and NBD1. We pursued this further by showing that peptide binding partners, intramolecular interactions, and peptide mimetics and form the basis for successful two-hybrid screening strategies and peptide ELISAs (19–25), including studies of CFTR (26). In our experiments, we used several independent protocols (immunoprecipitation of recombinant NBD1, CL1 binding to isolated and properly folded NBD1, and cell-free ELISA) to demonstrate a strong and specific affinity between CL1 and NBD1. We pursued this further by showing that the interface is critical for CFTR ion transport, regulates ATP hydrolysis, and can be utilized to identify small molecules that modulate the ion channel activity of the full-length protein. Our findings point to a CFTR gating mechanism in which the correct orientation of CL1 serves as a prerequisite not only for pore formation but also for heterodimerization of NBD1 with NBD2.

**Experimental Procedures**

**Reagents and Constructs**—C-terminally biotinylated peptides were obtained from Genemed Synthesis as shown in Fig. 1. A biotinylated and scrambled control peptide for CL1-1B had the sequence MFYKGIMRKSTMLKQLAK. The peptides were synthesized by solid phase methodology with Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. Peptide purity was confirmed by C-18 column HPLC and mass spectrum analysis (purity >90% in all cases). Anti-HA antibodies were purchased from Covance Research Products. Antiserum against the NBD1 domain of human CFTR was generated in rabbits. cDNAs for human WT or F508del NBD1 (amino acids 389–673) and full-length WT or F508del CFTR were cloned into pcDNA3.1 with amino-terminal HA tags. These constructs were termed pCHA NBD1 WT, etc. Constructs without the regulatory extension (RE, amino acids 635–673) or RI (amino acids 405–436) were also subcloned. The identities of all constructs were verified by sequencing. Recombinant purified and properly folded NBD1 protein (amino acids 389–678) used in the experiments shown in Figs. 2D and 4C was provided by SGX Pharmaceuticals and bound ATP as described previously (27). This protein contained the solubilizing mutations F494N/Q637R. The NBD1 protein used in the pilot screen (Figs. 8–10) was provided by Dr. Phil Thomas.

**Pulldown Assays**—The pCHA constructs were used to transiently transfected HEK293 cells, with empty pcDNA3.1 and pCHA M-Ras as controls. Two days post-transfection, cells were harvested and lysed in pulldown buffer (200 mM NaCl, 50 mM Tris HCl (pH 7.5), 5 mM MgCl2, 15% glycerol, and 1% Nonidet P-40) supplemented with protease inhibitors (Roche). 10 μg of biotinylated peptide was immobilized on streptavidin-Sepharose per pulldown sample. 400–500 μg of protein from cell lysates was rotated with peptide-bound streptavidin-Sepharose for ~1 h at 4 °C. Samples were washed three times with pulldown buffer before addition of 5× SDS loading buffer for analysis by Western blot. For pulldown assays with purified NBD1 protein, 0.2 μg of NBD1 was added to peptide-bound Sepharose in a total volume of 200 μl of buffer, and samples were rotated for ~45 min at 4 °C. In NBD1/CL1 dissociation experiments, 2 μg of CL1-1B or other peptides was immobilized on streptavidin-Sepharose beads were blocked by a 1-h incubation with excess free biotin. The blocking efficiency was checked and found to be complete (data not shown). Cell lysate (500 μg) was added to the blocked beads and rotated for 1 h at 4 °C. After three washes with pulldown buffer, free biotinylated CL1-1B peptides were added in 200 μl, and samples were rotated at 4 °C for 1 h. Samples were washed with pulldown buffer, and SDS loading buffer was added for Western blot analysis. Kinetic pulldown data were analyzed using curve-fitting software (GraphPad Prism). Where quantitation by densitometry was performed, p values were calculated by t test.

**NBD1 ELISA to Study Binding Kinetics**—Streptavidin-coated black 96-well plates (Nunc) were incubated with a 50 μM solution of CL1 peptides in PBS-Tween and blocked with 0.5% BSA in PBS-Tween. 100 μl of a 100 nM NBD1 WT or F508del protein solution (containing a single solubilization mutation) in 20 mM Tris HCl (pH 7.4) at 4 °C (with 3 mM ATP, 150 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine, and 10% glycerol) was added to the wells and serially diluted 2-fold to a concentration of 1.6 nM. The plate was then incubated at 4 °C for 2 h. After thorough washing, bound NBD1 was detected using the LI2B4 anti-NBD1 antibody and an HRP-labeled secondary antibody in the presence of Amplex Red reagent (Molecular Probes). Fluorescence was measured at 560/590 nm (excitation/emission). The reading from wells without NBD1 protein was subtracted from all data points. Curves were fitted using y = V_max × x / (k_d + x) and GraphPad Prism software.

**Membrane Patch Analysis**—Macroscopic currents from inside-out membrane patches excised from baby hamster kidney cells stably expressing wild-type CFTR were evoked by a ramp protocol from −80 to +80 mV. CFTR channels were activated by exposure to 1.5 mM ATP and 110 units/ml PKA (control). Further phosphorylation by PKA was blocked by adding protein kinase inhibitor (1.4 μg/ml) while PKA was still present. This maneuver stabilizes the phosphorylation status of CFTR (28) so that the effects of CFTR inhibitors are not attributable to indirect modulation by PKA. CL1-1 peptide was tested at 14 μg/ml.
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(∼3.73 μM, added to the cytosolic membrane), and the CFTR inhibitor Inh-172 (Sigma) was used at 10 μM to block all remaining channel activity. In single-channel studies, currents were recorded at +60 mV across patches obtained using small-tip pipettes (10–12 MΩ). Channels were activated with a lower dose of PKA (27.5 units/ml) to resolve unitary currents. Both the open probability of the patch and open rate (openings per second per patch) were monitored as indicated, with data normalized to the open probability of the patch or opening rates before the addition of peptide. Modulator compounds added to the cytosolic compartment were tested in a similar fashion.

ATPase Measurements—ATPase activity was measured by determination of the production of [γ-32P]P, from [γ-32P]ATP exactly as described previously by Ramjeesingh et al. (29).

Compound Screening—Compounds (2200) were chosen randomly as a subset from the NCI/National Institutes of Health Open Chemistry Repository Library (http://www.dtp.nci.nih.gov/) and tested by an adapted (proof of concept-type) ELISA to study alterations in binding of biotinylated CL1-1B peptide to recombinant NBD1 protein. Properly folded human NBD1 at 5 μg/ml in PBS 0.5% Tween was added to 96-well plates overnight at 4 °C, followed by blocking with BSA-Tween. 100 μl of 20 mM biotinylated CL1-1B with 20 μM of test compound (in PBS-Tween) was added in duplicate for 1 h at room temperature. After washing, bound CL1-1B peptide was detected using horseradish peroxidase-conjugated Neutravidin (Invitrogen) with Quantablu fluorogenic peroxidase substrate (Pierce). Fluorescence was measured at 320/405 nm (excitation/emission). Wells without NBD1 (in the presence of peptide but absence of compound) were considered negative controls, and wells with NBD1 and peptide in the absence of test compound were considered positive controls. Z factors were calculated for each plate using these controls. The average value was Z = 0.87 ± 0.054. A subset of compounds that altered binding of biotinylated CL1-1B peptide to NBD1 precipitated with similar efficiencies on CL1 under steady-state conditions (Fig. 2). We observed high-affinity CL1 binding site on NBD1. A direct interaction of CL1 with NBD1 bound CL1-1 and CL1-1B but did not interact efficiently with other CL1 peptides, providing a further indication of specificity (Fig. 3). These results indicated the presence of a strong binding site within CL1 between Phe-157 and Lys-166 (FSLYKKTLLK) to the first NBD of CFTR. We also noted that CL1 peptides might be useful as “decoy” molecules for disrupting the intramolecular interaction between NBD1 and CL1 within the full-length protein (see below) and that these peptides may be used in place of an antibody for affinity precipitation of CFTR.

Results

Binding of NBD1 to CL Peptides—We designed partially overlapping peptides to span each of the four intracellular loops of CFTR (Fig. 1). NBD1 WT or F508del were affinity-precipitated after recombinant expression from HEK293 cell lysates. Both NBD1 WT and F508del precipitated with the greatest efficiency from samples containing the most N-terminal peptide derived from the first CL, CL1-1, leading to robust recovery (∼12% of input NBD1 (Fig. 2, A, and E)). Given the strong evidence of binding, we focused further on the particular interaction of NBD1 with CL1. We designed shorter sequences, CL1-1A and CL1-1B, which covered the amino- and C-terminal regions of CL1-1, respectively. Although CL1-1A exhibited no binding whatsoever to NBD1 from cell lysates, CL1-1B was even more efficient than CL1-1, precipitating ∼20% of input WT or F508del NBD1 from samples (Fig. 2, B and E). The interaction was selective because a control protein of similar size and overall configuration, the GTPase M-Ras, did not bind to any CL1 sequence (Fig. 2C) even though it was expressed at higher levels than NBD1. Other CLs also bound to NBD1 but with lower efficiency, supporting both specificity and the presence of a high-affinity CL1 binding site on NBD1. A direct interaction of CL1 with NBD1 was also shown by experiments in which recombinant, isolated, and correctly folded NBD1 (following prokaryotic overexpression and protein purification) was precipitated on CL1-1 or CL1-1B (Fig. 2D).

We next asked whether CL1 peptides could be used to precipitate full-length CFTR. Like the isolated NBD, wild-type and F508del CFTR bound CL1-1 and CL1-1B but did not interact efficiently with other CL1 peptides, providing a further indication of specificity (Fig. 3). These results indicated the presence of a strong binding site within CL1 between Phe-157 and Lys-166 (FSLYKKTLLK) to the first NBD of CFTR. We also noted that CL1 peptides might be useful as “decoy” molecules for disrupting the intramolecular interaction between NBD1 and CL1 within the full-length protein (see below) and that these peptides may be used in place of an antibody for affinity precipitation of CFTR.

NBD1 WT and F508del Exhibit Similar Affinities for CL1—The wild-type and the mutant NBD1 precipitated with similar efficiencies on CL1 under steady-state conditions (Fig. 2). We also determined whether there were differences in association...
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or dissociation rates attributable to F508del. When assessed by pulldown from HEK293 cell lysates, both WT and F508del NBD1 associated with CL1-1B rapidly, within minutes (Fig. 4A). Although the association of F508del NBD1 with CL1-1B was slightly slower than that of the WT NBD1 ($p = 0.04$ for the 1-min time point), we did not observe statistically significant differences beyond 1 min (Fig. 4A). Likewise, binding to CL1-1B (or CL1-2) peptide, as measured by ELISA, was very similar for both WT and mutant NBD1 (Fig. 4C). Virtually identical amounts of excess free peptide were required to dissociate bound NBD1 from the immobilized peptide (Fig. 4B).

**The Role of RE and RI Segments in CL1 Binding**—The regulatory extension (CFTR amino acids 635–673) lies at the C terminus of NBD1, is sometimes viewed as part of the R-domain, and contains the phosphorylation site Ser-660. Studies by NMR indicate that phosphorylated RE undergoes a conformational change that promotes NBD heterodimerization with NBD2 (31). It has also been suggested that the NBD1 regulatory insertion (amino acids 405–436) restricts access of CL1 to its binding site, although aspects of this model were not confirmed by chemical cross-linking (15, 32). Because both RE and RI have been implicated in CFTR gating and because CL1 strongly inhibits channel activity (see below, Figs. 6 and 7), we deleted RE segments, either alone or in combination with RI, from WT or mutant NBD1 to study the effects on binding to CL1-1B peptide. The association kinetics for WT or mutant NBD1 were not significantly different from the full-length NBD1 (Fig. 5, A and B). However, in the absence of the RI segment, WT NBD1 showed slower binding kinetics at the earliest time points tested ($p = 0.006$ at 1 min and $p = 0.0043$ at 2 min.), whereas NBD1 F508del binding was not affected significantly by the absence of the RI ($p$ values all $>0.5$; Fig. 5, C and D). This suggests that small conformational differences in the spatial orientation of the RI segment between WT and mutant NBD are mediated allosterically and affect overall domain structure (as reported by others) (33). Additional biochemical, functional, enzymatic, and drug screening experiments were conducted next to test the relevance of the NBD1/CL1 interaction within full-length CFTR.

**CL1 Peptides Block CFTR Channel Activity**—To study the relationship between NBD1/CL1 binding and the activity of CFTR in its full-length conformation, we added CL1 peptides to excised inside-out membrane patches from baby hamster kidney cells expressing wild-type human CFTR. We observed a pronounced inhibition of membrane currents by $\sim 80\%$ because of the presence of the CL1-1 peptide (Fig. 6, A and B; $p < 0.001$). We also studied unitary CFTR currents in single-channel patches. There were far fewer channel openings per time span when CL1-1 was added, whereas unitary currents, *i.e.* single-channel conductances, were unaffected (Fig. 6C). Notably, quantification of these data showed that addition of CL1-1 greatly inhibited the open probability of the patch and the opening rate (openings per second per patch) (Fig. 6, D and E). Because we were unable to wash out the CL1-1 peptide in these experiments, we could not determine the number of channels in a patch or values for open probability ($P_o$). However, we estimated that $P_o$ was reduced to $\sim 50\%$ in the presence of CL1-1 peptide on the basis of the assumption that the total number of channels ($N$) in each patch equaled the maximum number of simultaneous openings (mean control $P_o = 0.13 \pm 0.04$ versus mean peptide $P_o = 0.061 \pm 0.01$). These data suggest that a binding interaction between NBD1 and CL1 is an important requirement for channel gating and that excess CL1-1 disrupts this interface in full-length CFTR.

**CL1 Peptides Inhibit CFTR ATPase Activity**—We also determined whether blocking NBD-CL1 interactions could affect the intrinsic ATPase activity of CFTR. Fig. 7 shows that the CL1-1B peptide inhibited ATPase activity by $\sim 50\%$ at 10 $\mu M$ and by $\sim 75\%$ when used at 160 $\mu M$ ($p < 0.05$ in both cases). One interpretation of this finding is that addition of CL1 peptide elicits a conformational change in purified full-length CFTR that disrupts ATP binding. That the peptide did not completely abolish ATPase activity suggests that proper alignment of the NBD1/2 dimer is necessary but not sufficient for maximal ATP hydrolysis.

**Compounds That Influence NBD1/CL1 Binding**—Cell-based compound library screening to discover CFTR potentiators or inhibitors using intact epithelial cells probed with fluorescence...
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FIGURE 2. NBD1 interactions with peptides derived from the cystolic loops of CFTR. A and B, HA-tagged NBD1 WT or F508del (∆F) transiently expressed in 293 cells were precipitated on CL peptides. One-tenth of the amount of protein that was used for a pulldown sample was loaded in the whole cell lysate (wcl) lanes. b.o., beads only. C, HEK293 lysates containing HA-tagged M-Ras were used in pulldown assays with CL1 peptides. D, purified recombinant NBD1 WT was precipitated on CL1 peptides. E, pulldown data quantified by densitometry presented as percent of total protein expressed. HA-tagged proteins and recombinant NBD1 were visualized by Western blotting. Error bars show mean ± S.E. (n = 3). The unbiased pulldown protocol revealed a comparatively weak interaction of NBD1 with CL4, which may reflect the specific protocol used to monitor affinity (i.e., protein domains studied here versus extensively cysteine-substituted, full-length CFTR tested previously) (15).

FIGURE 3. Interactions of full-length CFTR with CL1 peptides. Full-length HA-CFTR WT or F508del from lysates of HEK293 cells were precipitated on CL1 peptides. Wcl, whole cell lysates; b.o., beads only.

FIGURE 4. Association and dissociation kinetics of NBD1 WT and F508del. A, lysates from HEK293 cells expressing HA-NBD1 WT or F508del were incubated with CL1-1B peptide immobilized on Sepharose for the indicated amounts of time. The level of NBD1 bound to CL1-1B-Sepharose was quantified by Western blot and densitometry. The value of the 0X sample (no free competing peptide (or no peptide) was added for one additional hour. NBD1 that remained bound to Sepharose beads with CL1-1B was quantified by Western blot analysis and densitometry. The value of the 0X sample (no free competing peptide added) was set to 100%, and all other data are expressed in relation to that sample. Error bars in A and B show mean ± S.E. (n = 3), except for the data points at 25X in B, where n = 2. (C) ELISA assays were performed using CL1 peptides immobilized on 96-well plates and recombinant NBD1 WT or F508del proteins at different concentrations, as indicated.

basis of purified protein domains has not been reported previously but could furnish a valuable adjunct to test new chemical space and identify agents that directly bind CFTR, minimizing off-target (including cellular kinase or other) effects. In this context, very limited information is available regarding modulators that directly bind CFTR, and no previous binding epitope on CFTR for any modulator has yet been described (40). Discovery of new modulator compounds is relevant for therapy of cystic fibrosis and a number of diseases associated with acquired CFTR dysfunction (see below). Because our data indicate that CL1 binding to NBD1 is important for channel gating
and ATP hydrolysis (Figs. 6 and 7) and that the contact interface is tractable by a cell-free assay (Fig. 4C), we investigated whether a similar ELISA-based system could be configured to test drugs that directly modify NBD1/CL1 binding. Fig. 8A shows dose-dependent binding of CL1-1B peptide to immobilized NBD1 protein (EC50/H11015 20 nM) and poor binding of a scrambled peptide. A study of 2200 small molecules obtained from the NCI/National Institutes of Health Open Chemistry Repository Library was conducted in a pilot fashion and led to the identification of four new agents that augment NBD1/CL1 binding and 13 inhibitors (Fig. 8B). Among the inhibitors, four discovered independently (on different plates and days) were found to share a similar chemical structure (Fig. 8C).

Functional data for an active enhancer (D7) and inhibitor (F8) are shown in Figs. 9 and 10. The D7 compound enhanced binding of NBD1 to CL1-1B in a dose-dependent fashion by ELISA (Fig. 9A) and augmented CFTR chloride channel activity in excised, inside-out membrane patches 1.6-fold at 20/H9262 M (Fig. 9B). The drug also effectively stimulated short-circuit current across monolayers of human bronchial epithelial cells expressing WT CFTR (Fig. 9C). In addition, D7 enhanced chloride transport of F508del CFTR ~6-fold (following overnight incubation with 5 μM C18, a pharmacologic corrector of misprocessing (Fig. 9D)). To test corrected F508del potentiation by an independent protocol, Fischer rat thyroid cells encoding a single integrated copy of the F508del mutation (using methods described in Refs. 41, 42)) were evaluated. These cells have been utilized widely for compound library screening and evaluation of CFTR modulators (41–43) and also exhibit abrogation of F508del rescue because of chronic potentiator exposure (Fig. 9E). F508del activity following 24-h treatment with D7 (a compound believed to influence NBD1/CL1 binding in a manner relatively independent of F508del (Figs. 2 and 4)) produced modest but significant effects on total activity of the mutant protein.

In experiments to probe NBD1/CL1 interface disruption, inhibitor F8 reduced CL1-1B peptide binding to NBD1 to 30% when tested at 1.6/H9262 M by ELISA, with further inhibition at higher concentrations (Fig. 10A). CFTR currents across membrane patches were also inhibited by 75% (Fig. 10B), as were transepithelial currents measured in Ussing chambers (~40% inhibition at 20 μM and ~80% inhibition at 40 μM (Fig. 10C)).

It should be noted that the screen conducted here does not exclusively identify agents specific for CFTR (as opposed to other ABC proteins). More extensive compound library analysis, together with medicinal chemistry, functional optimization, etc., will be necessary to establish drugs that directly bind the NBD1/CL1 interface (see below) and are suitable for clinical advancement.

**Discussion**

In this study, we tested regions that connect the NBDs to MSDs using a series of cytosolic loop probes and determined
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FIGURE 6. CL1 peptides block CFTR channel activity. A, example of a tracing obtained from inside-out macropatches for which addition of CL1-1 peptide (3.7 μM) inhibited CFTR currents. PKI, protein kinase inhibitor. B, data are mean ± S.E. for inhibition of CFTR currents by the scrambled control peptide (ctrl. pep.) and CL1-1 peptide in macropatches (n = 11; *** p < 0.001). C, an example of recordings of unitary currents in the absence (top panel) or presence (bottom panel) of CL1-1 peptide. D and E, quantification of unitary current recordings showing the inhibition by CL1-1 of (D) open probability of the patch (NP0) and (E) opening rate (openings per second per patch) for seven individual patches. The individual patches are represented by the same symbols as those in D and E.

whether there were differences in binding attributable to wild-type or mutant NBD1. A similar approach has been used to characterize binding interactions among numerous integral membrane proteins (19–25), including CFTR binding partners (26). NBD-CL binding has emerged as an area of critical importance with regard to ABC protein regulation, including CFTR ion transport. These findings point to a portion of CL1 that contributes to the MSD/NBD1 interaction in CFTR. The data shown here provide a perspective on full-length CFTR domain binding and function that would not be ascertainable by structural analysis alone or by other methods, such as homology directed protein cross-linking (15).

The well established functional coupling between substrate binding and ATP hydrolysis among ABC proteins suggests that a conformational change elicited by substrate is often communicated to the NBDs. Although direct structural evidence for CFTR regarding this point is not yet available, the observation that a peptide mimetic configured to interrupt the NBD-CL interface also ablates ion channel gating and blocks hydrolysis may offer a clue to a mechanism. The R domain appears to decrease its helical propensity upon phosphorylation by PKA (31, 44), which has been suggested to confer elongation of the domain and loss of an interaction with NBD1, improved NBD1/2 dimerization, and enablement of the binding/hydrolysis cycle that regulates CFTR. Therefore, we believe that the phosphorylated R domain may assume the role of a “pseudo-substrate” to control the overall gating process, allowing CFTR to bind ATP and initiate chloride transport by adopting a mechanism common to other ABC transporters regulated by substrate binding. Our data suggest that a high-affinity interaction between NBD1 and CL1 plays a crucial but downstream role in this process because the connection appears to be vital for reorientation of the transmembrane helices and opening of the channel. On the basis of data presented here and by others (31, 45), we speculate that a conformational signal (the interaction between NBDs and MSDs to gate CFTR) flows both ways. A well formed interface is required for optimal association of the NBDs to permit ATP binding and/or hydrolysis and, at the same time, allows the power of NBD dimerization to constrain the transmembrane domains and regulate channel gating.

The Sav1866 structure was the basis for cysteine cross-linking studies that identified possible NBD1 contacts with the cytosolic loops in CFTR (15, 16). It should be noted that CL1-1B studied here ends approximately five residues before the corresponding sequence in Sav1866 is predicted to insert within a groove between the α-helical and Rec-A-like subdomains of NBD1. We suggest, therefore, that the specific conformation necessary for CFTR gating may not be fully represented by the structure of proteins such as Sav1866. In the ABC transporter Bmr A, for example, there is evidence for physical disengagement of CL1 from NBD1 during ATP hydrolysis-driven realignment of the transmembrane α helices, a finding compatible...
with data shown here (Figs. 6 and 7) that might otherwise not be captured by a fixed structural model (46). The mechanism of CFTR opening itself has been argued to differ fundamentally from many other ABC proteins, with the outward facing conformation (and NBD dimerization) associated with pore closure (47). There is also growing appreciation of the importance of NBD1 allostery, rather than direct contacts defined by in silico modeling, being responsible for key aspects of CFTR gating and processing. Complete omission of the RI, for example, substantially overcomes both the F508del gating and processing defects, although RI is not expected to structurally assume any direct contact with Phe-508 that can readily explain these findings (33). Allosteric modulation of CFTR channel activity has also been shown in studies of specific transmembrane domain mutations that facilitate CFTR opening in an ATP-independent fashion (48–49). On the basis of its unique ion channel function, therefore, we suggest that CFTR gating may adhere only in part to previous structural models derived from other ABC solute transporters.

There has been recent clinical progress with regard to CFTR potentiators identified by cell culture-based compound library screening (34–37). New agents discovered in this fashion have served a therapeutic role in cystic fibrosis and may also provide benefit in “acquired” CFTR deficiency states such as chronic obstructive pulmonary disease, chronic sinusitis, and pancreatitis (50–55). On the other hand, conditions such as cholera and enterotoxigenic diarrhea mediated by CFTR overactivity might benefit from therapeutic interventions designed to block CFTR ion transport (56). To date, primary screening for CFTR modulators has largely been confined to drug library analysis using Fischer rat thyroid cell lines expressing recombinant CFTR. Potentiator compounds discovered in this fashion have stimulatory activity against some, but not all, clinically important CFTR conductance defects (41) and may impair pharmacologic correction of F508del CFTR (38, 39).

The importance of allosteric mechanisms that regulate CFTR opening suggests that designation of a binding site for

![Diagram](image_url)
FIGURE 9. Evaluation of the CFTR activator D7. A, D7 augmentation of CL1-1B binding to NBD1 in a dose-dependent fashion by ELISA. a.u., arbitrary units; conc., concentration. B, D7-stimulated Cl⁻ channel activity in excised, inside-out membrane patches. Right panel, representative tracing of macroscopic current. CFTR Inh-172 (10 μM) was used to block CFTR activity. ***, p < 0.0016 (n = 7). PKI, protein kinase A inhibitor, 1.4 μg/ml; ctrl, control. C, D7-stimulated short-circuit current (∆Isc) with standard error across monolayers of human bronchial epithelial cells expressing wild-type CFTR in Ussing chambers. Right panel, example of short-circuit current tracing. **, p < 0.01 (n = 3). D, the function of CFTR F508del expressed by cystic fibrosis bronchial epithelial cells and corrected by prior overnight incubation with 5 μM C18 is enhanced by D7. ***, p < 0.01 (n = 3). DMSO, dimethyl sulfoxide. E, effect of co-incubation of lumacaftor (VX-809) with potentiators of F508del rescue in Fischer rat thyroid cells. Cells were treated for 24 h at the concentrations shown. CFTR was activated acutely with forskolin (20 μM), and data were analyzed by one-way analysis of variance with Bonferroni’s multiple comparisons test (VX809 treatment alone in comparison, F(10,22) = 18.65, p < 0.0001). *, p < 0.05; **, p = 0.0063; ***, p ≤ 0.0005; ****, p < 0.0001 (n = 3).
modulators described here may be premature. Charged heterocyclics are known to bind transmembrane regions and modulate the activity of other ABC proteins, and the compounds shown in Fig. 8 might therefore interact with NBD1, the NBD1/CL1 interface, and/or other regions of MSD1. This includes the possibility of direct association with (or even passage through) the MSDs themselves prior to entry into cytosol. Although this study cannot exclude more than one CFTR binding position(s) or allosteric effects on gating, our findings provide new information relevant to CFTR regulation, including a drug assay system on the basis of novel mechanistic data.

The results from this report indicate the feasibility of large-scale pharmaceutical protocols to identify modulators that bind directly to purified CFTR domains. The same approach might be used to discover inhibitors of other clinically important ABC proteins. We were particularly encouraged by the observation that one of the compounds studied here strongly enhanced the physiological activity of CFTR F508del in combination with a pharmacologic corrector molecule. The ability to modify protein activity with agents that directly bind CFTR and influence conformation suggest a means by which specific compounds for treatment of ABC protein-related diseases might be developed in the future.

Author Contributions—E. J. S. conceived and coordinated the study, conducted data analyses for all figures, and wrote the paper. A. E. designed, performed, and analyzed the experiments shown in Figs. 2–5. W. W., K. N., and K. L. K. designed, performed, and analyzed the experiments shown in Figs. 6, 9, and 10. C. M. M., M. R., C. E. B., and G. L. L. designed, performed, and analyzed the experiments shown in Figs. 4 and 7. W. J. C., L. C. P., J. H., and S. E. V. designed, performed, and conducted the experiments shown in Figs. 8–10. H. A. L., S. Atwell, and S. Aller contributed purified peptides for the experiments shown in Fig. 2 and performed structural analyses of CFTR domains. All authors analyzed the results and approved the final version of the manuscript.

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