Conserved Allosteric Hot Spots in the Transmembrane Domains of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Channels and Multidrug Resistance Protein (MRP) Pumps*

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Background: Multidrug resistance proteins (MRPs) and the cystic fibrosis transmembrane conductance regulator (CFTR) are thermodynamically distinct ATP-binding cassette (ABC) transporters.

Results: Structural elements that couple ATP binding to channel opening in the CFTR channel also facilitate MRP drug export.

Conclusion: MRPs and CFTR share components of a conserved activation mechanism.

Significance: Allosteric hot spots suggest mechanistic similarities between thermodynamically distinct ABC transporters.

ATP-binding cassette (ABC) transporters are an ancient family of transmembrane proteins that utilize ATPase activity to move substrates across cell membranes. The ABC subfamily of the ABC transporters includes active drug exporters (the multidrug resistance proteins (MRPs)) and a unique ATP-gated ion channel (cystic fibrosis transmembrane conductance regulator (CFTR)). The CFTR channel shares gating principles with conventional ligand-gated ion channels, but the allosteric network that couples ATP binding to its nucleotide binding domains (NBDs) with conformational changes in its transmembrane helices (TMs) is poorly defined. It is also unclear whether the mechanisms that govern CFTR gating are conserved with the thermodynamically distinct MRPs. Here we report a new class of gain of function (GOF) mutations of a conserved proline in the pore-lining TM6. Multiple substitutions of this proline produced ATPase-active MRP pumps (Yor1p) with ATP binding that couples ATP binding at its nucleotide binding domains (NBDs) to conformational changes in its transmembrane helices (TMs), thereby promoting channel opening (CFTR) or substrate efflux (MRPs). Subsequent ATP hydrolysis resets the transport cycle. CFTR opening also requires phosphorylation of a unique regulatory domain by cyclic nucleotide-dependent kinases, but this mechanism is poorly understood in MRPs.

The cystic fibrosis transmembrane conductance regulator (CFTR) plays an essential role in cilia, and the MRPs and CFTR have the added distinction of being ions that passively transport their substrates (small anions, such as chloride) down their prevailing electrochemical gradients. In contrast, the MRPs and CFTR are employed in both the transport of ions and substrates. The MRPs and CFTR share components of a conserved mechanism for controlling ATP binding, and this is the focus of this study.

The translocation pathways of CFTR and the MRPs are composed of multiple transmembrane helices (TMs) linked by intracellular loops (ICLs) to two cytosolic nucleotide binding domains (NBDs). The NBDs dimerize in response to binding two ATP molecules at their dimer interface, causing a conformational shift to propagate from the NBDs to the TMs, thereby promoting channel opening (CFTR) or substrate efflux (MRPs). Subsequent ATP hydrolysis resets the transport cycle. CFTR opening also requires phosphorylation of a unique regulatory domain by cyclic nucleotide-dependent kinases, but this mechanism is poorly understood in MRPs. The MRPs and CFTR have the added distinction that their two ATP binding sites are asymmetric. Site 1 in ABCD1 proteins is catalytically deficient due to a non-canonical signature sequence in NBD2 and, in the case of CFTR, an absent hydrolytic glutamate in the NBD2 Walker B motif.

Consequently, ATP binds stably to site 1 in CFTR (and probably the

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MRPs) and remains bound to that site during multiple rounds of channel opening and closing (11–13).

There is emerging evidence that ATP binding gates open CFTR by an allosteric mechanism that qualitatively resembles the gating of a conventional ligand-gated ion channel (i.e. a channel that reversibly binds rather than consumes its ligand, such as the nicotinic acetylcholine receptor channel) (14, 15). Like other ligand-gated channels for which ligand binding is allosterically coupled to pore opening, CFTR channels do open in the absence of ligand (albeit infrequently), and gain of function (GOF) mutations that increase ATP-free activity have been reported (16, 17). Thus, ATP binding biases the equilibrium toward the CFTR open state but is not absolutely required for channel opening. This is more consistent with the classical allosteric activation schemes that have been proposed for conventional ligand-gated channel gating, as opposed to strict coupling between ATP binding and channel activation (14, 18). The notable difference is that CFTR normally consumes its ligand (ATP) by hydrolysis to promote most channel closings (9, 11). An allosteric activation mechanism implies that GOF mutations that increase ligand-free activity should reciprocally enhance ligand sensitivity because ligand affinity is coupled to channel conformation. This concept of reciprocity is based on the fact that the open conformation has a higher ligand affinity than the closed conformation, a fundamental feature of allosteric activation schemes (14, 19, 20). Previously, we showed that a GOF mutation near the base of TM9 reciprocally increases ATP sensitivity in the NBDs, as predicted by the allosteric model (16).

The extent to which CFTR channel gating and MRP-mediated drug efflux share operational principles is unknown. Given the emerging view that CFTR channel gating by ATP binding shares allosteric properties with the gating of conventional ligand-gated channels, we postulated that aspects of this activation mechanism might also be shared with the evolutionarily related MRP efflux pumps. ABC exporters like the MRPs presumably utilize an alternating access mechanism in which the transporters shift between “inward-facing” and “outward-facing” conformations, alternately exposing substrate to the intracellular and extracellular environments (21, 22). CFTR channels may also alternate between inward- and outward-facing conformations upon ATP binding and hydrolysis with the distinction that one of these conformational states must have a leaky or degraded gate to permit the formation of a pore that is accessible to both sides of the membrane (23). In regard to this pore, TM6 and TM12 are considered the major pore-lining TMs for the CFTR channel, based on the results of mutational studies and cysteine accessibility experiments (24, 25). The homologous TMs in the MRPs have also been implicated in substrate binding and translocation (26).

Here we provide evidence that allosteric mechanisms for coupling conformational changes in the TMs to the ATP occupancy of their NBDs are indeed conserved between CFTR and a yeast MRP, Yor1p (yeast oligomycin resistance protein 1). We report a new class of GOF mutation of a proline near the base of pore-lining TM6 that is conserved in the ABC transporter subfamily. Multiple substitutions at this position in CFTR exhibited GOF effects, including enhanced ATP-free channel activity, increased ATP sensitivity, and stronger activation by the weak agonist, AMP-PNP. CFTR mutations at this TM6 position had additive effects with a previously reported GOF mutation in an outer collar of TMs that surrounds the major pore-lining helices (16), indicating that these two structural elements function together to control CFTR gating. Each class of GOF mutation in the TMs functionally rescued ATP binding mutants both of CFTR and of the Yor1p oligomycin exporter, presumably by enhancing ATP occupancy of the NBDs (i.e. by allosteric rescue of ATP binding defects in the NBDs). Our results indicate that CFTR channels and MRP efflux pumps share a conserved allosteric mechanism for coupling ATP binding to the translocation pathway, and reinforce the view that the CFTR channel gating mechanism is similar to the activation mechanisms of conventional ligand-gated channels.

**EXPERIMENTAL PROCEDURES**

Cell Culture, CFTR Transfections, Immunoblotting, and Patch Clamp Electrophysiology—HEK-293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen/Invitrogen) supplemented with 10% fetal bovine serum (FBS) and transiently transfected with the relevant CFTR cDNAs, as described previously (16, 27). CFTR mutants were generated by PCR mutagenesis, verified by DNA sequencing, and subcloned into the pCDNA3 mammalian expression vector (Invitrogen). For CFTR protein detection by immunoblotting, the cells were washed once in PBS 48 h post-transfection and lysed in lysis buffer (130 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1% (w/v) Triton X-100, 2 mg/ml iodoacetamide, 0.5 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, pH 7.3) on ice. The lysates were cleared by centrifugation, and protein concentration was determined using the Micro BCA protein assay kit (Thermo Scientific). 100 μg of protein for each lysate were resolved by SDS-PAGE using 6% Tris-glycine gels, and transferred to nitrocellulose for Western blotting. CFTR proteins were probed with anti-CFTR monoclonal antibody (MM13-4; Millipore). Blots were developed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Cells were typically grown for 24–48 h at 37 °C prior to patch clamp recordings. CFTR-mediated macroscopic and single channel currents were recorded in the excited, inside-out configuration as described (16, 27). Patch pipettes were pulled from Corning 8161 glass to tip resistances of 1.5–3.5 megohms for macroscopic recordings and 7–12 megohms for single channel current recordings. The same recording solution was used in the bath and pipette with the following composition: 140 mM N-methyl-d-glucamine, 3 mM MgCl₂, 1 mM EGTA, and 10 mM TES, adjusted to pH 7.3 with HCl. Stock solutions of ATP and AMP-PNP were prepared in water and adjusted to neutral pH with NaOH. Macroscopic currents were recorded using a ramp protocol (±80 mV) with a 10-s time period. Single channel recordings were performed at a holding potential of ~60 mV. All experiments were performed at 21–23 °C. Single channel current signals were analog-filtered at 110 Hz and then digitally filtered at 50 Hz with Clampfit 10 software (Axon Instruments). Estimates of single channel open probability (Pₒ) values and channel opening rates (openings/s-channel) were generated.
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from records containing fewer than eight detectable channels using Clampfit 10. Signal durations briefer than 50 ms were ignored to minimize the contribution of flickery closings due to pore block (e.g. by the pH buffer).

Yeast Phenotypic Assay of Yor1p-mediated Oligomycin Export—All assays were performed in YPEG medium (1% yeast extract, 2% peptone, 3% ethanol, 3% glycerol), which requires respiration for energy production and thus renders cells dependent on Yor1p function for growth in the presence of the ATP synthase inhibitor, oligomycin. For phenotypic analysis, the BY4741 genetic background was used, and plasmids were transformed by the lithium acetate method (28). The BRY01 (MATα his3Δ1 leu2Δ0 ura3Δ0 can1Δ0::Pgali-Tadh1-Pmfa1-hisS+ lyplΔ0 hmrΔ0::URA3ca yor1-Δ0::NatMx) strain was obtained by knocking out the entire YOR1 open reading frame in Y11578-1.2b (MATα his3Δ1 leu2Δ0 ura3Δ0 can1Δ0::Pgali-Tadh1-Pmfa1-hisS+ lyplΔ0 hmrΔ0::URA3ca (29) and then back-crossing by SGA (30) to the ho-Δ0 knock-out strain from the Saccharomyces cerevisiae genomic gene deletion strain collection (Open Biosystems) to create BRY02. Plating BRY02 for his5 mutations were constructed by plasmid-based PCR mutagenesis of pEAE93 (31), after first reverting the ΔF670 mutation, and verified by DNA sequencing. BRY03 plasmid transformants were then selected on uracil dropout media. Strains transformed with desired plasmids were pregrown for 48 h at 30 °C in uracil dropout media with G418 and ClonNat. For cell array printing, cultures were transferred to a 384-round well plate (Evergreen Scientific, Los Angeles, CA) with one strain per row and diluted 1:50 in sterile glass-distilled water before transferring to agar media. Culture dilution and printing were performed with a 384-FP6 pin tool, using a Sciclon ALH3000 liquid handling robot (Caliper Life Sciences). Oligomycin gradient plates were created by first pouring 15 ml of YPEG media with 0.2 μg/ml oligomycin into the bottom of a Nunc monowell plate, which was tipped up by overlapping 1½ inches of the opposite end of the plate on a plate lid, allowing it time to solidify, and then laying the monowell plate horizontally and topping it with 25 ml of YPEG. The yeast strains were robotically printed from the diluted 384-well plate onto the surface of the oligomycin gradient plates using a 384-FP6 pin tool. The plates were incubated at 30 °C and robotically scanned to acquire images every 2 h until carrying capacity was reached. Image analysis was performed to obtain culture density (average pixel intensity) at each time point, and density versus time was fitted to a logistic growth function as described previously, to obtain growth parameters for each spotted culture (32). Changes in Yor1p function were assessed by comparing consecutive row positions across the oligomycin gradient media. Phenotypic differences were quantified based on the change in the cell proliferation parameters, K and l, after fitting the time series of average pixel intensity to Equation 1,

\[ G(t) = \frac{K}{1 + e^{-r(t-t_0)}} \]  

(Eq. 1)

where \( K \) functionally represents the carrying capacity (maximum culture density), \( I \) is the time to reach half-carrying capacity, and \( r \) is the maximum specific cell proliferation rate (33). Phenotypic differences were visualized by comparing growth curves between mutants in a particular column of the cell array (a single concentration) or comparison of the change in a cell proliferation parameter across the cell array. For gradient analysis of cell proliferation parameters, a no-gradient control plate was obtained to subtract from the gradient plate to more accurately assess the interaction between the mutation and the oligomycin concentration.

Molecular Dynamics Simulations—Previously published “closed” and “open” CFTR models based on the empirical structures of inward-facing Msba (Vibrio cholerae lipid flippase) and outward-facing Sav1866 (Staphylococcus aureus multidrug exporter), respectively, were used in the construction of the simulation systems (34, 35). The outward-facing model originally contained a molecule of ADP within each binding pocket, which was substituted with Mg-ATP via root mean square deviation-based superimposition. The inward-facing model contained one molecule of bound ADP, which was replaced by a molecule of Mg-ATP by superimposition, whereas a second Mg-ATP molecule was placed into the second binding pocket using Mg\(^{2+}\) and phosphate-coordinating side chains as a guide. A 180 × 180-Å pre-equilibrated patch of POPC bilayer was generated using the Membrane Builder plugin in VMD 1.9 using the CHARMM36 topology (36). All water molecules were removed from the patch to facilitate insertion of the CFTR models. The CFTR model was inserted into the POPC bilayer using surface tryptophan residues as a guide for depth placement. This entire process was repeated for each simulation replicate. After insertion, all overlapping lipid molecules within 2.4 Å of the protein were removed, and the entire system was minimized in the absence of solvent for 15,000 steps for membrane packing. The system was then explicitly solvated with TIP3 water using the VMD Solvate plugin and ionized with 0.15 M/liter of NaCl to neutralize surface charges. The system was minimized for an additional 15,000 steps and then slowly heated from 0 to 310 K over 15,000 steps. All molecular dynamics and time-independent minimization were performed in NAMD 2.8–2.9 using the NPT ensemble and CHARMM36 force fields using a 2-fs time step and rigid bonds between hydrogen atoms and heavy atoms (37). The simulations employed particle mesh Ewald long range electrostatics, Berendsen pressure control, and temperature coupling at 310 K under periodic boundary conditions. Long range cut-offs were set at 12 Å using a switching function beginning at 10 Å. Velocities were reinitialized every 1 ns, and each inward-facing CFTR system was simulated for 120 ns on the Cheaha high performance computing cluster at the University of Alabama at Birmingham. One outward-facing CFTR system was simulated for 30 ns for comparison. Trajectory output was generated every 4–20 ps depending on the simulation time scale.
RESULTS

A Conserved TM6 Proline in the ABCC Transporter Subfamily—Fig. 1 shows the conserved TM6 proline in a multiple sequence alignment of ABCC transporters and its location in the presumed CFTR structure. The left panel in Fig. 1A depicts a previously published homology model of an inward-facing conformation of CFTR (putative closed state) (34) with a cut-out view of the transmembrane domains that are known to be the major pore-lining TMs (TM6 and TM12 in yellow) and the "outer collar" TM3/4 and TM9/10 (green) pairs as predicted by an inward-facing CFTR model (left, middle) and an outward-facing CFTR model (right, middle) (34). The locations of the Pro-355 residue (red) and the site of a previously reported class of constitutive mutation (Lys-978, purple) are both shown in Van Der Waals representations. Pro-355 is located at the base of pore-lining TM6 near the inner leaflet, whereas Lys-978 is located in the "outer collar" at the base of TM9 in ICL3. The outer collar TMs are predicted to expand and twist outward during channel activation, allowing the pore-lining TMs more room for movement. Bottom, a T-COFFEE multiple sequence alignment (67) of CFTR, the human MRPs, two yeast ABCC homologs, and two representative non-ABCC bacterial ABC transporters. The Pro-355 CFTR residue is highly conserved among the human MRPs and their yeast homologs but is absent in the bacterial transporters.
Table 1), a poorly hydrolyzable ATP analog that is a very weak agonist for wild type CFTR (38, 39). The latter result is similar to the effects of GOF mutations that increase the activation of conventional ligand-gated channels and hormone receptors by partial or weak agonists (14, 19, 40). The increased $P_o$ values of the Pro-355 mutants in the absence of ATP or following AMP-PNP activation were due in large part to increases in the frequency of channel openings relative to the wild type channel (Table 1). These effects on channel opening rate indicate that the TM6 proline plays a role in stabilizing the CFTR closed state.

The GOF effects of the Pro-355 mutations were also apparent in macroscopic current recordings obtained from excised macropatches containing many channels (Fig. 3). Wild type channels open so infrequently following ATP removal that the macroscopic current decreases to virtually zero when an ATP scavenger (hexokinase/glucose) is added to the bath (12, 16) (see Ref. 39 for an example WT-CFTR record). In contrast, each of the Pro-355 mutants exhibited detectable macroscopic currents following ATP removal that varied between 1 and 10% of the currents measured in the presence of ATP (see representative record in Fig. 3A, scatter plot of all results in Fig. 3B, and mean data in the figure legend). The WT-CFTR currents that are activated by 2 mM AMP-PNP also are quite small even in macropatch recordings (Fig. 3D; see representative macroscopic current recordings in Ref. 39) as opposed to the Pro-355 mutants, each of which exhibited substantially greater macroscopic current activation by AMP-PNP (Fig. 3, C and D). Based on these macroscopic and single channel data, we conclude that each of the three Pro-355 substitutions is a GOF mutation that would be classified as an isomerization mutation in the nomen-
The P355A Mutation Rescues Channels That Are Normally ATP-unresponsive—We next determined whether a Pro-355 GOF mutation could promote the activities of channels that normally cannot be stimulated by ATP, namely a truncation mutant lacking NBD2 (Δ1198-CFTR) and an NBD1 signature sequence mutant (G551D-CFTR). When combined with the P355A substitution, these constructs also expressed sufficiently well for patch clamp analysis, although, like the single mutants, their expression was lower than wild type CFTR (see immunoblot in Fig. 4G). Δ1198-CFTR lacks one of the two NBDs that are essential for ATP-dependent channel opening and normally exhibits very low, ATP-independent currents in excised macropatches (Fig. 4, A and C) (16, 27). The Δ1198-CFTR currents can be strongly activated by the natural compound curcumin, which provides a useful indicator of the approximate numbers of gating-defective channels in the patch (Fig. 4, A and D) (16, 27). Fig. 4, B–D, shows that introducing the P355A mutation into the Δ1198-CFTR background created channels with...
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A. \( \Delta 1198 \)

B. \( P355A/\Delta 1198 \)

C. Control current (pA)

D. Relative curcumin activation

E. Control current (pA)

F. Relative curcumin activation

G. Expos: 5 sec
greater macroscopic currents under control conditions (albeit still ATP-independent) and correspondingly lower relative activation by curcumin (mean data in Fig. 4D).

The P355A mutation also partially rescued the activity of G551D-CFTR, a severely defective gating mutant that is common in the CF patient population (Fig. 4, E and F). The G551D mutation disrupts the NBD1 signature sequence that is essential for ATP-dependent gating and strongly inhibits ATP hydrolysis (41, 42). G551D-CFTR channels in excised macropatches behave much like the /H90041198-CFTR truncation mutant; very low ATP-independent currents under control conditions and strong activation by curcumin (16, 27). Like for the truncation mutant, the Pro-355 substitution increased the control currents and reduced the relative activation by curcumin (Fig. 4, E and F).

**Additive GOF Effects of the P355A Mutation and a Mutation in the Outer TM Collar**—We previously reported another class of GOF mutation that locates to the base of TM9 (Lys-978) in the putative outer TM collar that surrounds the principal pore-lining TMs (see Fig. 1A for predicted location of Lys-978 in CFTR structural models) (16). We reasoned that, given the different locations of the two classes of GOF mutations (pore versus outer collar), a double mutant (P355A/K978C) may exhibit an additive GOF effect. The data in Fig. 5 show that this is the case.

**FIGURE 5. GOF mutations in the pore-lining TM6 (P355A) and in the outer TM collar (K978C) have additive effects on CFTR channel activity.** A, macroscopic current record showing the relatively large ATP-independent current and robust activation by 2 mM AMP-PNP for the P355A/K978C double mutant. For the ramp protocol, conditions were the same as for Figs. 2–4, B and C, mean fractional ATP-free current and relative AMP-PNP activation normalized to the control current at 1.5 mM ATP for the indicated single and double mutants. n values are indicated in parentheses. *, p < 0.05 compared with double mutant by unpaired t test. Error bars, S.E.

**FIGURE 4. The P355A substitution increases the channel activities of CFTR mutant constructs that cannot be activated by ATP.** A and B, macroscopic currents mediated by Δ1198-CFTR without or with the P355A substitution. Conditions were identical to those in Fig. 3. Curcumin (30 uM) was added where indicated to provide a rough measure of the number of gating-defective channels in the patch (16, 27). Note the much larger control current for P355A/Δ1198-CFTR that was insensitive to the addition of the ATP scavenger (i.e. was ATP-independent). C and E, scatter plots showing the generally larger macroscopic control currents at −80 mV for Δ1198-CFTR channels and G551D-CFTR channels containing the P355A substitution. The scatter is due in part to variable numbers of channels in the macropatches. Note the break in the y axis for C, D and F, mean relative activation by curcumin normalized to control currents for the indicated constructs. The much lower relative activation of the P355A mutants is due to their higher control or baseline currents. n values are indicated in parentheses. *, p < 0.05 by unpaired t test. G, immunoblot of P355A-Δ1198, P355A-G551D, and P355A/K978C double mutants transiently expressed in HEK-293T cells. Two exposures of the same blot are shown. 100 ug of total protein were loaded in each lane. The upper band in each lane represents the mature (post-ER) form. Error bars, S.E.
case. GOF effects were again operationally defined as elevated channel activity in the absence of nucleotide (~ATP) and stronger activation by the weak agonist AMP-PNP. The double mutant (P355A/K978C-CFTR) exhibited relatively high ATP-independent currents in excised macropatches (~35% of the ATP control current) and strong activation by AMP-PNP that approached that by ATP (~90% of the ATP control current). These GOF effects of the double mutant were substantially greater than for either single mutant. It is also evident from the single mutant data in Fig. 5, B and C, that the K978C mutation had a somewhat stronger GOF effect than the P355A mutation. The additive effects of the two GOF mutations can be explained by their locations in different structural regions of the channel (i.e. near the base of the pore (Pro-355) and in the surrounding TM collar (Lys-978)).

The P355A TM6 Mutation Increases the ATP Sensitivity of Channel Gating—A GOF or isomerization mutation is predicted by a classical allosteric activation scheme to enhance ligand occupancy at normally subsaturating concentrations. This concept of reciprocity (or allosteric coupling) between the pore and the ligand binding domains is based mechanistically on the premise that the open channel has the higher ligand affinity and is a central feature of all cyclic allosteric activation schemes for ligand-gated channels (14, 18). The data in Fig. 6 confirm this prediction both for the single P355A mutant (Fig. 6A) and for a double mutant in which the Pro-355 mutation was introduced into an NBD2 mutant that has a markedly reduced ATP affinity (Y1219G-CFTR; Fig. 6B). The tyrosine at position 1219 locates to the A-loop of NBD2, where it stacks against the adenine ring of ATP at site 2 in CFTR (Fig. 6C). Mutating the A-loop tyrosine has been shown to inhibit ATP binding to P-glycoprotein (43) and to strongly reduce the ATP sensitivity of CFTR channel gating (44). As reported previously by the latter authors, the Y1219G mutant of CFTR exhibited a marked rightward shift in the ATP dose-response curve relative to wild type CFTR with an EC50 of 1.5–2 mM. Introducing the P355A mutation increased the apparent ATP affinity of the Y1219G mutant (leftward shift in Fig. 6B). The K978C mutation in the outer TM collar (TM9) also increased the apparent ATP affinity of the Y1219G mutant and to a greater degree than the Pro-355 substitution, as would be expected if the former is a stronger GOF mutation (see also Fig. 5). The data of Fig. 6 support the idea that Pro-355 mutations are isomerization mutations that increase ligand occupancy/sensitivity at normally subsaturating concentrations. These results also indicate that both classes of GOF mutations (Pro-355 in TM6; K978C in the outer TM collar) can compensate for a partial defect in ATP binding by allosteric coupling between the TMs and the NBDs (i.e. by “allosteric rescue” of the ATP binding defect).

Homologous TM Mutations Rescue Defective Substrate Export by ATP Binding Mutants of a Yeast MRP—Our finding that an ATP binding mutant of CFTR (Y1219G in NBD2) was rescued by GOF mutations in the TMs motivated us to determine whether such allosteric coupling between the TMs and NBDs could also be observed for an MRP drug exporter. The yeast oligomycin exporter Yor1p was chosen for this analysis because it is an MRP4 homolog whose transport activity can be scored as cell growth on oligomycin media (33, 45). In addition, Yor1p possesses the conserved proline at the base of TM6 that is a feature of the ABCC subfamily (Pro-485 in Yor1p), a lysine in the outer TM collar at the same position as Lys-978 in CFTR.
FIGURE 7. Homologous “outer collar” substitutions reverse oligomycin growth defects of Yor1p constructs with ATP binding defects. A, T-COFFEE single-pair alignment of human CFTR and yeast Yor1p (MRP4 homolog) showing the conserved NBD1 signature sequence and Walker B aspartate (top), the conserved A-loop tyrosine in NBD2 (middle), and the lysine near the base of TM9 (bottom).

B, representative image of end point growth of indicated Yor1p constructs after 140 h on an oligomycin gradient (0 – 0.2 μg/ml).

C and D, representative growth rates of the indicated Yor1p constructs at the highest oligomycin concentration tested (0.2 μg/ml). E and F, quantitative analysis of oligomycin growth curves of the indicated Yor1p constructs. Data are means ± S.E. (error bars) averaged over three independent experiments for each construct.

G, Lys-997 substitutions do not rescue G713D (NBD1) and G1370D (NBD2) signature sequence mutants of Yor1p.

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(Lys-997 in Yor1p), and an A-loop tyrosine in NBD2 at the same location as Tyr-1219 in CFTR (Tyr-1222 in Yor1p; see alignments in Fig. 7A). Yor1p mutations at positions Pro-485 (TM6) and Lys-997 (TM9) that are homologous to the CFTR GOF mutations described above were assayed both as single mutants and as double mutants when combined with one of two NBD mutations that are expected to inhibit Mg-ATP binding to Yor1p: (i) Y1222G, an A-loop mutation homologous to the Y1219G mutation of CFTR, and (ii) D734N, a Walker B mutation that is predicted to reduce Mg-ATP binding because the conserved aspartate helps coordinate the metal cofactor in ABC exporters (13, 46, 47). Unfortunately, the corresponding CFTR Walker B mutant (D572N) is a severe endoplasmic reticulum processing mutant that could not be analyzed by patch clamping in HEK cells (48). The Lys-997 mutations were also introduced into NBD1 and NBD2 signature sequence mutants (G713D-Yor1p and G1370D-Yor1p), which are expected to be severely defective ATPases (41, 49).

The results in Fig. 7 confirm that the ATP binding mutants of Yor1p (Y1222G and D734N) exhibit defective growth on oligomycin and show that this growth phenotype was strongly rescued by either of two Lys-997 substitutions (K997C or K997S). Fig. 7B depicts the end point growth of the indicated mutants across a gradient of oligomycin concentration. Fig. 7, C and D, shows representative growth curves for each Yor1p construct at the highest oligomycin concentration tested. These experiments were performed independently three times with nearly identical results. The reproducibility of the rescue by the Lys-997 substitutions is illustrated in Fig. 7, E and F, which shows the results of a quantitative analysis of the growth of each construct on oligomycin media averaged over three such experiments. Importantly, although the Lys-997 substitutions robustly rescued the oligomycin growth phenotype of each of the Yor1p ATP binding mutants, neither Lys-997 substitution reversed the strong growth defects exhibited by the Yor1p signature sequence mutants that are expected to have negligible ATPase activity (see Fig. 7G). The failure of the Lys-997 substitutions to reverse the growth defects of these signature sequence mutants is presumably due to the fact that these Yor1p constructs lack sufficient ATPase activity to energize active oligomycin export (see “Discussion”).

Fig. 8 shows that the Pro-485 substitutions in TM6 also rescued the oligomycin growth defect of the Walker B mutant (D734N-Yor1p) and, to a lesser extent, the A-loop mutant (Y1222G-Yor1p). Representative results are shown in Fig. 8, A–C. Mean growth curve data averaged over three independent experiments are presented in Fig. 8, D and E. Rescue by the Pro-485 substitutions was less than that observed for the Lys-997 substitutions, which might be related in part to the stronger GOF effects we observed for the corresponding Lys-978 mutations in CFTR. Of note, the single Pro-485 mutants exhibited partial growth defects in oligomycin that were more obvious for the P485S substitution. This suggests, as was the case for CFTR, a partial reduction in transporter expression by these mutations (Fig. 2F). The P485A substitution more strongly rescued the growth defect of the Y1222G A-loop mutant (e.g. see Fig. 8C), which is presumably due to the lesser effect of the single P485A substitution on Yor1p function. The mean growth curve data in Fig. 8, D and E, indicate that three out of four of the Pro-485/NBD double mutants reproducibly exhibited improved growth on oligomycin media when compared with the single NBD mutants despite the fact that the Pro-485 substitutions by themselves moderately reduced oligomycin resistance. These data, combined with the Lys-997 Yor1p results in Fig. 7, indicate that homologous TM substitutions can allosterically rescue ATP binding mutants of both an MRP drug pump and the CFTR channel. This supports the hypothesis that these thermodynamically distinct transporters share a conserved mechanism for allosterically coupling conformational changes in their TMs to ATP binding at their NBDs.

**DISCUSSION**

The present results implicate the conserved proline at the base of TM6 as a structural element that helps to stabilize the closed state of the CFTR channel. Each of several mutations at this position exhibited GOF effects on channel gating that were evidenced as follows: (i) higher $P_o$ values and more frequent channel openings in the absence of nucleotide; (ii) substantially stronger activation by the normally weak agonist, AMP-PNP; (iii) functional rescue of CFTR channel constructs that normally cannot be activated by ATP (i.e. Δ1198- and G551D-CFTR), and (iv) increased ATP sensitivity of channel gating either alone or when combined with an NBD2 mutation that partially inhibits ATP binding. These multiple GOF effects of the Pro-355 mutations on CFTR gating are characteristic of isomerization mutations that increase channel activity ($P_o$) in the absence of ligand. As argued by Changeux and colleagues (18, 19) in the context of their classical allosteric activation models, isomerization mutations are expected to have pleiotropic effects on channel gating or receptor activation because they bias the equilibrium to the open (active) conformation under all conditions. The consequences of such a mutation include stronger activation by normally weak agonists and increased sensitivity to the physiologic ligand, as observed here for the Pro-355 mutations. The fact that these CFTR mutants behave similarly to isomerization mutations that have been described for conventional ligand-gated channels reinforces our earlier argument that ATP binding promotes CFTR channel opening by an allosteric mechanism that is qualitatively similar to those proposed for more typical ligand-gated channels that reversibly bind their ligands (15, 16, 39).

**How Pro-355 Mutations May Destabilize the CFTR Closed State—GOF effects at position 355 were observed for multiple amino acid substitutions with very different side chain chemistries. Thus, it appears that it was the lack of a proline residue at this location that destabilized the CFTR closed state. Due to their unique cyclic side chains, proline residues confer conformational rigidity to protein backbones and break hydrogen bond networks in $α$-helices. Also, unlike all other amino acids that overwhelmingly adopt a trans conformer of their preceding peptidyl-prolyl bonds, proline residues can dynamically switch to the cis conformer by undergoing cis-trans isomerization (50, 51). The trans and cis conformers are energetically comparable due to trans state steric clashing, although the energy barrier to isomerization is quite high and is normally overcome by prolyl isomerases or by mechanical forces (51).
Given that TM6 rotation has been argued to underlie channel opening (24), it is possible that isomerization of the Pro-355 proline may contribute to the overall energy barrier to CFTR channel activation. This possibility is consistent with the inward-facing model of CFTR from Mornon et al. (i.e. the putative closed state based on MsbA) in which Pro-355 exists as a cis conformer, whereas Pro-355 exists as a trans conformer in the outward-facing model (i.e. the putative open state based on Sav1866) (34, 35). The Pro-355 cis conformer in the latter model is consistent with other published outward-facing CFTR homology models (25, 52–54). Because the Pro-355 cis conformer is a unique feature of the Mornon et al. inward-facing model, we performed atomistic molecular dynamics (MD) simulations of both the outward-facing and inward-facing models of CFTR under physiologic conditions (inserted in POPC bilayers, 150 mM NaCl, 310 K). Fig. 9 shows that the cis Pro-355 conformer was stable in the inward-facing model over 100 ns in triplicate simulations and that the trans Pro-355 conformer was stable in the outward-facing model over 30 ns (Fig. 9B).

Inspection of the region adjacent to the conserved proline (Pro-355) in the CFTR inward-facing model revealed a related and potentially interesting structural feature, namely a type VI β-turn between residues 353 and 356 (Fig. 9C). Type VI β-turns are structural motifs uniquely confered by cis prolines (57, 58). The type VI β-turn also was stable over our triplicate 100-ns simulations of the inward-facing CFTR model. Such turns can be augmented by stacking interactions between flanking aromatic residues and the proline side chain (55). Pro-355 is pre-
ceded by a phenylalanine residue (Phe-354), which, in the original Mormon et al. model and in our MD simulations of that model, appears to stabilize the cis conformer by stacking with the Pro-355 residue. This turn is lacking in all published outward-facing models of CFTR (34, 54, 56) and did not form in our 30-ns simulation of outward-facing CFTR. The stability of the type VI H9252-turn could add to the energy barrier to activation conferred by a cis proline. In sum, we propose that the proline at position 355 contributes to the formation of a structural element at the base of TM6 that helps to stabilize the CFTR closed state. One plausible structural element that could serve this role is a cis conformer of Pro-355 with an associated type VI β-turn, which would be consistent with (but not proven by) our simulation results. Overcoming the energy barrier to channel opening that is conferred by this and other structural elements would typically require the energy conferred by ATP binding and consequent NBD dimerization.

Different Classes of GOF Mutations Reveal Distinct Structural Elements That Contribute to the Energy Barrier to CFTR Channel Opening—Previously, we reported that certain mutations at position 978 near the base of TM9 have GOF effects on CFTR gating (16). GOF effects of the P355A and K978C mutations were additive, implying that they influence channel gating by different mechanisms. The results of cysteine-scanning and pore-blocking studies of CFTR indicate that TM6 and TM12 are the principal pore-lining helices (25, 59, 60). As noted above, Pro-355 is located at the base of TM6 along this putative translocation pathway. In contrast, the previously identified constitutive mutations map to the long intracellular loops that extend cytosolically from TM3/4 and TM9/10, respectively. Along most of their lengths, these TMs lie peripherally to the pore-lining TM6 and TM12 to form what we have termed the outer “collar” that surrounds the pore region. This collar appears to restrict the space available to the major pore-lining TMs for rotation or bending in the inward-facing model and in our MD simulations of this model (e.g. see Fig. 1A). The strongest GOF substitutions at position 978 within this outer collar (Cys, Ser, and Pro) (16) also happen to be residues that are known to introduce kinks and bends in β-helices (61). We speculate that these mutations destabilize the inward-facing conformation by distorting the outer collar, causing it to shift away from TM6 and TM12 with a consequent increase in the space available to these pore-lining TMs for whatever movements they undergo during channel opening (e.g. TM rotation; see Fig. 1A). The Pro-

FIGURE 9. Molecular dynamics simulations predict a stable Pro-355 cis conformer in inward-facing CFTR and a stable Pro-355 trans conformer in outward-facing CFTR. A, root mean square deviation backbone equilibration during MD simulations of the outward-facing CFTR model (30 ns) and inward-facing CFTR (representative 30-ns snapshot from triplicate 100-ns simulations). B, the peptidyl-prolyl bond dihedral angle preceding the proline is defined as cis at or near 0° and trans at or near 180°. Both the cis and trans Pro-355 conformers remained stable over the entirety of the MD simulations in the inward-facing and outward-facing CFTR models, respectively. C, structural features of the cis and trans Pro-355 residues in both models. Side chains are hidden from flanking residues to highlight the isomerization of the peptidyl-prolyl bond.

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355 substitutions probably operate by a discrete mechanism, such as a direct effect on TM6 conformation, as noted above. By identifying different classes of GOF mutations, it appears to be possible to reveal different structural elements that coordinately establish the energy barrier to CFTR channel activation.

**GOF Mutations in the TMs Increase the ATP Sensitivity of CFTR Gating and Allosterically Rescue an ATP Binding Defect in the NBDs—Isomerization mutations of conventional ligand-gated channels increase ligand sensitivity because they bias the equilibrium to the conformation with the higher ligand affinity (i.e. the open state) (14, 19, 20). The two classes of GOF mutation that we have identified for CFTR both increased the ATP sensitivity of channel gating (Fig. 6) (16), presumably by a qualitatively similar mechanism. The open pore conformation of the CFTR channel is coupled to a tight NBD dimer with two ATP molecules bound at the interface of this dimer (3). We argue that GOF mutations in the TMs that bias the equilibrium toward the open conformation should increase ATP occupancy at the NBD dimer interface at normally subsaturating ATP concentrations by allosteric/reciprocal coupling between the pore and the NBDs. This is supported by the strong effects of the two classes of GOF mutations on the ATP sensitivity of Y1219G-CFTR activity. The Y1219G mutation is located within a conserved aromatic region known as the A-loop that lies just upstream of the Walker A motif in NBD2. The A-loop is critical for binding ATP, and the conserved tyrosine in particular has been determined to coordinate the adenine ring of bound ATP in several ABC transporters, including CFTR (62–66) (see also Fig. 6C). Studies by Hwang's group (44) demonstrated that a glycine substitution at this position dramatically decreases the ATP sensitivity of CFTR channel activation. Both the P355A and K978C GOF mutations increased the ATP sensitivity of the Y1219G mutant with the K978C substitution restoring the ATP sensitivity of channel gating to nearly wild type levels. This apparent allosteric rescue of an ATP binding mutant by GOF mutations in the TMs is further evidence for a CFTR activation mechanism that is similar to that proposed for conventional ligand-gated channels.

**MRPs and CFTR May Share a Conserved Allosteric Network for Coupling TM Conformational Changes to ATP Binding—**At present, little is known about the shared operational principles between the CFTR channel and the MRP efflux pumps. Because they are thermodynamically distinct transporters, they conceivably could employ different mechanisms for coupling ligand binding to channel gating or drug transport. However, the results of our comparative analysis of CFTR and Yor1p, an oligomycin exporter and yeast MRP4 homolog, support the notion that these transporters use similar mechanisms for linking ATP binding at the NBDs to conformational changes at the TMs. Two different ATP binding mutants of Yor1p were functionally rescued by TM6 and TM9 mutations that were identified as GOF mutations in our CFTR experiments. One of these ATP binding mutants was an NBD1 Walker B mutant (D734N) that lacks a conserved aspartate that helps to coordinate the binding of the magnesium cofactor (13, 46, 47). The other ATP binding mutant (Y1222G-Yor1p) was homologous to the A-loop Y1219G mutant in CFTR NBD2 that exhibits markedly reduced ATP sensitivity. As discussed above, the ATP sensitivity of Y1219G-CFTR was enhanced by both classes of GOF mutation, an effect we interpreted as allosteric rescue of an ATP binding defect by such TM mutations. We propose that the same mechanism underlies the rescue of the corresponding ATP binding mutants of Yor1p. In each case, GOF mutations in the TMs would be expected to increase ATP occupancy of the NBDs at normally subsaturating concentrations. This should lead to increased drug export by Yor1p constructs for which ATP binding is limiting.

It should be noted that we would not expect all defective Yor1p mutants to be rescued by this mechanism. For example, Yor1p mutants that are severely defective for ATP hydrolysis or the coupling between the NBDs and translocation pathway should be difficult, if not impossible, to rescue by these types of TM mutations. This probably explains our inability to detect rescue of signature sequence mutants in Yor1p (Fig. 7G) that should have impaired ATPase activity (41, 49). Yor1p-mediated drug export requires ATP hydrolysis, whereas CFTR channel opening does not. This thermodynamic distinction probably explains why TM mutations can enhance the activities of G551D-CFTR channels (Fig. 4), whereas the homologous mutations cannot functionally rescue the corresponding Yor1p signature sequence mutant (Fig. 7G).

**A Proline “Switch” That Is Unique to the ABCC Subfamily?**—The proline at the base of TM6 in CFTR channels and in the MRP pumps is not conserved outside of the ABCC subfamily. The apparent uniqueness of this proline among the ABCC transporters raises the interesting question of whether its functional role is specific to members of this subfamily. We speculated above that cis-trans isomerization of Pro-355 might contribute to the energetics of CFTR channel opening. Whether proline isomerization also underlies the conformational switching (inward-to-outward-facing) of an MRP exporter is unknown, but mutating this proline had GOF effects in Yor1p as well. One possibility is that this proline is an adaptation to limit CFTR channel opening or MRP conformational switching when only one ATP molecule is bound. The ABCC transporters, unlike the bacterial exporters, contain a degenerate hydrolytic site (5, 10) that is predicted to stably bind ATP for extended periods of time (e.g. ATP reportedly binds to site 1 in CFTR for many seconds and multiple gating cycles) (11, 12). Stable ATP binding at one of the two sites might induce continual strain on the TMs that would increase the probability of CFTR opening or MRP conformational switching unless there are mechanisms in place to further stabilize the CFTR closed state or the inactive (inward-facing) conformation of the MRPs. A proline conformer at the base of TM6 that stabilizes the CFTR closed state or the MRP inactive state in the absence of ATP binding to both sites might be one such mechanism.

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