

not move markedly during the gating cycle, consistent with only partial NBD-dimer separation after ATP hydrolysis.

At the NBD-TMD interface we studied positions M961 (site of CF-mutation in coupling helix 3) and A1256 (NBD2). Although single mutations M961L and A1256V decreased apparent ATP-affinities, these effects were additive, indicating no change in coupling between these positions upon ATP binding to closed channels. P_o and τ_b were unaffected by these mutations. He et al. (JBC 283:26383) showed that L1260 and L1261 are in close contact with M961, and chemical crosslinking of L1261C with M961C lowers P_o . Gating of mutants L1260A and L1261A paired with mutation M961L is under investigation.

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Helical Rotation Associated with the Gating of the CFTR Chloride Channel

Yonghong Bai, Min Li, Tzyh-Chang Hwang.

CFTR, a member of the ABC transporter superfamily, is an ATP-gated chloride channel. Its two transmembrane domains each encompassing six transmembrane segments (TMs), form the ion permeation pathway. However, which TMs contribute to the pore-lining and how conformational changes in the TMs lead to the opening and closing of the pore remain largely unknown. Our previous cysteine scanning studies reveal that TM6, a potential pore-lining candidate, is likely involved in a rotational movement during gating. Here, we presented the cysteine scanning results of TM12 (residues 1129-1150), which is the equivalent TM to TM6 based on crystallographic studies that show a pseudo two-fold symmetry in several ABC proteins. Three cysteine residues, S1141C, W1145C and N1148C, react with a negatively charged MTS reagent at a rate approaching that of free thiols with the same reagent, suggesting that they face the aqueous pore. Consistent with this idea, cysteine modification by a positively charged MTS reagent affects the single-channel current amplitude as well as the potency of the anionic, hydrophobic open-pore blocker, glibenclamide. Furthermore, blockade of the pore by glibenclamide completely protect cysteines at 1141 and 1145 from modification by MTS reagents, further supporting the idea that these residues line the pore. Interestingly, by measuring the reaction rates when the channel is gated by high (2 mM, $P_o \sim 0.60$) and low (30 μ M, $P_o \sim 0.15$) concentrations of ATP, we inferred that whereas 1145C reacts faster in the open state, 1141C and 1148C react faster in the closed state. Thus, our results suggest that the cytoplasmic gate has been degenerated during evolution, and that helical rotations of TMs, which are proposed to be important for the function of ABC transporters, also play a role in the gating transitions of the CFTR channel.

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Identification of a Novel Post-Hydrolytic State in CFTR'S Irreversible Gating Cycle

Kangyang Jih, Min Li, Tzyh-Chang Hwang.

CFTR is a chloride channel belonging to the ABC transporter superfamily. After activated by phosphorylation, CFTR is opened by ATP-induced NBD dimer formation. Once opened, ATP will soon be hydrolyzed in composite site 2 formed by the head of NBD2 and the tail of NBD1. It was proposed that closing of the channel involves partial separation of the NBD dimer and dissociation of the hydrolytic products. This "partial NBD dimer" closed state (C2), which can be locked open by pyrophosphate (PPi), has a lifetime of ~ 30 s before it dissipates to the C1 closed state with complete separation of NBDs. The lock-open rate for the C2 state is relatively slow (0.19 s⁻¹ for 10 mM PPi) and lock-open currents reach a steady state that is $\sim 32\%$ of ATP-induced current (IATP). Interestingly, if the perfusate is changed directly from ATP to 10 mM PPi (a deadtime of ~ 30 ms), lock-open current reaches 61% of IATP in 1 s. However, if the ATP is removed for 3 s before applying PPi, the current increases slowly as locked open from the C2 state. These results indicate the existence of a short-lived state X from which the lock-open rate is much faster than that from the C2 state. Previous studies revealed that the lock-open time for W401Y-CFTR is prolonged by ~ 2 fold. Surprisingly, the C2 state of W401Y-CFTR is extremely unstable. We could barely see lock-open events even only 5 s after ATP washout. Strikingly, if we exchange solution directly from ATP to PPi, lock-open current is 75% of IATP for this mutant. These results suggest the W401Y mutation can discriminate state X from the C2 state. Single-channel experiments are under way to elucidate the biophysical and biochemical features of state X.

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Constitutive Mutations Strongly Promote CFTR Channel Activation by Nonhydrolyzable ATP Analogs

George O. Okeyo, Wei Wang, Binli Tao, Kevin L. Kirk.

Normal CFTR channel activation depends on ATP binding at the NBD1-NBD2 dimer interface. Nonhydrolyzable ATP analogs minimally activate WT-CFTR (i.e., they act only as very weak agonists); therefore, gating typically requires nucleotide hydrolysis. Earlier we discovered CFTR cytosolic loop mutations that promote ATP-free channel opening (PNAS 107, 3888-3893, 2010). These constitutive mutations imply an allosteric gating mechanism whereby ATP shifts the equilibrium between closed and open states without being absolutely required for channel opening. An allosteric gating mechanism predicts that weak agonists, e.g. nonhydrolyzable ATP analogs, should strongly activate constitutive mutants, thereby promoting nonhydrolytic gating. We tested this prediction using the nonhydrolyzable nucleotides, AMP-PNP and ATP γ S, to activate constructs with constitutive mutations at position K978 in cytosolic loop 3. In excised inside-out membrane patches, K978C-CFTR was strongly activated by 2.0 mM AMP-PNP and 1.5 mM ATP γ S with estimated open probabilities of 0.49 ± 0.01 and 0.63 ± 0.05 respectively, compared to 0.84 ± 0.03 observed under maximal ATP activation. As expected, these nonhydrolyzable nucleotides only weakly activated WT-CFTR with estimated P_{os} of 0.02 ± 0.01 and 0.01 ± 0.001 for ATP γ S and AMP-PNP, respectively. Activation of K978C by both ATP analogs was dose-dependent, although the EC_{50} for activation by ATP γ S was lower (0.1 ± 0.001 mM) compared to AMP-PNP (0.3 ± 0.1 mM). Neither AMP-PNP nor ATP γ S activated CFTR channels bearing the K978C mutation in combination with the disease-associated G551D mutation in NBD1 or in a construct lacking NBD2 (Δ 1198). This implies that activation by these ligands requires NBD dimerization, consistent with the slow deactivation kinetics ($t_{1/2} \sim 150$ sec) observed upon nucleotide removal. Our findings indicate that constitutive mutations enhance nonhydrolytic gating of CFTR by ATP γ S and AMP-PNP, presumably by reducing the energy barrier for channel opening.

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Two Salt Bridges Differentially Contribute to Maintenance of CFTR Channel Function

Guiying Cui, Chengyu Z. Prince, Nael A. McCarty.

Two salt bridges in human CFTR, R352-D993 and R347-D924, have been identified and both contribute to the native architecture of the open channel pore. Disease-related mutations R352W/Q/G and R347C/P/L/H destroy the salt bridges and result in destabilized pore structure and function in channels expressed heterologously. Questions remaining include whether these two interactions are formed sequentially or coincidentally and whether the residues move similar distances during channel gating. R352C-D993C-CFTR expressed in oocytes can be latched into the open state by the bifunctional crosslinker MTS-2-MTS, suggesting that the distance between the two amino acids in the open state could be $<4\text{-}6\text{\AA}$. We are currently testing the R347C-D924C salt bridge with a similar technique. Results thus far suggest that the two interacting pairs contribute to stabilizing different phases of the gating cycle. Furthermore, some transmembrane residue mutations in CFTR have been shown to affect protein maturation such that channels fail to traffic to the plasma membrane (PM). Therefore, we transiently transfected GFP-tagged R352A-, D993A-, R347C-, and D924C-CFTR into HEK293 cells and found that R352A-GFP-CFTR was retained in the endoplasmic reticulum, similarly to the well-characterized Δ F508-GFP-CFTR, while other salt bridge-destroying mutants including D993A-GFP-CFTR were trafficked to the plasma membrane, like WT-GFP-CFTR. Western blots of immunoprecipitated CFTR showed immature (band B) and mature (band C) forms for WT-GFP-CFTR and all salt bridge mutations except R352A-GFP-CFTR which only exhibited band B, like Δ F508-GFP-CFTR. R352A-GFP-CFTR was not able to be rescued to the PM by low-temperature incubation, although Δ F508-GFP-CFTR was rescued under identical conditions. These results suggest that the abnormal trafficking behavior of R352A-CFTR is not related to the salt bridge function of R352, supporting the idea that the R352-D993 interaction only forms in the open state upon channel activation. Support: NIH-R01-DK056481.

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On the Mechanism of CFTR Inhibition by a Thiazolidinone Derivative Zoia Kopeikin, Min Li, Tzyh-Chang Hwang.

We employed inside-out patch-clamp technique to investigate the mechanism of CFTR inhibition by a thiazolidinone derivative, CFTRinh-172. We found that application of CFTRinh-172 results in an increase of the mean closed time, and a decrease of the mean open time of WT-CFTR channels. A hyperbolic relationship between the closing rate and [CFTRinh-172] suggests that CFTRinh-172 does not act as a simple pore blocker. We have shown