Probing the structure and function of cystic fibrosis transmembrane conductance regulator using chemical modification

PhD Theses

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Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel and a member of the large, ABC (ATP Binding Cassette) Transporter superfamily. The predicted domain architecture includes two membrane spanning domains (MSDs), each with six transmembrane (TM) domains, two nucleotide-binding domains (NBDs), and a unique regulatory (R) domain.

Although it is well known that CFTR functions as a chloride channel, the composition of the minimal functional unit remains unclear; neither the number of CFTR polypeptides required, nor the number of pores per functional channel, are known. Three alternative scenarios have been proposed: (i) one-polypeptide/one-pore, (ii) two-polypeptides/one-pore, and (iii) one-polypeptide/two-pores. Evidence for each of these possibilities has been derived from biochemical, structural, and/or electrophysiological studies of wild-type CFTR (WT-CFTR) and selected mutants. In the first objective of this thesis study, we made use of mutants

containing cysteines engineered at putative pore-lining positions in TM6 to determine the minimal functional unit of the CFTR channel. These mutants were readily covalently modified by the sulfhydryle (SH) modifying reagents from the extracellular side without altering the gating properties of CFTR

Several lines of evidence suggest that CFTR pore experiences more than two conformations, *e. g.* closed, sub- and full-conductance states, including evidence that changes in anion selectivity and susceptibility to blockade are associated to the ATP-dependent gating cycle. However, how hydrolysis of ATP at the NBDs induces either the movement of or conformational changes in the TMs, that leads to opening or closing the channel pore, are not understood. In the first objective of this thesis study, we never observed that $MTSET^+$-induced modification of engineered cysteine at position R334 occurred during an open burst. Therefore, we hypothesized that modification of R334C-CFTR by $MTSET^+$ is favored by the closed state.

Objectives

The goals of the present study were (1) to determine the oligomeric structure of functional CFTR chloride channel and (2) to probe the dynamic architecture of the outer vestibule of CFTR pore using expression in *Xenopus* oocytes, covalent labeling, and high-resolution electrophysiological analyses.

Methods

For mutants R334C, site-directed mutagenesis used a nested PCR strategy in which the mutation was designed into antiparallel oligomers. R334C was prepared from a construct carrying the full coding region of CFTR in the pBluescript vector. The rest of the mutants used in this study were prepared with Quick-Change protocol (Stratagene; La Jolla, CA) using oligonucleotide-mediated mutagenesis. All mutant constructs were verified by sequencing across the entire open reading frame before use. Stage V-VI oocytes were injected in a

6) Zhang Z-R, G. Cui., S. Zeltwanger, and N. A. McCarty (2004). Time-dependent interactions of Glibenclamide with CFTR: kinetically complex block of macroscopic currents. ***J. Membrane Biol***, 201(3): 139-155.

List of publication related to the thesis

1) Zhang Z-R, G. Cui, X. Liu, B. Song, D. C. Dawson and N. A. McCarty. (2005) Determination of the functional unit of the CFTR chloride channel: One polypeptide forms one pore. ***J. Biol. Chem.*** 280(1):458-468.

2) Zhang Z-R, Binlin Song, and McCarty N. A. (2005) State-dependent modification of R334C by $MTSET^+$ reveals conformational change in the outer vestibule of CFTR. ***J. Biol. Chem.*** 280(51):41997-42003.

3) Cui G., **Z-R Zhang,** B. Song, and N. A. McCarty. (2007) Mutations at arginine 352 alter the pore architecture of CFTR (in press for ***J. Membrane Biol.***)

4) Fuller, M. D., **Z-R Zhang,** G. Cui, and N. A. McCarty. (2005) The block of CFTR by scorpion is state dependent. ***Biophys. J.*** 89(6):3960-75
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5) Thompson, C.H., D.M. Fields, P.R. Olivetti, M.D. Fuller, **Z-R Zhang**, J. Kubanek, and N.A. McCarty. (2005) Inhibition of ClC-2 Cl- channels by a peptide component of scorpion venom. ***J. Membrane Biol***. 208(1):65-76.

range of 5-100 ng of CFTR cRNAs dependent upon the purpose of experiments. Excised, inside- out patches, outside-out macropatches and two-electrode voltage-clamp configurations were used to generate data using an Axopatch 200B amplifier operated by pClamp 8.0 software, filtered at 100 Hz and analyzed using Clampfit 9.0. Unless otherwise noted, values given are mean ± SEM. Statistical analysis was performed using the *t*-test for unpaired or paired measurements by Sigma Stat 2.03 (Jandel Scientific; San Rafael, CA), with $p < 0.05$ considered indicative of significance.

Results

In the first objective of this thesis study, we first used the inside-out single channel recording configuration to study the amplitude and distribution of sub- and full-conductance states of channels before and after modification in single- and double-site cysteine engineered mutants. We found that within an open burst it was possible to distinguish three, distinct conductance

states referred to at the full conductance (**f**), subconductance 1 (**s1**) and subconductance (**s2**). Amino acid substitutions in TM6 altered the duration and probability of occurrence of these subconductance states, but did not greatly alter their relative amplitudes. Covalent modification of single R334C-CFTR channels by $MTSET^+$ (a SH-modifying agent that deposits positive charge at accessible cysteine residues), monitored in real-time, resulted in simultaneous modification of all three conductance levels in what appeared to be a single step, without changing the proportion of time spent in each state suggesting that modification of R334C-CFTR by $MTSET^+$ did alter gating property of CFTR. This behavior suggested that at least a portion of the conduction path is common to all three conducting states (one-pore). The time course for the modification of R334C-CFTR, measured in outside-out macropatches using a rapid perfusion system, was also consistent with a single modification step suggesting each pore contained only a single copy of the cysteine at position 334. Our results are consistent with a model for the CFTR

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conduction pathway in which a single anion-conducting pore is formed by a single CFTR polypeptide.

In the second objective of this thesis, we used covalent modification of CFTR channels bearing a cysteine engineered at position 334 to investigate changes in pore conformation that might accompany channel gating. In single R334C-CFTR channels studied in excised patches, modification by $MTSET^+$, which increases conductance, occurred only during channel closed states. This suggests that the rate of reaction of the cysteine was greater in closed channels than in open channels. R334C-CFTR channels in outside-out macropatches activated by ATP alone were modified with first-order kinetics upon rapid exposure to $MTSET^+$. Modification was much slower when channels were locked open by addition of non-hydrolyzable nucleotide, or when the R334C mutation was coupled to a second mutation, K1250A, which greatly decreases channel closing rate. In contrast, modification was faster in R334C/K464A-CFTR channels, which exhibit prolonged interburst closed states. These data indicate that the

reactivity of the engineered cysteine in R334C-CFTR is state-dependent, providing evidence of changes in pore conformation coupled to ATP binding and hydrolysis at the NBDs. The data also show that maneuvers that lock open R334C-CFTR do so by locking channels into the prominent s2 subconductance state, suggesting that the most stable conducting state of the pore reflects the fully-occupied, pre-hydrolytic state of the NBDs. *The results provide direct evidence that conformational changes in the outer vestibule of CFTR are linked to the ATP-dependent gating cycle.*

Conclusions

Objective 1: 1) CFTR exhibits subconductances. 2) Single channel amplitude is dramatically decreased by mutant R334C and potentiated by double mutant R334C/T338A. 3) R334C lies in the conduction pore of CFTR. 4) The functional CFTR channel confers a single-pore, and is formed as a monomer by a single CFTR polypeptide.

Objective 2: 1) The rate of covalent modification at R334C differs dramatically between closed and open channels. 2) The state-dependent modification rate coefficient at position R334C most likely reflects a difference in reactivity of that cysteine during the gating cycle rather than physical obstruction that reduces accessibility. 3) Changes in the rate coefficient for SH-modifying reagent at R334C-CFTR under different experimental conditions likely reflect conformational changes in the outer vestibule of the CFTR pore, which is associated with ATP-dependent gating. 4) Transitions between the three major conductance states of R334C-CFTR channels are linked to ATP-dependent gating events at NBDs