Direct Comparison of the Functional Roles Played by Different Transmembrane Regions in the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel Pore*

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The cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel contains 12 transmembrane (TM) regions that are presumed to form the channel pore. However, little is known about the relative functional contribution of different TM regions to the pore. We have used patch clamp recording to investigate the functional consequences of point mutations throughout the six transmembrane regions in the N-terminal part of the CFTR protein (TM1-TM6). A range of specific functional assays compared the single channel conductance, anion binding, and anion selectivity properties of different channel variants. Overall, our results suggest that TM1 and -6 play dominant roles in forming the channel pore and determining its functional properties, with TM5 perhaps playing a lesser role. In contrast, TM2, -3, and -4 appear to play only minor supporting roles. These results define transmembrane regions 1 and 6 as major contributors to the CFTR channel pore and have strong implications for emerging structural models of CFTR and related ATP-binding cassette proteins.

Cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a member of the ATP-binding cassette (ABC) family of transport ATPases (1) that functions primarily as a cAMP-regulated, ATP-dependent Cl⁻ channel in epithelial cell apical membranes (2, 3). Over 1000 genetic mutations that disrupt the synthesis, maturation, trafficking, or function of CFTR have been shown to be associated with clinical cystic fibrosis.

The CFTR molecule is formed from two homologous repeats, each consisting of six transmembrane (TM) regions followed by a cytoplasmic nucleotide binding domain (Fig. 1A). These two halves are joined by a cytoplasmic regulatory domain that contains multiple consensus sequence sites for phosphorylation by protein kinase A (PKA) and protein kinase C (Fig. 1A). The originally proposed roles of the nucleotide binding domains and the regulatory domain in regulation of CFTR function by ATP and by PKA-dependent phosphorylation (4) have been borne out by a wealth of experimental evidence (2, 3, 5). In contrast, the primary structure of CFTR gave little clue as to the location of the pore region through which Cl⁻ ions pass, although the walls of this pore are presumed to be lined by multiple TM regions (3, 6–8). Indeed, it has become clear that TM6 plays a key role in forming the pore and determining its functional properties (6, 7, 9–11). However, strong evidence supporting the involvement of any other TM is currently lacking. Although limited evidence has been put forward supporting a pore-forming role for TM1 (Refs. 12–15, but see Ref. 16), TM3 (17), TM5 (15), TM8 (18), TM11 (19), and TM12 (20–23), a comprehensive comparison of the functional roles of different TM regions has not previously been reported.

A low resolution crystal structure of CFTR was recently reported (8). It shows two distinct conformational states with apparently very different TM region organizations, possibly reflecting a large structural rearrangement of the TMs between channel open and closed states (8). Unfortunately, the identity of TM regions contributing to the pore region, or even the number of TMs likely to line the pore, could not be identified in the CFTR crystal structure. However, one of the low resolution CFTR structures showed strong similarity to the high resolution crystal structure of the bacterial ABC protein MsbA (8, 24), which also contains 12 TM regions. Furthermore, the two CFTR structures identified are homologous to two low resolution structures previously produced for the human ABC protein P-glycoprotein (25). Thus, the TM organization of these different ABC proteins may be fairly well conserved, potentially allowing structural insights to be made by homology modeling. However, the large structural rearrangement that apparently occurs in the TMs of CFTR (8) and P-glycoprotein (25), and the resulting uncertainty concerning the structure of the open, Cl⁻ -conducting form of the CFTR pore, make it difficult to predict which TM regions will be important in forming the open pore and in potential interactions with permeating Cl⁻ ions. All of the ABC crystal structures produced to date suggest multiple TM regions form α-helices that surround a central pore region in a reasonably parallel fashion, a situation that contrasts starkly with the haphazard-looking arrangement of membrane-spanning segments in CIC chloride channels (26).

Previously we compared the effects of mutations in TM6 and -12 of CFTR on a number of functional pore properties and concluded that TM12, in the C-terminal “half” of CFTR, played a very minor role compared with its N-terminal counterpart, TM6 (23). Based on the idea that the N-terminal half is more important in determining the functional properties of the CFTR pore, we compared the functional effects of point mutations in each of the six TM regions in this part of the protein. To comprehensively screen multiple mutants across several TMs,
we have used relatively straightforward functional measures of different, well defined functional channel properties. Our results suggest major roles for TM1 and -6, with perhaps a lesser role for TM5.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis of CFTR—**Mutations were constructed at each of six consecutive amino acid residues in the mid-to-outer regions of TMs 1–6 (Fig. 1B), based on alignment with a region of TM6 that our previous results suggest major roles for TM1 and -6, with perhaps a lesser role for TM5.

**FIG. 1.** Location of the region of CFTR mutated in the present study. A, overall topology of CFTR, comprising 12 TMs (arranged in two sets of six), two cytoplasmic nucleotide binding domains, and the cytoplasmic regulatory domain. B, location of mutated residues within N-terminal TMs 1 to 6: Thr-95-Pro-99 (TM1), Ala-120-Ile-125 (TM2), Val-208-Gly-213 (TM3), Ala-223-Gly-228 (TM4), Val-317-Val-322 (TM5), and Leu-333-Thr-338 (TM6). TM regions aligned as described previously (6, 7).

**RESULTS**

**Single Channel Properties—**Expression of wild type or mutant CFTR in baby hamster kidney cells led to the appearance of PKA- and ATP-dependent Cl− channel currents in excised membrane patches (Fig. 2). Most mutant forms of CFTR exam-

**Electrophysiological Recording—**Patch clamp analysis of CFTR was carried out using inside-out membrane patches excised from transiently transfected baby hamster kidney cells prepared as described previously (27). Following patch excision, channels were activated by exposure to PKA catalytic subunit plus MgATP (1 mM). Unitary currents were recorded following weak stimulation of channel activity with a low concentration of PKA (1–10 nM). Experiments in which the PKA concentration was subsequently increased suggest that single channel recordings were made from patches that, in fact, contained tens or even hundreds of quiescent CFTR channels. In contrast, macroscopic currents were activated using a high concentration of PKA (~20 nM). To study the open channel blocking effects of intracellular Au(CN)2− (Fig. 5), channels were “locked” in the open state by addition of 2 mM sodium pyrophosphate to the intracellular solution following attainment of full PKA-stimulated current amplitude as described previously (10, 27, 29, 30). Both intracellular (bath) and extracellular (pipette) solutions were based on one containing (mM) 150 NaCl, 2 MgCl2, 10 N-tris[hydroxymethyl]methyl-2-aminoethanesulfonate. Single channel recordings (Fig. 2) were carried out with a low extracellular Cl− concentration (28), with 150 mM sodium gluconate substituted for NaCl in the pipette solution. To estimate SCN− permeability (Fig. 9), NaCl in the intracellular solution was replaced by 150 mM NaSCN. In all cases the intracellular solution contained 1 mM MgATP. All solutions were adjusted to pH 7.4 with NaOH. Given voltages have been corrected for liquid junction potentials calculated using pCLAMP8 software (Axon Instruments, Union City, CA).

Current traces were filtered at 50–200 Hz using an 8-pole Bessel filter, digitized at 250 Hz-1 kHz, and analyzed using pCLAMP8 software (Axon Instruments). Macroscopic current-voltage (I-V) relationships were constructed using depolarizing voltage ramp protocols as described previously (31, 32). Background (leak) currents recorded before addition of PKA were subtracted digitally, leaving uncontaminated CFTR currents (32, 33). Rectification of the I-V relationship was quantified (Fig. 6) as the “rectification ratio,” the slope conductance at −50 mV as a fraction of that at +50 mV as described previously (10). The macroscopic current reversal potential was estimated by fitting a polynomial function to the leak-subtracted I-V relationship and was used to calculate the permeability of SCN− relative to that of Cl− (PSCN/PCL) as described previously (34). Experiments were performed at room temperature, 21–24 °C. Mean values are given as mean ± S.E.

**FIG. 2.** Examples of CFTR single channel currents. Currents were recorded from different CFTR variants in inside-out membrane patches at a membrane potential of −60 mV in each case. For each trace the line to the left represents the current level when all channels are closed.
ined showed similar unitary I-V relationships to that of wild type CFTR (Fig. 3) with a slope conductance of approximately 5.5 pS (Fig. 4). However, the unitary conductance was drastically reduced by some mutations in TM1 (K95Q, Q98A, P99A) and TM6 (R334K, F337A) (Figs. 2–4). Significant, but smaller, reductions in unitary conductance were observed in G213A, V318A, S321A, V322A, K335A, and I336A (Figs. 3 and 4). Only one mutation studied, T338A, led to a significant increase in channel conductance (Figs. 3 and 4) as described previously (35). No channel activity was observed following transfection of M212A cDNA.

Macroscopic Current Properties—The macroscopic current properties of wild type and mutant CFTR channels were studied in inside-out patches following maximal channel activation with PKA and pyrophosphate (see “Experimental Procedures”). Examples of leak-subtracted macroscopic CFTR I-V relationships are shown in Fig. 5 (Control currents). In most cases macroscopic I-V relationships were linear or weakly inwardly rectifying in the presence of symmetrical high Cl⁻ concentrations (as quantified in Fig. 6), although particularly strong inward rectification was observed in Q98A, P99A, and R334K. Interestingly, one mutant, K95Q, showed strong outward rectification (Figs. 5 and 6). Macroscopic currents were not large enough for analysis in L210A or M212A.

Previously we have used Au(CN)₂⁻ as a high affinity probe of anion binding sites in the CFTR pore (29, 30) and to identify TM6 residues contributing to intrapore anion binding (10, 27). As shown in Fig. 5, 100 μM Au(CN)₂⁻ caused a voltage-dependent block of wild type and mutant forms of CFTR. The voltage dependence of block, as well as the effect of several mutations, are clearly seen in the mean fractional current-voltage relationships shown in Fig. 7. The blocking effects of 100 μM Au(CN)₂⁻
Au(CN)$_2$ at $-100$ mV were significantly altered in 22 of 34 mutants studied (Fig. 8), consistent with the previous finding that anion binding in the CFTR pore is relatively sensitive to the effects of mutagenesis (15, 23). Mutations that affect block were identified in all TM regions studied; however, mutagenesis of TM1 and -6 appeared the most likely to be associated with weakened Au(CN)$_2$ block. The weakest block by Au(CN)$_2$ was observed in K95Q, T338A, R334K, and Q98A, consistent with these residues perhaps being associated with permeant anion binding sites inside the pore. Block was particularly strong in L333A, V97A, and I336A.

Anion Selectivity—Previous mutagenic studies of CFTR have suggested that the selectivity of anion permeation is relatively resistant to the effects of mutagenesis within the putative pore region (15, 23, 27). We therefore restricted our investigation of anion selectivity to those TM regions that were associated with significant changes in both single channel conductance and Au(CN)$_2$ block of Cl$^-$ permeation, TM1, -5, and -6.

CFTR is associated with a lyotropic anion selectivity pattern (6) that has been shown to be disrupted by mutagenesis within TM6 (27, 35–37). Changes in lyotropic anion selectivity are reported by changes in the relative permeability of highly lyotropic anions such as Au(CN)$_2$ (27) and SCN$^-$ (11). As a rapid screen for selectivity-altering mutants, we therefore estimated SCN$^-$ permeability from measurements of the macroscopic current reversal potential in inside-out patches with SCN$^-$-containing bath solutions (Fig. 9). Thiocyanate permeability was strongly increased in A96V and T338A, suggesting enhancement of lyotropic selectivity in these mutants, and dramatically reduced in F337A, which we previously suggested reflects the role of Phe-337 in contributing to an anion selectivity filter in the pore (11, 36). Smaller effects on SCN$^-$ per-
meability were associated with other mutations in TM1, -5, and -6 (Fig. 9). Some mutants that significantly affect both unitary conductance and Au(CN)$_2^-$ block were found to be without effect on SCN$^-$ permeability (Q98A, V318A, R334K, K335A).

DISCUSSION
Numerous prior studies have illustrated the role of TM6 in controlling CFTR pore properties such as unitary conductance (9, 20, 28, 35, 38, 39), binding of permeant anions (10, 15, 27, 39), and anion selectivity (35–37). Previous work by ourselves (11, 27) and others (9, 37) has emphasized the role of the mid-to-outer region of TM6 (Arg-334-Ser-341) in determining the functional properties of the pore. We proposed that anion selectivity is determined primarily by anion binding around Phe-337 and Thr-338 and that this central “selectivity filter” is flanked by other binding sites involving Arg-334 and Ser-341 (11). Based on the idea that the CFTR pore is lined by multiple TM regions in a roughly parallel orientation, we have compared the effects of mutagenesis in this region of TM6 with aligned regions of TMs 1–5.

To obtain as objective as possible an overview of the functional roles of different TMs, we have examined three different aspects of pore function: single channel conductance (Figs. 2–4), permeant anion binding (quantified using Au(CN)$_2^-$ block of macroscopic Cl$^-$ current; Figs. 5, 7, 8), and anion selectivity (quantified using SCN$^-$ permeability; Fig. 9). The sum of all of these investigations is summarized in Fig. 10. Conductance is strongly affected by mutagenesis of residues in TM1 (Lys-95, Gln-98, Pro-99) and TM6 (Arg-334, Phe-337, Thr-338) and, to a lesser extent, TM5 (Val-318, Ser-321, Val-322). Permeant an-
ion binding is affected by mutations throughout TM1–6, but based on the data shown in Fig. 8, TM1 (Lys-95, Gln-98) and TM6 (Arg-334, Thr-338) appear most likely to contribute to anion binding sites inside the pore. Interestingly, mutagenesis of these same residues is associated with some of the greatest changes in the shape of the macroscopic I-V curve (Fig. 6), again consistent with altered anion binding inside the pore. Anion selectivity shows an apparently unique dependence on Phe-337 in TM6 (Fig. 9) as discussed previously (11, 36); no additional selectivity-disrupting mutations were identified in the present study. Nevertheless, anion selectivity is influenced by other residues in TM1 (Ala-96) and TM6 (Thr-338). Based on this survey of different permeation properties, we feel that TM1 and TM6 play quite dominant roles in forming the CFTR pore and determining its functional properties, with TM5 perhaps playing a more minor role; TM2, -3, and -4 seem to play only supporting roles.

Although the present results concerning the role of TM6 are reminiscent of much previous work (see above), TM1 has previously received little attention. Subtle changes in anion selectivity were previously observed following mutagenesis of Lys-95 (12) and Gly-91 (which is located more cytoplasmically than the residues mutated in the present study) (15), and reduced unitary conductance following mutagenesis of Pro-99 has been reported previously (14). A number of amino acid residues in the outer half of TM1, including Lys-95 and Gln-98, were suggested to have side chains that were in contact with the pore lumen based on substituted cysteine accessibility mutagenesis (SCAM) (13). Although this latter finding would appear consistent with our suggestion that TM1 makes a significant contribution to the pore, the SCAM method also suggested a pore-forming role for TM3 (17). Based on the finding that CFTR could still form a cAMP-regulated Cl− channel following deletion of TM1–4, it was suggested that these TMs were not essential parts of the channel pore (16). Our present results suggest that TM1 does make an important contribution to the pore when it is included in an intact CFTR molecule; possibly, deletion of so many TMs forces the CFTR molecule to fold into a non-native conformation that includes a pore with very different structure yet broadly similar functional properties to that of the native molecule.

Recent molecular models of human ABC proteins closely related to CFTR, such as P-glycoprotein (40) and the multidrug resistance-associated protein MRP1 (41), do not predict an important role for TM1, instead emphasizing the potential roles of TM5, -6, -11 and -12 in lining the central pore. Although the structures of CFTR and P-glycoprotein appear similar (8), our results implicating TM1 and -6 in forming the CFTR pore suggest important differences may exist and caution against over-reliance on homology modeling between these related proteins.

Our results suggest that N-terminal TM1 and -6 play an important role in forming the CFTR Cl− channel pore and in determining its functional properties. We suggest that permeating Cl− ions approach close enough to both of these TM regions to interact with the amino acid side chains of Lys-95, Gln-98, Arg-334, Phe-337, and Thr-338 and that these interactions are important in controlling the rate of Cl− permeation.

2 The numbering of TM regions here is as for CFTR and P-glycoprotein; because MRP1 has an additional 5 TM regions at the N-terminus, this in fact corresponds to TM10, -11, -16, and -17 in MRP1.
through the pore. We believe that these functional investigations are important in interpreting emerging structural data (8) to understand the structure and permeation mechanism of the CFTR Cl\(^{-}\) channel pore.

Acknowledgments—We thank Susan Burbridge and Jeremy Roy for technical assistance.

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