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Extent of the selectivity filter conferred by the sixth transmembrane region in the CFTR chloride channel pore

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Summary

Point mutations within the pore region of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel have previously been shown to alter the selectivity of the channel between different anions, suggesting that part of the pore may form an anion 'selectivity filter'. However, the full extent of this selectivity filter region and the location of anion binding sites in the pore are currently unclear. As a result, comparisons between CFTR and other classes of Cl^- channel of known structure are difficult. We compare here the effects of point mutations at each of eight consecutive amino acid residues (arginine 334–serine 341) in the crucial sixth transmembrane region (TM6) of CFTR. Anion selectivity was determined using patch-clamp recording from inside-out membrane patches excised from transiently transfected mammalian cell lines. The results suggest that selectivity is predominantly controlled by a single site involving adjacent residues phenylalanine 337 and threonine 338, and that the selectivity conferred by this 'filter' region is modified by anion binding to flanking sites involving the more extracellular arginine 334 and the more intracellular serine 341. Other residues within this part of the pore play only minor roles in controlling anion permeability and conductance. Our results support a model in which specific TM6 residues make important contributions to a single, localized anion selectivity filter in the CFTR pore, and also contribute to multiple anion binding sites both within and on either side of the filter region.

Keywords: Anion selectivity; anion permeability; chloride channel; CFTR; lyotropic selectivity.

Introduction

Ionic selectivity in voltage-gated cation channels is determined over a discrete, narrow region of the pore referred to as the selectivity filter [1–5]. Selective binding of the ion of physiological interest within this region is thought to underpin the high ionic selectivity of these channel types [1, 3–6]. In spite of the localized nature of the selectivity filter [1–3], multiple ions bind within this region concurrently [1,2,4,5]. Mutual electrostatic repulsion between simultaneously bound ions then allows these channels to combine strong selectivity with high flux rates [1,4,5,7,8].

Much less is known about the physical nature of selectivity and permeation in anion channels [9,10]. These channels are relatively poorly selective, usually allowing most small anions to permeate to some extent [9,11–14]; this may reflect a lack of evolutionary pressure to establish and maintain strong selectivity, since Cl^- is the predominant anion in most biological fluids. In fact, the selectivity properties of Cl^- channels could be obtained without the need for a 'selectivity filter' of the kind described above [11]. Nevertheless, we have previously provided evidence that selectivity between different anions within the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel is controlled primarily over a physically restricted region of the pore [12,15]. These functional results are consistent with the assignment, on structural grounds, of a region of a prokaryotic ClC Cl^- channel pore as an 'anion selectivity filter' [10]. However, the limited scope of previous structure–function studies precludes definition of a selectivity filter region in the CFTR pore, and therefore makes structural and mechanistic comparisons with channels of known structure impossible [10]. Furthermore, although discrete anion binding sites undoubtedly exist in Cl^- channel pores [9,10,13,15,16], their importance in determining selectivity is unclear [9–11,16].

The CFTR molecule contains 12 transmembrane regions, several of which are presumed to come together to form the pore [9,17,18]. Previous structure–function investigations of the CFTR Cl^- channel pore have focused on the sixth transmembrane region (TM6), which clearly plays a key role in forming the pore and determining its permeation properties [9,15,17–20]. Mutation of residues in the central portion of TM6 — at K335 [19,21], F337 [12,15,16], T338 [15,16,20,22] and S341 [15,20] — have previously been associated with changes in relative anion permeability and conductance under different experimental conditions. Point mutations within this same key region of TM6 also affect other pore properties such as unitary conductance [16,22–26] and binding of permeant [15,16,21] and blocking anions [25,27–29]. However, since nothing is known about the roles of other nearby residues, it is difficult to put the effects of these mutations into any kind of structural context. Fuller understanding of the relative roles of different residues is critical for understanding the mechanism of selectivity and conduction in the CFTR pore, and for comparison of this mechanism with that of other classes of Cl^- channels of known structure [10]. In order to obtain some information concerning the extent of TM6 important for controlling interactions with permeant anions, we have compared channels bearing mutations at each of eight consecutive residues (R334–S341) centred around previously identified key residues. Since TM6 presumably runs the length of the pore, we hoped in this way to gain some spatial information concerning the shape and extent of the pore region important for controlling CFTR anion selectivity.

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Results

To investigate the relative roles of different TM6 residues in controlling CFTR channel selectivity, mutations were constructed at each of eight consecutive TM6 residues (R334–S341). These residues flank the important F337, previously described as central in determining selectivity between different anions [12,15]. In most cases alanine substitutions were employed, except where we have previously determined that mutation to alanine results in a lack of functional channel expression in BHK cells (R334, T339; see Experimental procedures). Transient co-expression of each of these mutants along with enhanced green fluorescent protein (GFP) (see Experimental procedures) led to the appearance of PKA- and ATP-dependent CFTR Cl^- currents in visibly identifiable GFP-expressing cells (Figure 1A). However, in contrast to the linear macroscopic I–V relationship observed for wild-type CFTR under symmetrical ionic conditions, most mutants showed some degree of inward rectification (Figure 1B); this rectification was particularly strong for R334C.

Anion selectivity of different CFTR variants was examined under bi-ionic conditions, with Cl^- present in the extracellular solution and different anions present in the intracellular

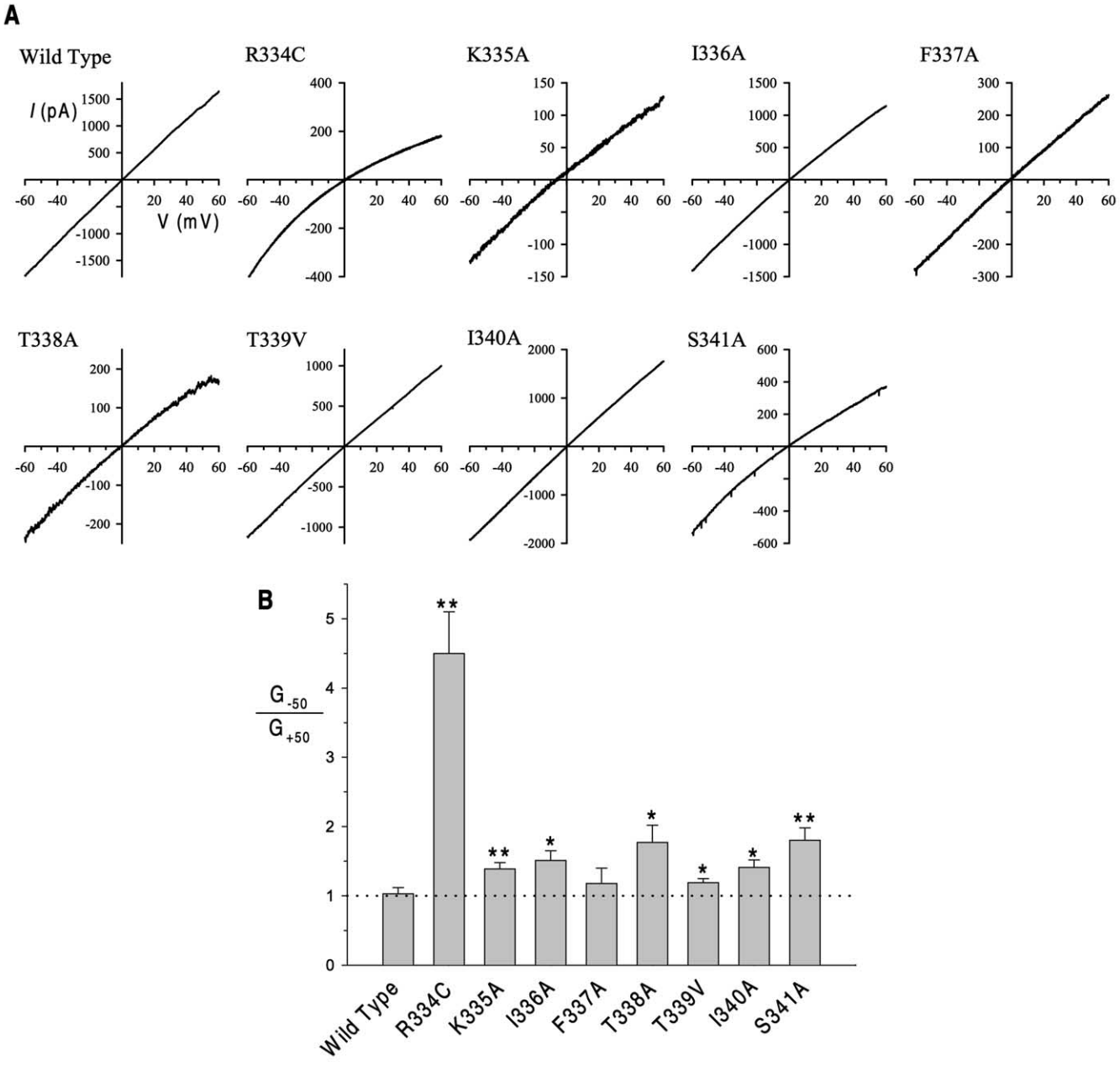

Figure 1. Macroscopic chloride currents carried by wild-type and mutant CFTR channels. (A) Example leak-subtracted macroscopic I–V relationships for each CFTR variant studied recorded with symmetrical 154 mM Cl^--containing solutions as described in the Experimental procedures. (B) Rectification of the macroscopic I–V relationship quantified as described in the Experimental procedures and in Table 3. Mean of data from five to eight patches. *Significant difference from wild-type (*$p < 0.05$; **$p < 0.005$).

solution, as described in detail previously [12,18,22] (see Experimental procedures). Example leak-subtracted $I-V$ relationships obtained with different intracellular anions are shown for wild-type, R334C, F337A, T338A, T339V and S341A in Figure 2. As described previously [12], experiments with different anions were carried out on different patches, and as such no information is contained in the relative current amplitudes with different anions in Figure 2. Other mutants studied (K335A, I336A, I340A), like T339V, had only minor effects (data not shown). Of eight mutants studied, only T339V was without any significant effect on anion permeability (Table 1), and five mutations (R334C, K335A, F337A, T338A, I340A) led to changes in the permeability sequence among halides (Figure 2 and Table 2). The relative permeability of the lyotropic SCN^- anion, which is high in the wild-type ($P_{SCN}/P_{Cl} = 4.75 \pm 0.30$, $n = 6$) (Table 1) was significantly altered in six out of eight mutants studied (Table 1 and Figure 3), with P_{SCN}/P_{Cl} being greatly reduced in F337A and most strongly increased in T338A and S341A.

Anion relative conductance was also estimated from the slope of the $I-V$ relationship for inward versus outward current (Figure 2), as described previously [16] (see Experimental procedures). Although this method tends to overestimate the relative conductance of some anions due to *trans*-ion effects on Cl^- conductance [16], the anion conductance sequence for wild-type CFTR ($Cl^- > Br^- > SCN^- \sim F^- > I^-$) (Table 3) is the same as that estimated from unitary current measurements under symmetrical ionic conditions [16]. A number of significant changes in the apparent relative conductance of different anions were observed in some mutants (Table 3). Perhaps most significantly, the relative conductance of Br^-, I^- and SCN^- were all increased in S341A, leading to a change in the conductance sequence to $Br^- > I^- \sim Cl^- > SCN^- > F^-$ (Table 3). For all other CFTR variants studied, Cl^- remained the anion with the highest apparent conductance (Table 3). Furthermore, there was no apparent correlation between the observed changes in anion permeability (Table 1) and anion conductance (Table 3).

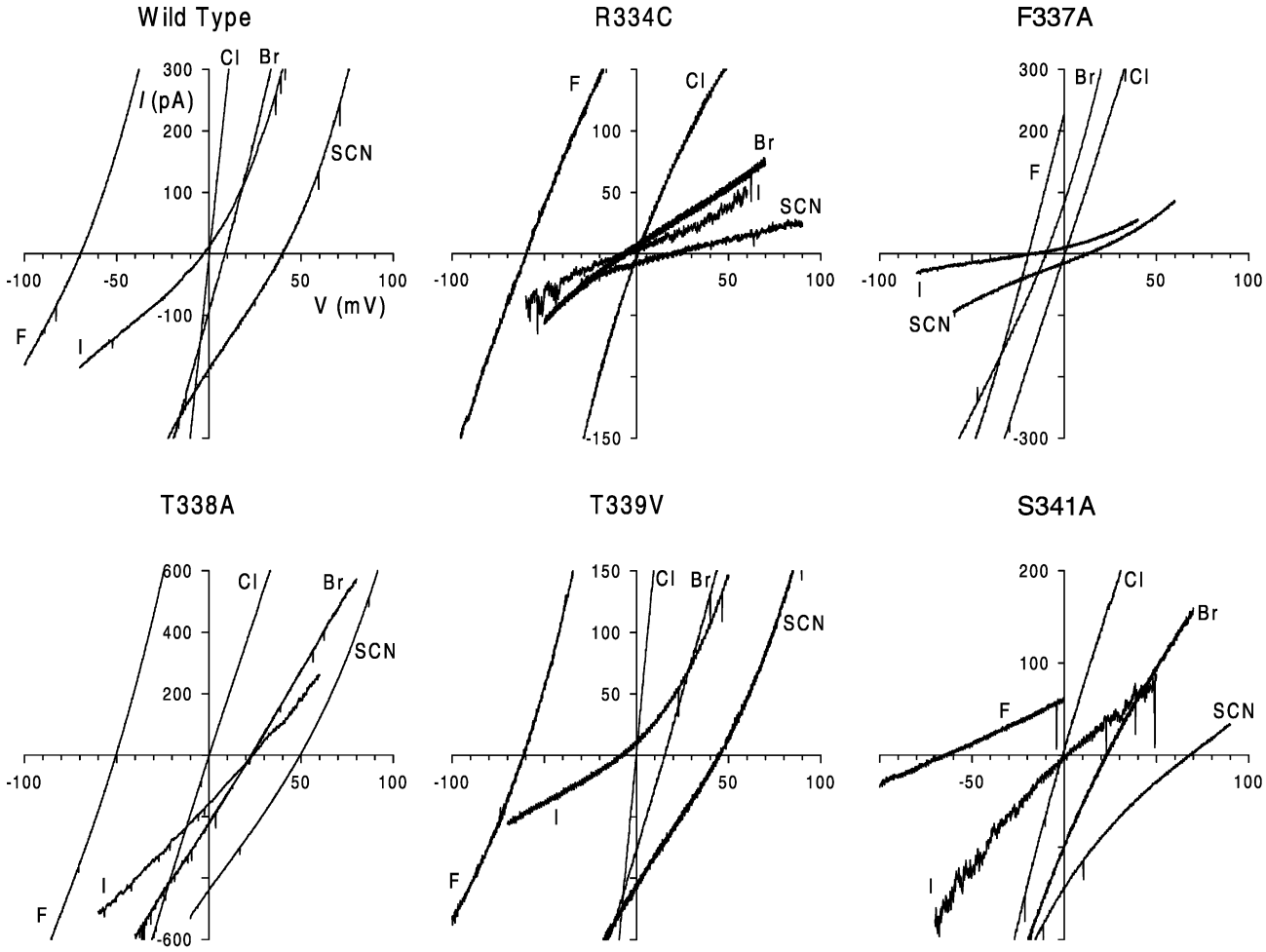

Figure 2. Anion selectivity of macroscopic CFTR currents. Leak-subtracted macroscopic $I-V$ relationships were constructed as described previously [12] (see the Experimental procedures). Each $I-V$ relationship is from a different patch (see the Results). Currents were recorded under bi-ionic conditions, with Cl^- in the extracellular solution and the named anion present in the intracellular solution.

Table 1. Relative permeabilities of intracellular anions in wild-type and mutant CFTR channels.

	Wild type	R334C	K335A	I336A	F337A	T338A	T339V	I340A	S341A
Cl	1.00 ± 0.00 (6)	1.00 ± 0.01 (6)	1.00 ± 0.05 (5)	1.00 ± 0.01 (5)	1.00 ± 0.02 (6)	1.00 ± 0.02 (8)	1.00 ± 0.03 (6)	1.00 ± 0.02 (5)	1.00 ± 0.01 (6)
Br	1.47 ± 0.06 (6)	0.96 ± 0.00 (5)**	1.52 ± 0.03 (5)	1.35 ± 0.05 (5)	0.66 ± 0.03 (6)**	2.20 ± 0.05 (5)**	1.82 ± 0.24 (5)	1.40 ± 0.09 (6)	2.45 ± 0.20 (5)**
I	0.81 ± 0.04 (6)	0.72 ± 0.05 (3)	1.57 ± 0.06 (4)**	0.58 ± 0.02 (4)*	0.38 ± 0.15 (3)*	2.79 ± 0.26 (7)**	0.76 ± 0.02 (6)	1.24 ± 0.07 (6)**	0.73 ± 0.06 (6)
F	0.11 ± 0.01 (6)	0.09 ± 0.01 (3)	0.13 ± 0.02 (3)	0.07 ± 0.01 (5)	0.40 ± 0.02 (4)**	0.13 ± 0.01 (6)	0.07 ± 0.00 (5)	0.06 ± 0.01 (5)	0.05 ± 0.01 (6)*
SCN	4.75 ± 0.30 (6)	2.76 ± 0.38 (6)**	3.98 ± 0.16 (5)	3.70 ± 0.11 (5)*	1.26 ± 0.12 (5)**	7.50 ± 0.29 (6)**	4.82 ± 0.40 (5)	4.18 ± 0.14 (7)*	10.0 ± 1.8 (6)*

Relative permeabilities for different anions present in the intracellular solution under bi-ionic conditions were calculated from macroscopic current reversal potentials according to Eq. (1) (see Experimental procedures). For all CFTR variants, the current reversed close to 0 mV in symmetrical Cl^--containing solutions (Figure 1A). Numbers in parentheses indicate the number of patches examined in each case. *Significant difference from the corresponding value in wild-type (*$p < 0.05$; **$p < 0.005$).

Table 3. Relative conductances of intracellular anions in wild-type and mutant CFTR channels.

	Wild-type	R334C	K335A	I336A	F337A	T338A	T339V	I340A	S341A
Cl (G_{-50}/G_{+50})	1.03 ± 0.09 (6)	4.50 ± 0.60 (6)**	1.39 ± 0.09 (5)**	1.51 ± 0.14 (5)*	1.18 ± 0.22 (6)	1.77 ± 0.25 (8)*	1.19 ± 0.06 (7)*	1.41 ± 0.11 (5)*	1.80 ± 0.18 (5)**
Cl (G_{Cl}/G_{Cl})	1.00 ± 0.08 (6)	1.00 ± 0.13 (6)	1.00 ± 0.07 (5)	1.00 ± 0.09 (5)	1.00 ± 0.22 (6)	1.00 ± 0.14 (8)	1.00 ± 0.06 (7)	1.00 ± 0.09 (5)	1.00 ± 0.10 (5)
Br	0.64 ± 0.05 (6)	0.32 ± 0.02 (6)**	0.66 ± 0.05 (5)	1.07 ± 0.10 (5)*	0.35 ± 0.06 (6)**	0.49 ± 0.03 (5)	0.65 ± 0.09 (5)	0.66 ± 0.08 (6)	1.52 ± 0.30 (4)*
I	0.29 ± 0.05 (6)	0.74 ± 0.02 (3)*	0.27 ± 0.01 (4)	0.10 ± 0.02 (4)*	0.34 ± 0.08 (3)	0.38 ± 0.03 (5)	0.30 ± 0.05 (7)	0.27 ± 0.03 (6)	1.04 ± 0.16 (7)**
F	0.37 ± 0.04 (6)	0.32 ± 0.04 (3)	0.34 ± 0.03 (3)	0.70 ± 0.10 (4)*	0.12 ± 0.02 (3)*	0.23 ± 0.02 (6)*	0.50 ± 0.10 (4)	0.30 ± 0.02 (5)	0.51 ± 0.07 (6)
SCN	0.38 ± 0.02 (6)	0.33 ± 0.03 (6)	0.66 ± 0.10 (5)*	0.27 ± 0.02 (6)*	0.39 ± 0.04 (5)	0.26 ± 0.02 (5)*	0.26 ± 0.02 (4)*	0.35 ± 0.04 (6)	0.83 ± 0.14 (6)*

Relative conductances for different anions were calculated from the slope of the macroscopic $I-V$ relationship for inward versus outward currents (see Experimental procedures). For Cl^- itself, the ratio (G_{-50}/G_{+50}) represents this relative slope conductance (i.e. a measure of rectification of the $I-V$ relationship), while the ratio (G_{Cl}/G_{Cl}) represents this ratio for each individual patch as a fraction of the mean, and thereby acts as a control for comparison of the conductance of other intracellular anions relative to that of Cl^- under bi-ionic conditions. Numbers in parentheses indicate the number of patches examined in each case. *Significant difference from the corresponding value in wild-type (*$p < 0.05$; **$p < 0.005$).

Table 2. Halide selectivity sequences for different CFTR variants.

Halide permeability sequence	Eisenman sequence	CFTR variants
$I^- > Br^- > Cl^- > F^-$	I	K335A, T338A
$Br^- > I^- > Cl^- > F^-$	II	I340A
$Br^- > Cl^- > I^- > F^-$	III	wild-type, I336A, T339V, S341A
$Cl^- > Br^- > I^- > F^-$	IV	R334C
$Cl^- > Br^- > F^- > I^-$	V	F337A

Sequences were derived from the relative permeabilities given in table 1. Eisenman sequences (from the weak field strength sequence I to the relatively strong field strength sequence V) are as given by Wright and Diamond [31].

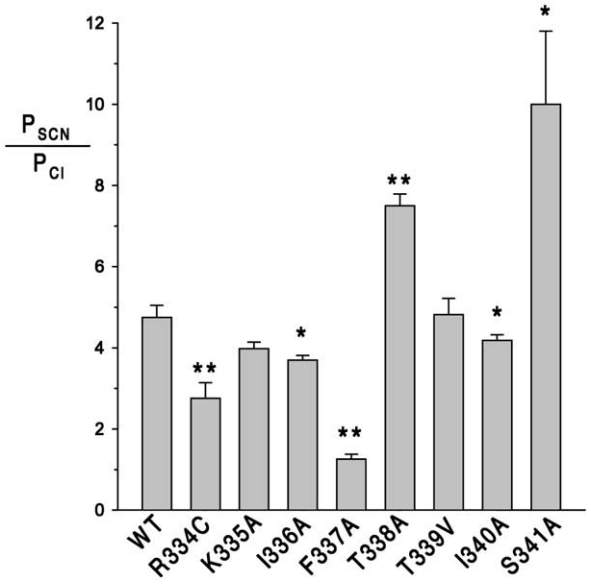

Figure 3. Thiocyanate permeability of wild-type and mutant CFTR channels. Relative SCN^- permeability (P_{SCN}/P_{Cl}), a marker of lyotropic anion selectivity in CFTR, was estimated as described in Table 1. Mean of data from five to seven patches. *Significant difference from wild-type (*$p < 0.05$; **$p < 0.005$).

Discussion

Wild-type CFTR shows a lyotropic anion selectivity sequence, with anions with low free energy of hydration (lyotropes) tending to show a higher permeability than those which retain their waters of hydration more strongly (kosmotropes) [11,30]. This dependence on energy of hydration suggests that anion dehydration is a limiting step in anion permeation through the pore [9,16]. In terms of selectivity between halides, wild-type CFTR shows a moderately weak field strength selectivity sequence (Eisenman sequence III, [31]) (Table 2). This halide selectivity sequence is changed to Eisenman sequence II in I340A, and Eisenman sequence I in both K335A and T338A (Table 2), consistent with a strengthening of lyotropic anion selectivity in these mutants. Conversely, the sequence is changed to Eisenman sequence IV in R334C and Eisenman sequence V in F337A, consistent with relative loss of lyotropic anion selectivity in these mutants. Loss of lyotropic selectivity in F337A is also

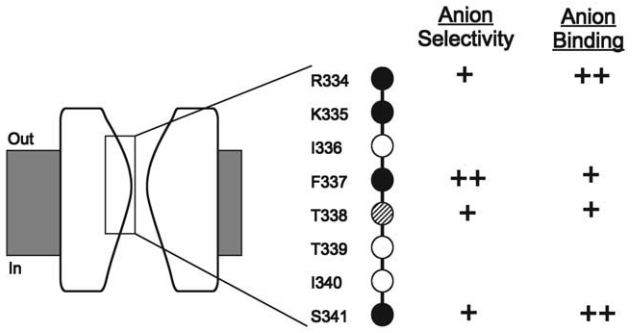

Figure 4. Simple cartoon model of TM6. The present study sought to assess the relative roles of residues in the mid-to-outer part of TM6 in controlling interactions between permeant anions and the pore. This region most likely contributes to the narrowest part of the pore [12,17,22], with R334 being in the outer pore vestibule [26]. Solid symbols represent those residues that have been proposed to have pore-lining amino acid side chains [26,32], while open symbols represent non-pore-lining residues [26]. Conflicting results concerning the orientation of T338 have been reported [32,33], hence this side chain is shaded. The relative effects of mutagenesis of different residues on lyotropic anion selectivity and anion binding observed in the present work are indicated to the right (see the Discussion). Our results are consistent with anion-binding sites located at each turn of an α-helix, with the central of these three sites forming the primary anion selectivity filter.

demonstrated by the fact that this is the only mutant studied in which selectivity for Cl^- over the kosmotropic F^- anion was somewhat compromised (Table 1).

We have also previously judged the lyotropic nature of CFTR anion selectivity by changes in the relative permeability of the highly lyotropic $Au(CN)_2^-$ anion [15]. In the present study, large increases in the permeability of the lyotropic SCN^- anion were observed in both T338A and S341A, and a dramatic decrease in SCN^- permeability was observed in F337A (Figure 3), consistent with previous results with $Au(CN)_2^-$ which suggest these residues are the main determinants of the permeability of strongly lyotropic anions [15]. Smaller but statistically significant decreases in P_{SCN}/P_{Cl} were observed in R334C, I336A and I340A.

Taken together, these anion permeability data suggest a relative loss of lyotropic anion selectivity in F337A and (to a lesser extent) R334C, strengthening of lyotropic selectivity in T338A and S341A, and only minor effects at other positions. R334, F337 and S341 have all been shown, using substituted cysteine accessibility mutagenesis, to have side chains which are in contact with the aqueous lumen of the pore [32] (Figure 4). Using this same method, T338 was originally described as non-pore lining [32], although more recently this finding has been refuted by others [33]. Our data suggest that interspaced non-pore-lining residues (I336, T339, I340) [32] may play only supporting roles. K335, which has been shown to be pore lining [26,32], also appears to be of only minor importance (Table 1), consistent with previous reports [19,21].

Mutations throughout TM6 also affected macroscopic Cl^- current rectification (Figure 1B and Table 3) and anion relative conductance (Table 3). Although the reasons for current rectification are not entirely clear [22,34], they likely signify altered Cl^- binding within or close to the pore

[16,25,26]. Current rectification was particularly striking in R334C (Figure 1B), consistent with the recently described role of the positive charge at this position in attracting anions to the mouth of the pore [26].

Changes in apparent anion relative conductance (Table 3), although difficult to ascribe to a single physical property of the pore [16], also likely reflect changes in intrapore anion binding. In particular, low relative conductance of lyotropic anions with high permeability (such as Br^- and SCN^-) most likely reflects tight binding of these ions, consistent with anion binding, like anion selectivity, being dependent on anion-free energy of hydration [11,16,35]. As previously described by others [20], mutation of S341 was particularly associated with changes in anion relative conductance, consistent with weakened lyotropic anion binding in S341A. Thus, while permeant anions do bind within the central 'selectivity filter' region (F337/T338) [15,16], the effects of mutations at R334 and S341 on different markers of anion binding suggest that this 'filter' region may be flanked by other permeant anion binding sites.

Previous work by ourselves [12,15,16,22] and others [20,26] has suggested that the mid-to-outer region of TM6 is particularly important in controlling interactions between the pore and permeant anions. Our present results allow us for the first time to ascribe relative roles of different residues within this region (Figure 4). Lyotropic anion selectivity is disrupted in F337A and modified in R334C, T338A and S341A. Permeant anion binding has previously been shown to involve both F337 and T338 [15,16], although the present results suggest stronger roles for R334 and S341. Our results therefore suggest that anion selectivity in CFTR is determined predominantly at a single, localized selectivity filter region and is modified by anion binding to sites which flank this region on either side. We hypothesize that these binding sites may be important in attracting permeant anions toward the selectivity filter region and/or allowing coordinated binding of multiple anions within this crucial pore region. Although only a single Cl^- ion binding site was observed in a ClC crystal structure, a second binding site was hypothesized [10]. Functional evidence exists that multiple anions may be able to bind within the CFTR pore simultaneously [24,36,37]; by analogy with cation-selective channels, multiple ion occupancy may serve to maximize ionic flux rates through the pore. The relative positions of the three binding sites hypothesized in Figure 4 are consistent with their being oriented along one face of an α-helix [32].

According to the model presented in Figure 4, the pore properties conferred by this region of TM6 are dominated by anion–amino acid side chain interactions. Thus, mutation of unequivocally non-pore-lining residues (I336, T339, I340) had only very minor effects. Interestingly, at the putative selectivity filter identified in a ClC Cl^- channel crystal structure, bound Cl^- ions make hydrophobic contacts with a phenylalanine side chain, and polar contacts with both serine and tyrosine side chain hydroxyl groups [10]. In CFTR, predominant interactions with the hydrophobic F337 and hydroxylated T338 side chains may be involved in forming a similar Cl^- ion binding site, although in the absence of direct structural information we cannot be certain that these two adjacent amino acid side chains can simultaneously interact with a single Cl^- ion. Finally, it must be noted that although the effects of mutagenesis at some sites were modest (Table 1), alanine substitution of five different residues in TM12 were without any effect on the anion selectivity sequence under similar conditions [18], demonstrating the primary role of TM6 in determining pore properties.

Experimental procedures

Mutagenesis and expression of CFTR

Experiments were carried out on baby hamster kidney (BHK) cells transiently cotransfected with CFTR and enhanced green fluorescent protein (GFP) [15]. Mutagenesis of CFTR was carried out as described previously [15] and verified by DNA sequencing. In the present study, we compared the effects of single mutations at each of eight consecutive residues (arginine 334–serine 341) in the sixth transmembrane region (TM6) of CFTR (Figure 4), an important determinant of pore properties including selectivity among anions [9,12,15]. In most (six of eight) cases, alanine substitution was employed; however, we have previously found that the mutants R334A [15] and T339A [22] fail to express in BHK cells, and for these residues mutants which gave adequate current expression (R334C, T339V) were studied.

Electrophysiological recordings

Macroscopic CFTR current recordings were made using the excised, inside-out configuration of the patch-clamp technique, as described in detail recently [38]. Briefly, CFTR channels were activated following patch excision by exposure to 40–80 nM protein kinase A catalytic subunit (PKA) plus 1 mM MgATP. For Cl^- current recording, both pipette (extracellular) and bath (intracellular) solutions contained (mM): 150 NaCl, 2 $MgCl_2$ and 10 *N*-tris-(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES). For anion selectivity experiments, the intracellular solution was replaced by one containing (mM): 154 NaX (where X is the anion being tested), 2 $Mg(OH)_2$ and 10 TES (i.e. bi-ionic conditions). All solutions were adjusted to pH 7.4 using NaOH. All chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada) except for PKA (Promega, Madison, WI, USA).

Macroscopic current–voltage (I–V) relationships were constructed following full activation of CFTR using a depolarizing voltage ramp protocol, with a rate of change of voltage of 75–100 mV s^{-1} [30,39]. Background (leak) currents recorded before addition of PKA were subtracted digitally, leaving uncontaminated CFTR currents [30,39]. Current traces shown are the response to a single voltage ramp. Currents were filtered at 200 Hz using an eight-pole Bessel filter, digitized at 1 kHz, and analysed using pCLAMP8 software (Axon Instruments, Union City, CA, USA). The current reversal potential (V_{rev}) was estimated by fitting a polynomial function to the I–V relationship and was used to estimate the permeability of different anions relative to that of Cl^- (P_X/P_{Cl}) according to the equation:

$$P_X/P_{Cl} = \exp(\Delta V_{rev} F/RT), \quad (1)$$

where ΔV_{rev} is the difference between ΔV_{rev} measured under bi-ionic conditions with a test anion X^- and the mean V_{rev} measured with symmetrical Cl^--containing solutions, and F, R and T have their usual thermodynamic meanings.

The relative conductance of intra- versus extracellular anions was estimated by measuring the relative slope of the I–V curve for inward versus outward currents, as described previously [16]. Relative conductance was estimated as the ratio of the slope conductance at a voltage 50 mV more negative than V_{rev} to that at 50 mV more positive than V_{rev}. Slope conductance was measured by fitting a straight line to all data points between 48 and 52 mV from the reversal potential. With symmetrical Cl^--

containing solutions, the relative conductance of intra- versus extracellular Cl^- ions gave a quantitative measure of the degree of rectification of the I–V relationship (Figure 1B). The conductance of an anion X^- relative to that of Cl^- (G_X/G_{Cl}) was then estimated as the conductance ratio under bi-ionic conditions as a fraction of the conductance ratio with symmetrical Cl^--containing solutions (Table 3).

Experiments were carried out at room temperature, 21–24°C. Data are mean ± SEM. Statistical comparisons between groups were carried out using a two-tailed t-test.

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