

Evolutionary and functional divergence between the cystic fibrosis transmembrane conductance regulator and related ATP-binding cassette transporters

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ATP-binding cassette (ABC) transporter superfamily, an ancient family of proteins found in all phyla. In nearly all cases, ABC proteins are transporters that couple the hydrolysis of ATP to the transmembrane movement of substrate via an alternating access mechanism. In contrast, CFTR is best known for its activity as an ATP-dependent chloride channel. We asked why CFTR, which shares the domain architecture of ABC proteins that function as transporters, exhibits functional divergence. We compared CFTR protein sequences to those of other ABC transporters, which identified the ABCC4 proteins as the closest mammalian paralogs, and used statistical analysis of the CFTR-ABCC4 multiple sequence alignment to identify the specific domains and residues most likely to be involved in the evolutionary transition from transporter to channel activity. Among the residues identified as being involved in CFTR functional divergence, by virtue of being both CFTR-specific and conserved among all CFTR orthologs, was R352 in the sixth transmembrane helix (TM6). Patch-clamp experiments show that R352 interacts with D993 in TM9 to stabilize the open-channel state; D993 is absolutely conserved between CFTRs and ABCC4s. These data suggest that CFTR channel activity evolved, at least in part, by converting the conformational changes associated with binding and hydrolysis of ATP, as are found in true ABC transporters, into an open permeation pathway by means of intraprotein interactions that stabilize the open state. This analysis sets the stage for understanding the evolutionary and functional relationships that make CFTR a unique ABC transporter protein.

ion channel | molecular evolution | CFTR | Type II divergence

Cystic fibrosis (CF) is the most common lethal, autosomal recessive disease affecting Caucasians in the United States. It arises from mutations in the gene encoding the CF transmembrane conductance regulator (CFTR), which is a member of the large superfamily of ATP-binding cassette (ABC) proteins (1, 2). The CFTR protein contains five functional domains: two transmembrane domains (TMD1 and TMD2), two cytoplasmic ATP-binding domains (ABC domains), and a cytoplasmic regulatory domain (R domain) [Fig. 1 and supporting information (SI) Fig. S1]. Activation of CFTR requires protein kinase A-mediated phosphorylation of the R domain followed by binding and hydrolysis of ATP at the ABC domains (2). CFTR is expressed in a variety of cells of epithelial origin; its loss or malfunction in epithelial cells of the small airways leads to alterations in the volume and composition of the airway surface liquid that are associated with chronic inflammation underlying CF lung disease (3).

ABC transporter proteins are membrane-associated proteins that possess the characteristic, and highly conserved, ABC ATPase domain. Proteins of the ABC transporter superfamily are engaged in numerous cellular processes and their regulation via the import and export of a variety of substrates. Phylogenetic analysis of the ABC transporter superfamily revealed that CFTR proteins belong to class I, which includes most known exporters,

and specifically within the OAD subclass that also includes the SUR proteins that regulate ion channel function (4). There are numerous ABC protein families (A–G) encoded in the human genome, and CFTR is a member of the ABCC family (1).

Although CFTR is a member of the ABC transporter superfamily, it clearly diverges from other ABC proteins in that it functions as an ion channel; no other member of the superfamily is known to bear channel activity. The ion channel function of CFTR has been directly demonstrated for three distantly related vertebrates: human, mouse, and *Xenopus*. For all three species, heterologous expression of a cDNA encoding the CFTR gene product has led to the appearance of CFTR-like channels (with characteristic chloride selectivity, activation by PKA, etc.) (5–7).

Channels and transporters are thought to operate by entirely divergent mechanisms. True transporters, such as the glucose transporter or excitatory amino acid transporters, move substrates across membranes down their electrochemical gradients by a mechanism commonly called the “alternating access” mechanism (8, 9). The transporter begins in a conformation permissive to binding substrate on one side of the membrane, undergoes a conformational change within the membrane domain, at least, and occupies a conformation permissive to release of substrate on the opposite side of the membrane; on release of substrate, the transporter returns to the initial conformation (see Fig. 1). At no time in this cycle is the substrate translocation pathway open at both ends. In secondary-active transporters, such as the Na⁺-coupled glucose cotransporter, and primary-active transporters, such as the ubiquitous Na⁺/K⁺-ATPase, the addition of chemical energy allows the transporter to also move substrate up its electrochemical gradient. Clearly, a leak in the substrate transport pathway, by way of being open simultaneously at both ends, would not support active transport.

In contrast, ion channels must occupy two major conformational states: one where the substrate transport pathway is blocked at one or multiple sites—the closed channel state—and one where the pathway is open throughout, thus allowing electrodiffusion—the open channel state. The membrane transport field has long considered transporters and channels to be fundamentally different, although there is some overlap in the absolute rates of substrate movement; some fast transporters move substrates at rates very similar to some very low-conductance ion channels. Indeed, the CIC family includes both

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Table 1. Type I and type II sequence divergence between CFTR and ABCC4 domains

Type I and type II sequences					
Vertebrates					
Type I divergence					
Domain	Residues	θ	SE	z	P
TMD1	281	0.70	0.07	9.65	5.1e-22
ABC1	190	0.53	0.08	6.57	4.9e-11
TMD2	300	0.62	0.06	10.98	4.7e-28
ABC2	185	0.79	0.12	6.69	2.3e-11
Type II divergence					
Domain	Residues	θ	SE	z	P
TMD1	281	0.49	0.08	6.42	1.3e-10
ABC1	190	0.16	0.10	1.68	0.09
TMD2	300	0.41	0.08	5.14	2.8e-7
ABC2	185	0.60	0.08	7.25	4.1e-13
Mammals					
Type I divergence					
Domain	Residues	θ	SE	z	P
TMD1		0.64	0.11	5.83	5.6e-9
ABC1		0.82	0.15	5.45	5.1e-8
TMD2		0.56	0.08	7.32	2.4e-13
ABC2		0.51	0.17	3.03	2.4e-3
Type II divergence					
Domain	Residues	θ	SE	z	P
TMD1		0.57	0.05	12.47	1.1e-35
ABC1		0.42	0.05	7.73	1.1e-14
TMD2		0.46	0.05	9.17	4.6e-20
ABC2		0.57	0.06	10.40	2.6e-25

erties; accordingly, the approach we used to illuminate functionally relevant evolutionary changes between CFTR and other ABCCs relies on the identification of these so-called type II sites (16). It should be noted that this approach is fundamentally distinct from more straightforward techniques that use evolutionary conservation alone as a surrogate for functional relevance.

An alignment of 20 orthologous vertebrate CFTR proteins with 18 orthologous ABCC4 proteins (Fig. S4B) was broken down into the four domains: TMD1, ABC1, TMD2, and ABC2, and position-specific sequence differences within and between groups (CFTR vs. ABCC4) were evaluated for evidence of type I and type II divergence. All four domains show statistically significant type I divergence, and three of four domains show significant type II divergence (Table 1). Overall, the signal of type I divergence is stronger than that for type II divergence. This indicates that the so-called early phase of duplicate evolution continued to occur long after the initial duplication that gave rise to the two paralogous CFTR and ABCC4 groups, and suggests that site-specific evolutionary rate changes occur more frequently because of relaxation of selective constraint rather than by adaptive fixation of variants. Nevertheless, sites that have undergone type II divergence are more likely to encode group specific functional properties because they are clearly adaptive in one or both lineages.

To further evaluate the role of adaptive divergence in the functional evolution of CFTR, we confined the type II divergence analysis to mammalian species only (Table 1). All four domains show statistically significant type II divergence in mammals, and the significance of type II divergence for mammals is greater than that for all vertebrates across all four domains. This is the opposite of what is seen for type I divergence, the significance of which decreases across all four domains when only mammals are considered. Furthermore, the number of individual type II sites increased substantially, for

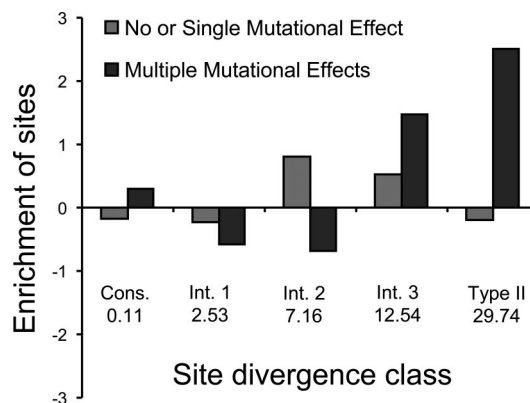


Fig. 2. Type II sequence divergence patterns compared with known mutational effects for the first transmembrane domain (TMD1) of CFTR. Analysis of conservation within and between paralogous groups (CFTR vs. ABCC4) yields five divergence bins ranging from sites that are conserved within and between groups to the type II divergence sites that are maximally divergent between groups and conserved within groups (see Table S3). The 5 bins are: Conserved, Intermediate Class 1, Intermediate Class 2, Intermediate Class 3, and Type II Divergent. The figure shows the observed (O) versus expected (E) proportions [calculated as $(O - E)/E$] of sites in each divergence bin with 0–1 known mutational effects (light gray bars) versus those with 2 or more known mutational effects (dark gray bars). Values beneath the bars are the average type II posterior probability per bin.

example, from 12 to 46 in TMD1, when mammals were considered alone. These data indicate that the CFTR and ABCC4 groups have continued to evolve functional refinements after mammals diverged from the other vertebrates. The increase of

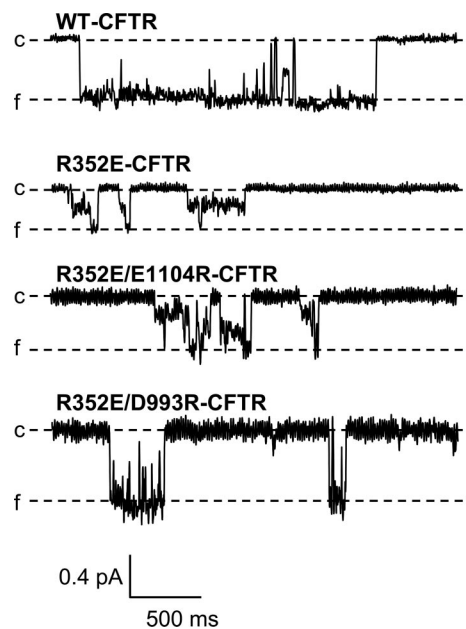


Fig. 3. Charge-destroying mutations at R352 alter CFTR single channel behavior. Isolated bursts of channel activity from oocytes expressing WT-CFTR, R352E-CFTR, R352E/E1104R-CFTR, and R352E/D993R-CFTR. Channels were studied in excised, inside-out patches in the continuous presence of 1 mM ATP and 50 U/ml PKA. In each trace, the closed level (c) is marked by a dashed line, and downward deflections represent channel opening. The full conductance level (f) for each trace is indicated. Channels bearing the R352E mutation, or the double mutant R352E/E1104R, exhibited frequent transitions to subconductance levels. In contrast, WT-CFTR channels, and channels bearing the revertant mutation R352E/D993R, primarily exhibit transitions to the full conductance level.

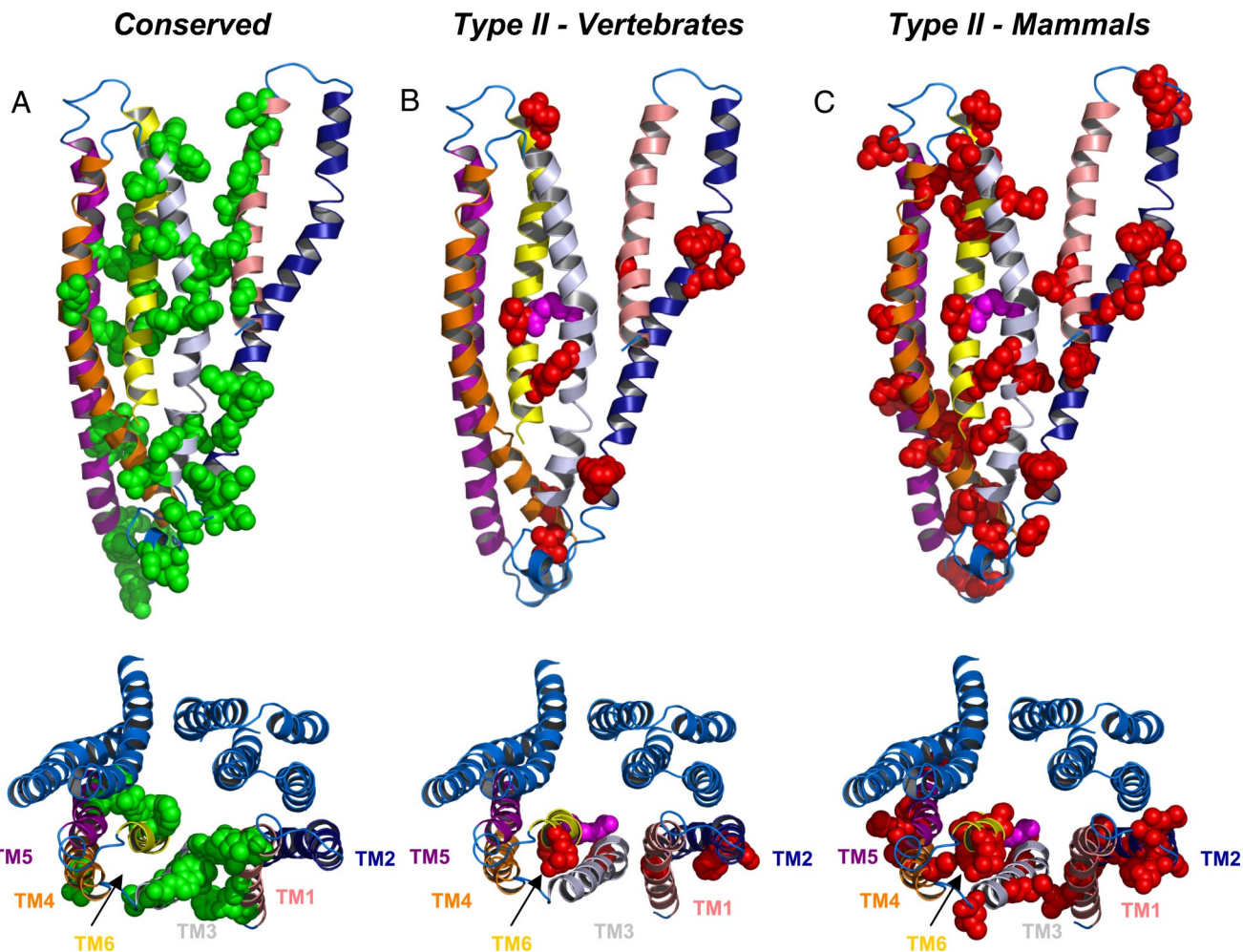


Fig. 4. Structural environment of conserved and type II divergent CFTR residues. Conserved and type II residues are highlighted on a homology model of the CFTR protein. Helices are colored as follows: TM1, salmon; TM2, dark blue; TM3, silver; TM4, orange; TM5, purple; and TM6, yellow. TMD2 is shown as blue helices. (A) TMD1 of the CFTR homology model with residues conserved between CFTR and ABCC4 shown in green space-filling representation. (B) TMD1 of CFTR with type II divergent residues across all vertebrates shown in red space-filling representation. (C) TMD1 of CFTR with type II divergent residues in mammals only shown in red space-filling representation. Lower images are the view from the extracellular face. R352 is shown in magenta.

type II and decrease of type I divergence among mammals, compared with all vertebrates, is consistent with the more prominent role of adaptive evolution in the late phase after duplication, as proposed by Gu (15).

The high type II divergence value (θ) for TMD1 was particularly interesting in light of experimental results, reported here and elsewhere (see Table S1), pointing to the functional relevance of specific TMD1 residues for ion channel activity. Across all vertebrates, there are 12 individual type II sites in TMD1 that are completely conserved within groups (CFTR vs. ABCC4) and radically different between them; these are the positions that are likely to contribute most substantially to the functional differences between groups. The characteristic amino acid residues and biochemical changes for each of these positions are shown in Table S2. We explored the functional relevance of these type II sites in TMD1 by comparing their identity to a list of sites with known mutational effects that was compiled from the CFTR literature (Table S1). To do this, individual sites were broken down into four divergence site classes based on the posterior probability value that indicates the likelihood of a site to contribute to type II divergence between groups (Table S3). For each of the four divergence site classes in TMD1, across all vertebrates, we calculated the observed versus expected proportion of residues in that class that had 0 or 1 mutational effects

and compared these with the class-specific proportions of residues that have multiple mutational effects (Fig. 2). Type II sites are the most substantially enriched class for residues that have multiple mutational effects, and intermediate class 3 sites, which are also highly conserved within and are radically different between groups (Table S3), are also highly enriched for residues that have multiple mutational effects. Residues with 0 or 1 mutational effects are most overrepresented in the intermediate class 2, which does not have a consistent pattern of within and between group variation. The enrichment distributions of the different mutational effect profiles are significantly different ($\chi^2 = 15.5$, $df = 4$, $P = 3.8e-3$). These patterns demonstrate the potential utility of type II sites for predicting functionally important CFTR sites. The predictive power of type II sites is underscored by the greater enrichment of sites with multiple mutational effects at type II versus absolutely conserved sites, which are typically used for functional predictions (Fig. 2). The functional relevance of type II sites is further supported by the finding that the number of known mutational effects per site is positively correlated with the average type II posterior probability, that is, the average type II divergence, per site ($r = 0.16$, $t = 2.6$, $df = 279$, $P = 8.8e-3$).

Example of a Divergent Site with Physiological Relevance. Within TMD1, one of the type II sites is R352 (L842 in ABCC4)

than the completely conserved sites, that we expect to be most relevant to ion channel specific function of CFTR. Type II sites and conserved sites are distributed throughout the protein (Movie S1 and Movie S2). Comparison of type II sites, identified by computational approaches, with specific experiments and structural features of CFTR further underscores their functional relevance; for example, mutation of R352, identified as a type II residue, led to destabilization of the open state critical to CFTR channel function. This approach may be useful in the design of experiments to identify the mechanisms by which CFTR, alone within the ABC superfamily, evolved to function as an ion channel.

Materials and Methods

CFTR Sequence Analysis. The CFTR protein sequence (RefSeq accession NP_000483.3) was used as a query in BLASTP (24) searches against the GenBank database (25) to identify 47 human paralogous members of the ABC transporter superfamily, along with CFTR and ABCC4 orthologs from a variety of vertebrate species including primates, more distantly related mammals, amphibians, birds, and fish. Information on all sequences analyzed in this study can be found in Table S4. Sequences were aligned by using the T-COFFEE program (26) and phylogenetic analysis was conducted by using the program MEGA (27). Protein domain architectures for sequences were characterized by using the Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>) (28).

CFTR Functional Divergence. The evolutionary determinants of the functional differences between CFTR and paralogous ABC transporter proteins were

evaluated by using the program DIVERGE (16). DIVERGE was used to identify sites of type II functional divergence, which occurs via changes in the biochemical properties of amino acids at specific positions between groups of related proteins. The rationale behind this approach to identification of sites of divergence, and the kinds of sites it identifies, is illustrated in Fig. S5. Type II functional divergence was evaluated by comparing 20 orthologous CFTR proteins to 18 ABCC4 orthologs (Fig. S4B). The strength of type II functional divergence was measured by the values of the parameter θ , where a θ value significantly greater than zero indicates functional divergence. For CFTR domains showing a statistically significant value of θ , position-specific amino acid differences that underlie the functional divergence of CFTR from paralogous ABC transporter proteins were determined.

Structural analysis of conserved and type II divergent residues was performed on the recently published CFTR homology model (17) obtained from <http://dokhlab.unc.edu/research/CFTR/>. The CFTR model was visualized by using Pymol software (<http://pymol.sourceforge.net/>). Specific residues are shown in space filling representation whereas backbone atoms are shown in diagram representation. All images were rendered in Pymol and resized for publication by using Adobe Photoshop v 8.0. Movie S1 and Movie S2 were created by using VideoMach software.

Functional Analysis. For single-channel recording, CFTR was expressed in *Xenopus* oocytes and studied in excised, inside-out patches at room temperature, as described (13, 29). Details are provided in the *SI Text*.

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