A Conserved Region of the R Domain of Cystic Fibrosis Transmembrane Conductance Regulator Is Important in Processing and Function*

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The R domain of cystic fibrosis transmembrane conductance regulator (CFTR) connects the two halves of the protein, each of which possesses a transmembrane-spanning domain and a nucleotide binding domain. Phosphorylation of serine residues, which reside mostly within the C-terminal two-thirds of the R domain, is required for nucleotide-dependent activation of CFTR chloride channel activity. The N terminus of the R domain is also likely to be important in CFTR function, since this region is highly conserved among CFTRs of different species and exhibits sequence similarity with the “linker region” of the related protein, P-glycoprotein. To date, however, the role of this region in CFTR channel function remains unknown. In this paper, we report the effects of five disease-causing mutations within the N terminus of the CFTR-R domain. All five mutants exhibit defective protein processing in mammalian HEK-293 cells, suggesting that they are mislocalized and fail to reach the cell surface. However, in the Xenopus oocyte, three mutants reached the plasma membrane. One of these mutants, L619S, exhibits no detectable function, whereas the other two, D614G and I618T, exhibit partial activity as chloride channels. Single channel analysis of these latter two mutants revealed that they possess defective rates of channel opening, consistent with the hypothesis that the N terminus of the R domain participates in ATP-dependent channel gating. These findings support recent structural models that include this region within extended boundaries of the first nucleotide binding domain.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ATP binding cassette (ABC) superfamily of membrane proteins, a family that includes several clinically important proteins such as P-glycoprotein, multidrug resistance-associated protein, and sulfonlurea receptor. The ABC family of membrane proteins, a family that includes several ABC transporters such as P-glycoprotein and multidrug resistance-associated protein (Fig. 1), whereas RD2 is unmatched in any ABC proteins (11, 12).

The R domain of CFTR was originally defined as amino acids 590–831, encoded by exon 13, which span the region between the C-terminal boundary of the first nucleotide binding fold and the second transmembrane domain (5). However, alignment of this entire sequence with CFTR from various species and with related ABC family members revealed that there may be two independent subdomains within this region (10). According to Dulhanty and Riordan (10), the N-terminal third of the R domain (RD1, i.e. residues 590–672) is highly conserved, whereas the large central region (RD2, i.e. residues 672–831) is not well conserved among different species. Further, the RD1 region corresponds to a homologous “linker” region in other ABC transporters such as P-glycoprotein and multidrug resistance-associated protein (Fig. 1), whereas RD2 is unmatched in any ABC proteins (11, 12).

Rich et al. (11) described RD2 as comprising the “functional R domain,” since it contains five or six of the sites phosphorylated by protein kinase A. Deletion of RD2 leads to partial channel activity of the unphosphorylated CFTR (13), supporting the hypothesis that phosphorylation of RD2 may cause a structural modification that is permissive to CFTR channel gating (14, 15). RD1, on the other hand, is unlikely to be significantly modified by phosphorylation. Of the two possible phosphorylation sites that reside in this region (Ser641 and Ser660), only Ser660 appears to contribute to channel activation by phosphorylation (16). Further, phosphorylation of the homologous “linker” region of P-glycoprotein does not appear to modify the transport function of this protein (17). Nevertheless, RD1 is likely to possess a critical role in CFTR maturation and/or function, since R domain deletions that extend to include this region lead to a total loss of channel activity (11). Significantly, several disease-causing mutations exist in RD1, highlighting the importance of this region.

The purpose of the present study was to study the role of RD1 in CFTR processing and channel function by examining the consequences of five disease-causing mutations in this region. We found that each of the variant proteins with mutations in RD1 exhibited defective processing in human embryonic kidney (HEK)-293 cells. Hence, it is probable that none, or only a fraction, of the mutant proteins are properly trafficked to the

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1 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; ABC, ATP binding cassette; HEK, human embryonic kidney; pS, picosiemens; NBD, nucleotide binding domain.

2 Cystic Fibrosis Consortium Genetic Database, coordinated by L.-C. Tsui, Hospital for Sick Children, Toronto.
cell surface, where they could contribute to measurements of chloride conductance across the surface of these mammalian cells. However, cell surface expression was detected by immunofluorescence in three of the RD1 mutants following expression in *Xenopus* oocytes, a more permissive expression system for CFTR processing mutants (19). One of these RD1 mutants, L619S, failed to exhibit chloride channel function in two-electrode voltage clamp studies and hence is likely to reside in a region critical for this activity. The two other mutants, D614G and I618T, exhibited partial function in two-electrode voltage clamp experiments and could be studied at the single channel level, wherein it was revealed that they exhibited altered rates of channel opening. These findings are consistent with a possible role for RD1 in modifying ATP-dependent opening of the CFTR chloride channel.

**MATERIALS AND METHODS**

*Site-directed Mutagenesis—*Mutations were introduced into CFTR cDNA in the Bluescript vector (pBQ6.2 version 2, kindly provided by Dr. J. M. Rommens) using the Transformer mutagenesis kit (CLONTECH, Mississauga, Canada) and specifically designed oligonucleotides. The smallest possible cassette containing the mutation, a *Bst*EI–HpaI fragment, was then subcloned into new vector pBQ6.2. This entire cassette was then sequenced to confirm the introduction of the specific mutation, was then subcloned into new vector pBQ6.2. This entire cassette was then sequenced to confirm chloride selectivity of the activated whole cell currents on the basis of the measured reversal potential. The magnitude of the cAMP-dependent currents was assessed continuously at a holding potential of −70 mV. The stimulatory effect of cAMP mixture on the whole cell currents was fully reversible. The difference in the whole cell currents before and after activation by the cAMP mixture was normalized to cyclic AMP-dependent currents recorded from oocytes from the same batch expressing wild type CFTR and reported as mean ± S.D. The unpaired Student’s *t* test was used to evaluate statistical differences between these groups.

The vitelline membrane on oocytes was removed in order to perform patch clamp studies of the single channel activity of wild type or variant CFTR. This membrane was manually detached using fine forceps after incubation of oocytes in hypertonic solution (standard oocyte solution plus 200 mM sucrose) for 10 min (24). After the removal of the vitelline membrane, the stripped oocytes were then placed in isotonic standard oocyte solutions. Single channel activities were recorded in the cell-attached configuration on oocytes stimulated with the cyclic AMP-activating mixture (same as in the two-electrode voltage clamp) for at least 5 min prior to the formation of the patch pipette seal. The pipette solution contained in mM 150 NaCl, 1.8 mM CaCl2, 1 mM MgCl2, 2 mM KCl, and 5 mM HEPES, pH 7.6. The bath solution was washed thoroughly with PBS and coverslipped using a mounting medium. Specimens were examined with an Olympus Vanox AHBT3 Reichert Polyvar epifluorescence microscope, and the images were captured using Imaging Pro/n (Media Cybernetics, Silver Spring, MD).

**Electrophysiological Studies in Xenopus Oocytes—CFTR channel activity was assessed 2 days after cRNA injection using the two-electrode voltage clamp technique (21). CFTR channel activity was stimulated by the addition of a cAMP activation mixture containing forskolin (10 μM), 3-isobutyl-1-methylxanthine (1 mM), and 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate (100 μM) to the bath perfusion solution. The isolation of a cRNA was performed in MOPS-buffered sucrose solution containing 100 mM NaCl, 1.8 mM CaCl2, 1 mM MgCl2, 2 mM KCl, and 5 mM HEPES, pH 7.6. The time course and magnitude of the cAMP-dependent currents was assessed continuously at a holding potential of −60 mV. In addition, current-voltage relationships were determined to confirm chloride selectivity of the activated whole cell current on the basis of the measured reversal potential. The stimulatory effect of cAMP mixture on the whole cell currents was fully reversible. The difference in the whole cell currents before and after activation by the cAMP mixture was normalized to cyclic AMP-dependent currents recorded from oocytes from the same batch expressing wild type CFTR and reported as mean ± S.D. The unpaired Student’s *t* test was used to evaluate statistical differences between these groups.

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Briefly, the open times for every single channel opening were sorted on were determined using methods described by Baukrowitz et al. (8). Open time constants for both wild type and mutant CFTR mutations, Inc.). Open time constants for both wild type and mutant CFTR were fit using TACFit program (Skalar Instruments, Inc.). For example, the probability of an event with the shortest defined \( t \) for an open state is 1. All data were fitted with either a single or double exponential function, yielding either one or two time constants, respectively. The same method was applied to analyze the single channel events for the closed time constants. Open and closed time duration histograms for wild type and mutant CFTR were fit using TACFit program (Skalar Instruments, Inc.). To obtain a sufficient number of events for mutant CFTR dwell time analysis, we pooled all of the open and closed times from multiple single channel recordings of CFTRD614G \((n = 6 \text{ patches})\) and CFTRI618T \((n = 12 \text{ patches})\).

**Disease-causing Mutations in the RD1 Region of CFTR Lead to Protein Misprocessing**—Western blot analysis of HEK-293 cells transiently transfected with CFTR-RD1 variants revealed that their processing is abnormal (Fig. 2). The mature, complex-glycosylated form of the CFTR protein that resides on the cell surface of T84 epithelial cells migrates on SDS gel as the high molecular mass species \((-170 \text{ kDa})\), commonly referred to as band C. As expected, the band C form of CFTR was detected by Western blot analysis of lysates from 293 cells transfected with cDNA encoding wild type CFTR. In addition, a broad, lower molecular weight band was detected. This broad band probably contains bands A \((135 \text{ kDa})\) and B \((150 \text{ kDa})\), thought to correspond to core glycosylated, immature forms of CFTR (2). While the lower molecular weight bands could be detected following Western blot analysis of HEK-293 cells transfected with the RD1 mutants, band C could not be detected. These results suggest that while the mutant proteins are being synthesized they are not properly processed in these mammalian cells and hence are unlikely to be effectively expressed at the cell surface.

**Cell Surface Expression of RD1 Mutants Following Expression in Xenopus Oocytes**—In an attempt to assay function of these mutant proteins, we expressed each mutant in Xenopus oocytes, since this system appears to be more permissive with respect to biosynthesis of CFTR mutant proteins (19). Wild type CFTR could be detected at the surface of Xenopus oocytes 3 days after injection of CFTR cRNA (Fig. 3c). This signal is specific for CFTR, since it is not detected in control oocytes injected with water rather than CFTR cRNA (Fig. 3a), nor was a signal detected if the anti-CFTR antibody was omitted (Fig. 3b).

Some of the RD1 mutants could be expressed at the surface of individual *Xenopus* oocytes. Immunofluorescence can be readily detected at the oocyte plasma membrane, 3 days after the injection of cRNA encoding CFTRD614G, CFTRI618T, and CFTRL610S and CFTRL619S (Fig. 3). On the other hand, the fluorescence signal was very weak in CFTRJ607–634 and CFTRL610S-injected oocytes. Hence, not all of the RD1 mutants appear to undergo similar processing and trafficking to the cell surface in *Xenopus* oocytes.

**Cyclic AMP-dependent Chloride Channel Activity Is Impaired for RD1 Mutants as Assessed in Xenopus Oocytes**—Using the two-electrode voltage clamp technique, we compared cyclic AMP-stimulated whole cell currents 2 days after oocyte injection of water (control), wild type, or mutant CFTR cRNA. Cyclic AMP-dependent currents were evoked by superfusion of the cAMP activating mixture described under “Materials and Methods.” As we previously reported (21), control (water-injected) oocytes do not exhibit cyclic AMP-stimulated whole cell currents \((n = 13 \text{ oocytes; data not shown})\). At 2 days after oocytes were injected with cRNA encoding wild type CFTR, we found that superfusion of a cyclic AMP-activating mixture led to large chloride currents \((1026 \pm 94 \text{ nA}, n = 33 \text{ oocytes, at } -140 \text{ mV})\). On the other hand, after the same expression period, oocytes injected with cRNA encoding the RD1 mutants CFTRJ607–634 and CFTRL610S failed to exhibit cAMP-activated chloride currents (Fig. 4). The lack of function of CFTRJ607–634 is probably related to its failure to be expressed at the cell surface (Fig. 3). On the other hand, CFTRL610S is expressed in the plasma membrane of *Xenopus* oocytes. Hence, the lack of functional expression suggests that mutation of the leucine in position 619 to serine severely impairs the channel activity of CFTR. Oocytes injected with CFTRL610S cRNA exhibited small cAMP-activated chloride currents \((about 10\% \text{ of the wild type currents, } p < 0.05)\), consistent with its very weak immunofluorescence signal at the
oocyte surface (Fig. 3). The mutants CFTRD614G and CFTRI618T were expressed on the oocyte plasma membrane and exhibited cAMP-activated currents that were about 75% and 50%, respectively, of the current measured in wild type CFTR (Figs. 3 and 4).

Single Channel Analysis Reveals Defects in Channel Opening by CFTR D614G and I618T—We know from the previous whole cell studies that the L619S mutation causes severe dysfunction of the CFTR channel activity because, despite expression of this mutant protein at the cell surface (Fig. 3), cyclic AMP-activated chloride currents were not detected (Fig. 4). The complete lack of measurable function for this mutant in the oocyte makes it impossible to determine by single channel analysis the molecular mechanisms underlying this defective activity. On the other hand, for the mutant proteins, Δ607–634 and L610S, insufficient protein reached the surface (Fig. 3) to perform single channel analysis. However, cAMP-dependent chloride currents conferred by expression of CFTRD614G and CFTRI618T (Fig. 3) could be analyzed at the single channel level and compared with those of wild type CFTR.

As described previously, 2 days after wild type CFTR, CFTRD614G, or CFTRI618T cRNA injection, the addition of a cAMP activation mixture leads to the stimulation of chloride currents in two-electrode voltage clamp studies (Fig. 4). Typically, those oocytes that express cAMP-activated currents of approximately 500–1000 nA (at the holding potential of −60 mV) exhibited clear single channel recordings in the cell-attached configuration. These single channel currents can be recognized as CFTR on the basis of their activation by cyclic AMP, unitary conductance of 7 pS, lack of voltage dependence, and linear I−V relationship (Figs. 5 and 6). CFTR channel activity can be readily distinguished from endogenous 12–20-pS chloride channels, since the endogenous chloride channels exhibit pronounced voltage dependence of gating and open only at depolarized potentials (data not shown).

Single and multiple CFTR channel openings were detected in cell-attached patches on cAMP-stimulated oocytes expressing the mutants CFTRD614G (n = 6 patches) and CFTRI618T (n = 12 patches) (Figs. 5 and 6). Single channel analysis revealed that the unitary conductances for the channel activity of CFTRD614G (7 ± 0.9 pS, n = 4 patches) and CFTRI618T (7 ± 0.6 pS, n = 4) were similar to wild type CFTR (7 ± 0.9 pS, n = 3). On the other hand, channel open probability of these mutant channels was abnormal. Whereas CFTR channel activity had an open probability (P_o) of 0.5, the CFTRD614G and CFTRI618T mutants exhibited a P_o of 0.29 and 0.33, respectively.

The reduced P_o of CFTRD614G and CFTRI618T mutants is probably due to a decreased rate of channel opening to a burst. It has been well documented that wild type CFTR in patches on mammalian cells stimulated with cyclic AMP-activating mixture exhibits a bursting pattern of channel activity (25, 26). Previous reports have documented that the CFTR channel opens to a burst, wherein channel openings within this burst are punctuated by sojourns to a short-lived closed state, and each burst is separated by a long closed state. Our analysis of dwell time histograms of single channel data obtained from CFTR expressed in Xenopus oocytes reveals that CFTR channel

![Image](https://example.com/image.png)
function in the Xenopus oocyte membrane also exhibits bursting kinetics (Fig. 6b). The open time histogram is fit with a single exponential with the open time constant ($\tau_1 = 0.19$ s and the closed time histogram fit with a double exponential function with two closed time constants: $\tau_1 = 0.024$ s and $\tau_2 = 0.120$ s, respectively. In the CFTRD614G and CFTRI618T mutants, the open time constants are prolonged to $\tau_1 = 0.430$ s and $\tau_2 = 0.330$ s, respectively, suggesting that the channel closing rate is altered by the mutations. In addition, while both CFTR mutants exhibit two closed time constants like the wild type protein, the longer of the two closed time constants is very prolonged in both cases, to $\tau_2 = 7.5$ s for CFTRD614G and $\tau_2 = 4.3$ s for CFTRI618T. The prolonged long closed times correlate to a remarkable slowing of the channel opening rate to a burst, i.e. from 8 openings/s for wild type CFTR to 0.1 openings for CFTRD614G and 0.2 openings/s for CFTRI618T.

**DISCUSSION**

The data shown in this report support the hypothesis that the N terminus of the R domain of CFTR participates in normal protein processing. Each of the disease-causing mutations in this region appears to cause a defect in protein processing in the mammalian HEK-293 cell expression system. Western blot analysis of HEK-293 cells expressing each R domain mutant revealed an electrophoretic mobility pattern that is common for all of the R domain mutants but is quite distinct from that observed for the wild type CFTR (Fig. 2). Band C, thought to represent the fully glycosylated form of the protein on the plasma membrane is not seen following expression of any of these RD1 mutants. For the most common CFTR processing mutant, CFTRAF508, protein misfolding is thought to underlie misprocessing (27). Therefore, while we lack direct proof, we speculate that the R domain mutant proteins may be misfolded during biosynthesis.

Our immunofluorescence and electrophysiological data support the suggestion that the Xenopus oocyte expression system is relatively permissive to CFTR processing mutants. Two RD1 mutants, CFTRL610S and CFTR3607–634, exhibit little or no channel function, respectively, and this is consistent with their low levels of surface immunofluorescence (Figs. 3 and 4). On the other hand, three of the CFTR R domain mutants are expressed at the plasma membrane of Xenopus oocyte, CFTRL619S, CFTRD614G, and CFTRI618T, where chloride channel function can be assessed. Mutation of the leucine residue at position 619 to serine has a profound inhibitory effect on channel activity, such that whole cell currents cannot be detected despite significant cell surface expression. In contrast, whole cell cyclic AMP-activated chloride currents can be detected in oocytes expressing CFTRD614G and CFTRI618T. Analysis of single channel currents conferred with these two latter variants suggests that the partially reduced whole cell currents reflect a defect in the rate of CFTR channel opening (Fig. 6). Hence, a region of RD1 encompassing residues 614, 618, and 619 fulfills an important role in the regulation of CFTR chloride channel activity.

Recently, we and others have speculated that the first nucleotide binding fold, possibly in cooperation with the second nucleotide binding fold, mediates CFTR channel opening (7, 9, 28). Since this function is impaired in disease-causing mutants in RD1, we suggest that this region is an important determinant of nucleotide affinity with the first nucleotide binding fold. Further, we have proposed that PKA phosphorylation of the R domain modulates the affinity of one or both nucleotide binding folds for nucleotides (28). Hence, the mutations studied at the single channel level in this report, CFTRD614G and CFTRI618T, may exhibit slow rates of channel opening because nucleotide affinity and/or phosphorylation has been impaired. This interpretation is compatible with other recent studies that employed recombinant R domain peptide and suggested that the regions within the R domain may mediate channel opening by facilitating the interaction of the nucleotide binding domains (NBDs) with ATP (15).

These functional data are also consistent with several recent structural predictions regarding the N terminus of the CFTR-R domain. As previously mentioned, the N terminus is highly conserved, emphasizing its probable significance for protein function. The aspartic acid residue at position 614 and hydrophobic residues at positions 610, 618, and 619 are conserved among different species of CFTR (Fig. 1). Three recent models of the first NBD of CFTR have extended the C terminus of the...
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NBD1 to include amino acid residues initially defined as part of the N terminus of the CFTR R domain (12, 29, 30). According to a model proposed by Hoedemaeker et al. (12), the first NBD forms a classical Rossmann fold, and RD1 may actually comprise part of the central β-sheet of this fold. The two β-strands formed by residues in RD1 are predicted to lie in close proximity to the Walker A or “P loop,” a structure that is thought to be critical for ATP binding and/or hydrolysis in proteins with similar function (5, 12, 31, 32). Therefore, our electrophysiological data showing a defect in channel opening for mutations in RD1 are consistent with this and similar structural models that place this region close to the nucleotide binding cleft of NBD1.

The five mutations studied in this paper lead to varying degrees of disease severity. Using the extent of pancreatic function as a benchmark for disease outcome, it has been reported that three mutations (CFTRΔ607–634, CFTRL619S, and CFTRI618T) are associated with pancreatic insufficiency and one mutation with (CFTRD614G) pancreatic sufficiency or mild disease. Unfortunately, the clinical status of the patient(s) with the mutation CFTRL619S is unknown. Our analysis of protein processing in HEK-293 cells suggests that all of these mutants are misprocessed. Hence, it is not possible to easily explain the above variation in patient phenotype on the basis of differences in protein folding or trafficking. On the other hand, the patient phenotypes appear to be more closely correlated with chloride channel activity of a given mutant as determined in the Xenopus oocyte system. For example, no channel function could be detected for the pancreatic insufficiency mutant, CFTRL619S, and partial channel function was detected for the pancreatic sufficiency mutant, CFTRD614G (Fig. 4). However, the relative activity of each mutant protein will impact on disease severity only if a proportion of this protein is expressed at the cell surface, and we have no evidence to support this possibility at present. Alternatively, because so few patients have been studied, it is possible that a secondary gene may be acting to modify disease severity in those individuals with the mutations studied in NBD1 because it forms part of this domain that do not involve phosphorylation sites. Instead, all mutations of the R domain in a region that is conserved among CFTR of particular mutations in NBD1 because it forms part of this domain as suggested in recent models of NBD1 (12, 29, 30).

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Note Added in Proof—Recently another report has been published (Vanekerberghen, A., Wei, L., Jaspers, M., Cassiman, J. J., Nilius, B., and Cuppens, H. (1998) Hum. Mol. Genet. 7, 1761–1769) that describes the importance of the N terminus of the R domain of CFTR on CFTR processing and function.

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