Disease-associated Mutations in the Fourth Cytoplasmic Loop of Cystic Fibrosis Transmembrane Conductance Regulator Compromise Biosynthetic Processing and Chloride Channel Activity*

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Fabian S. Seibert, Paul Linsdell§, Tip W. Loot, John W. Hanrahan§, David M. Clarke¶, and John R. Riordan**‡‡

From the 1Medical Research Council Group in Membrane Biology, Departments of Medicine and Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8; 2Department of Physiology, McGill University, Montreal, Quebec, Canada H3G 1Y6; and the **Department of Biochemistry and Molecular Biology, Mayo Foundation and 3S. J. Johnson Medical Research Center, Mayo Clinic Scottsdale, Scottsdale, Arizona 85259

A cluster of 18 point mutations in exon 17b of the cystic fibrosis transmembrane conductance regulator (CFTR) gene has been detected in patients with cystic fibrosis. These mutations cause single amino acid substitutions in the most C-terminal cytoplasmic loop (CL4, residues 1035–1102) of the CFTR chloride channel. Heterologous expression of the mutants showed that 12 produced only core-glycosylated CFTR, which was retained in the endoplasmic reticulum; the other six mutants matured and reached the cell surface. Some cases of substitution of one member of pairs of adjacent residues resulted in misprocessing, whereas the others did not. Thus, the secondary structure of CL4 may contribute crucially to the proper folding of the entire CFTR molecule. Cyclic AMP-stimulated iodide efflux was not detected from cells expressing the misprocessed variants but was from the other six, indicating that their mutations cause relatively subtle channel defects. Consistent with this, these latter mutations generally are present in patients who are pancreatic-sufficient, while the processing mutants are mostly from patients who are pancreatic-insufficient. Single-channel patch-clamp analysis demonstrated that the processed mutants had the same ohmic conductance as wild-type CFTR, but a lower open probability, generally due to an increase in channel mean closed time and a reduction in mean open time. This suggests that mutations in CL4 do not affect pore properties of CFTR, but disrupt the mechanism of channel gating.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel located in the apical membrane of many epithelia, where it plays a key role in the regulation of salt and water homeostasis (Welsh et al., 1992). CFTR was predicted to form a transmembrane pore with regulatory segments protruding into the cell cytoplasm (Riordan et al., 1989); several features of this model have been confirmed experimentally (Hanrahan et al., 1995). The channel consists of two structurally similar halves, each containing six transmembrane segments (TMs) and a nucleotide binding fold (NBF) with which ATP interacts. The two halves are connected by the R-domain, rich in consensus sequences for phosphorylation by several kinases, including cyclic AMP-dependent protein kinase and protein kinase C. On the cytoplasmically exposed side of the protein the TMs are linked to each other by cytoplasmic loops (CLs), which vary between 55 and 65 amino acids in length. There is evidence that the concerted action of ATP binding and ATP hydrolysis at the NBFs (Anderson et al., 1991; Hwang et al., 1994) and of phosphorylation at the R-domain (Cheng et al., 1991; Chang et al., 1993) is necessary to allow channel activation, however, little information is available regarding the importance of the CLs in CFTR function. Only recently, a report by Xie et al. (1995), which studied the effect of a 19-amino acid deletion in CL2 (residues 242–307), suggested that this region is involved in stabilizing the full conductance state of the CFTR chloride channel.

The CLs are highly conserved between CFTRs expressed in different species (Diamond et al., 1991) and moderately conserved in comparison to the CLs of other ABC transporters (Manavalan et al., 1993). Their possible significance is highlighted by the fact that 20% of all identified CF-associated mutations lie within CLs. Thus far in CF patients, 18 different point mutations have been described in exon 17b, which approximately corresponds to CL4 (amino acids 1035–1142); however, as yet the mechanistic impact of alterations in this segment of CFTR on its function and biosynthetic processing has not been investigated. The detailed analysis of amino acid substitutions in other CFTR domains has shown that the CF phenotype usually is attributable to either a lack of the channel at the cell surface or the production of a channel with impaired function (Welsh and Smith, 1993). The failure of many mutant forms of CFTR, including the most frequent ΔF508 variant, to be biosynthetically processed beyond the core-glycosylated form present in the endoplasmic reticulum (ER), has become a hallmark of CFTR (Cheng et al., 1990) and the focus of intense endoplasmic reticulum; NBF, nucleotide binding fold; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane helix; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.
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**Materials and Methods**

Construction of CL4 Mutants—The CFTR coding sequence, including nucleotides −72 to 4721 of the complete cDNA, was inserted into the expression vector pcDNA3 (Invitrogen) using the HindIII and Apal sites of the multicloning site. To facilitate rapid segment replacement, a silent mutation was created at position 3471. The resulting BstEII restriction site and the constitutive Apal restriction site allowed replacement of the intervening sequence by different polymerase chain reaction fragments that encoded 18 naturally occurring CF mutations in CL4. To allow efficient stable transfection, some of the mutations were cloned into pNUT-CFTR (Tabcharani et al., 1991), utilizing both endogenous DraI restriction sites of CFTR. Polymerase chain reaction was performed as described by Higuchi (1990). Sequences of the polymerase chain reaction fragments generated were verified after insertion into the vector using the T7 sequencing kit (Pharmacia Biotech Inc.).

Expression of CL4 Mutants—HEK-293, COS-1, and CHO (Chinese hamster ovary) cells were grown at 37°C in 5% CO2 in Dulbecco’s Modified Eagles medium containing 10% calf serum. Cells were transfected at a subconfluent stage with 2 μg/ml of the various vector constructs using the calcium phosphate precipitation method described by Chen and Okayama (1987). pcDNA-transfected HEK-293 and COS-1 cells were maintained for 48 h prior to further analysis. In the case of pNUT-transfected CHO cells, 50 μM methotrexate was added to the medium 24 h post-transfection, and individual colonies were selected as described (Chang et al., 1993).

Protein Detection—Cells were lysed in 1% sodium dodecyl sulfate (SDS) containing protease inhibitors (10 μg E-64, 12 μg/ml leupeptin, 100 units/ml aprotinin (ICN), 50 μg/ml AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride), 25 μg/ml benzamidine). The lysate was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting as described (Chang et al., 1993).

Endoglycosidase H digestion was carried out as described previously (Loo and Clarke, 1994).

Iodide Efflux Studies—Forty-eight hours post-transfection, COS-1 cells were loaded with NaI, and efflux was monitored as described previously (Tabcharani et al., 1991).

Patch-clamp Studies of CFTR-expressing CHO Cells—Recordings of single CFTR channels in inside out membrane patches excised from CHO cells were carried out as described previously (Tabcharani et al., 1991). CHO cells stably expressing wild-type or mutant CFTR were plated at low density on glass coverslips and cultured at 37°C in 5% CO2 for 2–5 days before use. Channels were activated following patch formation as described (Gray et al., 1995) and chloride channel activity of CFTR.

Measurements of channel open probability were made from recordings lasting 5–13 min at a membrane potential of −30 mV, as described previously (Tabcharani et al., 1991). To ensure a reliable estimate of the number of channels in the patch, 1 nA current was measured, and the ratio of the complex-glycosylated to core-glycosylated form of CFTR was calculated for patches containing more than one CFTR channel using the following equations:

\[ t_m = \frac{(N \cdot P_b \cdot t)}{n} \]  
\[ t_c = \left( \frac{(N - N \cdot P_b \cdot t)}{(n - 1)} \right) \]

where \( t_m \) and \( t_c \) are the mean burst duration and the mean closed duration between bursts of openings (interburst duration), \( N \) is the number of channels in the patch (estimated as described above), \( P_b \) is the open probability, \( t \) is the total length of the recording, and \( n \) is the number of identifiable bursts of openings. Bursts were defined as groups of openings separated by closings briefer than 10 ms. Although this method assumes only a single open state and a single interburst state, it has been used successfully to analyze CFTR kinetics from multichannel patches (Gray et al., 1988; Mathews et al., 1995) and is of some use in identifying gross kinetic differences between different channel variants.

All other details of data acquisition and analysis are as described previously (Tabcharani et al., 1991, 1993).

**Results**

Identification of Processing Mutants in CL4 of CFTR—Eighteen different point mutations have been reported in CL4 in DNA from CF patients (Fig. 1), but no information is available on their effects on the protein which give rise to the disease symptoms. Because the CFTR molecule is known to be extremely susceptible to the ER quality control system (Cheng et al., 1990; Lukacs et al., 1994), we first examined the capacity of each of the mutants to exit from that compartment and to acquire complex N-linked oligosaccharide chains in the Golgi apparatus. This can be readily assessed from mobility shifts in SDS-PAGE (Pind et al., 1994). Immunoblot analysis of wild-type CFTR and the CL4 mutants expressed transiently in HEK-293 cells are presented in Fig. 2A. For 12 CL4 mutants, only the core-glycosylated form of CFTR (band B) could be detected, indicating that these proteins were improperly processed. The mature forms of the other six mutants (F1052V, K1060T, A1067T, G1069R, R1070W, R1070Q) were produced in relatively normal amounts (band C), although for A1067T and R1070W CFTR the ratio of the complex-glycosylated to core-glycosylated bands was significantly lower than for wild-type CFTR. The same results were obtained when the mutants were transiently expressed in COS-1 cells and screened by Western blotting (data not shown).

To further confirm that the misprocessed CFTR mutants were core-glycosylated molecules, the protein samples were digested with endoglycosidase H prior to detection by Western blotting. All 12 processing mutants were endoglycosidase H-sensitive (Fig. 2B), suggesting that they were indeed retained in the ER.

Anion Permeability of Cells Expressing CL4 Mutants—Although the above observations suggested strongly that arrested processing and mislocalization was the primary cause of dysfunction in 12 of the CL4 mutants, it was possible that very low amounts of these reach the cell surface and provide some anion conductance. Similarly, it might be suspected that the other six which matured and reached the cell surface may be

![Fig. 1. Simplified representation of CF-associated point mutations identified in CL4.](http://www.jbc.org/)

**Fig. 1.** Simplified representation of CF-associated point mutations identified in CL4. CFTR was drawn according to the model proposed by Riordan et al. (1989). The enlarged CL4 corresponds to amino acids 1035–1102. Mutated residues are depicted as open circles. Each mutation is designated as the original residue (first letter), its location (number), and the amino acid to which it was changed (second letter). NBF, nucleotide binding fold; R, regulatory domain; TM, transmembrane sequence; CL, cytoplasmic loop.
defective in their chloride channel activity. To assess these possibilities, iodide efflux assays were used as a measure of the cyclic AMP-activated anion channel activity of CFTR. COS-1 cells were employed because of their better adherence to culture wells than HEK-293 cells. All six processed CFTR mutants were employed because of their better adherence to culture wells than HEK-293 cells. All six processed CFTR mutants could be activated by forskolin to various degrees (Figs. 3, A and B). To estimate the amount of CFTR protein present in each cell batch used in these experiments, the cells were lysed with equal volumes of 1% SDS and subsequently equal amounts of cell lysate were analyzed by Western blotting (Fig. 3E). This showed that the level of activity for cells expressing each CFTR mutant did approximately correspond to the amount of mature protein in the sample, i.e. in COS-1 cells, F1052V, K1060T, and G1069R produced efflux levels similar to wild-type CFTR in accordance with a level of protein expression similar to wild type. A1067T, R1070W, and R1070Q CFTR had significantly lower efflux levels and significantly lower protein expression than wild-type CFTR in COS-1 cells. Hence, none of the processed mutants were grossly defective in their ability to form CAMP-activated chloride channels. The same flux assay was performed with the 12 CL4 mutants that caused misprocessing of the protein. There was little or no response to forskolin in COS-1 cells expressing these mutants (Figs. 3, C and D), consistent with their lack of mature CFTR.

Patch-clamp Analysis of CL4 Mutants—Since the six processed mutants were detected in CF patients, some alteration in the normal function of CFTR might be expected. In order to identify more subtle changes in their activity, the mutant channels were stably expressed in CHO cells and analyzed by the single-channel patch-clamp technique. Other disease-causing CFTR mutants, which are appropriately processed and trafficked to the plasma membrane, show defective ion conductive properties (e.g. R334W, R347H, and R347P; Sheppard et al., 1993; Tabcharani et al., 1993) or defective regulation of channel activity (e.g. G551S, G1244E, S1255P, and G1349D; Anderson and Welsh, 1992). The conduction and gating properties of those CL4 mutants that matured correctly were similarly analyzed using single-channel patch-clamp recording. Stable expression of each of these CFTR variants in CHO cells led to the appearance of cyclic AMP-dependent protein kinase- and ATP-dependent channel activity in excised membrane patches (Fig. 4A). The unitary conductance of each of these channels, measured with symmetrical Cl− concentrations of 154 mM, were identical to that of wild type CFTR (−7.6 pS; Fig. 4, B and C), suggesting that these CL4 mutations have no effect on channel conductance. However, as is suggested by Fig. 4A, different CL4 mutations had distinct effects on channel gating. Mean channel open probability for each mutant was significantly reduced compared to wild type (Fig. 5A). As shown in Fig. 5, B and C, in some mutants this reduction in open probability was associated with a significant reduction in mean burst duration (F1052V, G1069R) or an increase in mean interburst duration (R1070W). In other cases, there were smaller changes in both of these parameters. These data suggest that residues in CL4 affect both the rate of channel opening and the stability of the open state and that mutations in this region may thereby lead to CF by causing a reduction in channel activity.

DISCUSSION

Current models of CFTR suggest that the molecule utilizes three major cytoplasmic domains (NBF-1, NBF-2, and the R-domain) in the regulation of its chloride channel activity (Hanrahan et al., 1996). An additional set of cytoplasmic domains is constituted by four fairly large CLs, connecting the TMs on the cytoplasmic side of the protein; little is known about their function. It would not be surprising if these loops also were involved in regulation of channel activity due to their position close to the putative pore-forming TM segments and their potential proximity to the major regulatory domains. Such a general view is consistent with the recent findings of Xie et al. (1995) who concluded from studies of a 19-residue deletion variant (amino acids 266–284) that CL2 is required to stabilize the full conductance state of the CFTR chloride channel. For the investigation presented here, a different strategy was applied to study CLs. Eighteen CF-associated point mutations in CL4 were reconstructed to determine which cause CF, because the channel cannot reach the plasma membrane, and which cause disease due to defective functioning at the cell surface.

Previous studies have shown that many mutations in CFTR, including the most frequent ΔF508 deletion, prevent biosynthetic maturation (Gregory et al., 1991). We find that this is also true of several mutations in CL4, in that 12 of the 18 resulted in misprocessing of the protein and its retention in the ER. Thus, for patients that carry these mutations, the CF phenotype most likely is a result of lack of channel expression at the cell surface. In the case of previously studied CFTR mutations known to result in ER retention, including ΔF508, it is believed that local misfolding prevents the attainment of the global cytoplasmic conformation of CFTR that is required for
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maturation and ER to Golgi transport. CL4 would be expected to contribute to this overall tertiary structure of the cytoplasmic aspect of the molecule on the ER surface. Disturbance of the local secondary structure of CL4 due to point mutations may prevent it from adequately making this contribution. It is not obvious why amino acid substitutions at 9 of the 14 different positions may compromise secondary structure, whereas those at the remaining 5 positions appear not to (see Table I). However, all of the substitutions resulting in misprocessing involve either charge changes or the introduction of a proline. Alterations at all of the positions in the C-terminal half of CL4 result in misprocessing, but only half of those in the N-terminal portion do so. Mutations at adjacent residues in that portion may either prevent maturation or not, viz. 1060 and 1061; 1066 and 1067; 1070 and 1071; 1075 and 1076.

Fig. 3. cAMP-stimulated I\(-\) efflux from COS-1 cells expressing CL4 variants. Wild-type, control (vector only), and CL4 variants of CFTR were transiently expressed in COS-1 cells for 48 h. Cells were incubated for 1 h in loading buffer containing 136 mM sodium iodide. Subsequently, the cells were washed with efflux buffer without sodium iodide. From time 0 onward, the efflux buffer contained 10 \(\mu\)M forskolin. The stimulated iodide effluaxes were determined with an iodide-sensitive electrode. A and B, iodide effluaxes of cells expressing mutants which allowed maturation of CFTR. C and D, iodide effluaxes of cells expressing mutants that inhibited processing of CFTR. E, immediately after the flux experiment, cells from A and B were lysed with an equal volume of 1% SDS plus protease inhibitors and an equal amount of cell lysate was analyzed by Western blotting (5% SDS-PAGE, M3A7 as primary antibody). These data are from representative experiments, each of which was repeated at least three times. Symbols are as follows: A: ○, WT; □, F1052V; △, K1060T; ■, A1067T; □, vector only control. B: ○, WT; ○, G1069R; □, R1070Q; △, R1070W; □, control. C: ○, WT; □, G1066R; ○, H1054D; ■, L1065P; □, control; △, G1061R; □, R1066H; □, R1066L. D: ○, WT; ○, Q1071P; □, W1098R; △, H1085R; □, M1101K; ■, M1101R; □, control; □, L1077P.
and 1067; 1070 and 1071. Different substitutions at the same residue always produced the same effect, i.e. R1066C, R1066H, and R1066L, as well as M1101K and M1101R all inhibited maturation, whereas R1070W and R1070Q were both normally processed. The impacts of the mutations on CFTR maturation are compared with the reported pancreatic status of the patients in which they were detected in Table I. For 8 of 11 mutations resulting in misprocessing and failure of maturation, the patients were pancreatic-insufficient and for two of three mutations allowing processing the patients were pancreatic-sufficient. This is consistent with the possibility that biosynthetic arrest of CFTR in the ER may cause severe disease, whereas some point mutations, compatible with transport of the protein to the cell surface, may result in less severe disease.

Those amino acid substitutions that do not interfere with maturation have relatively minor effects on channel activity. Bulk measurements of cyclic AMP-stimulated iodide efflux from entire populations of cells expressing these variants showed that they did not differ drastically from wild type. Patch-clamp analysis demonstrated that the CFTR channel in all six processed mutants had similar current-voltage relationships and conductances as the wild-type channel, indicating that once the channel was open, chloride permeation was not altered. This suggests that despite their close proximity to the putative pore entrance, these residues do not contribute to interactions between the channel and the permeant anion. However, all of these mutant channels had decreased open probability. Their residual partial activity correlates well with

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**Fig. 4.** Single channel activity associated with processed CL4 mutants. A, examples of wild-type, F1052V, K1060T, A1067T, G1069R, R1070Q, and R1070W CFTR single channel currents recorded from inside-out membrane patches at a membrane potential of −30 mV. In each case, the closed state is indicated by a horizontal line on the left. B, mean single-channel current-voltage relationships for wild type (○) and an example of a CL4 mutant (●, G1069R). Each point represents the mean of data from 3 to 12 patches. No error bars are shown, since in each case the S.E. was smaller than the size of the symbol. C, mean single-channel conductance of those CL4 mutants studied, showing mean data from 11 patches for wild-type and 4–10 patches for different mutants. In each case, error bars represent one S.E.
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Comparison of CFTR sequences among species shows that 13 of the 14 residues investigated in this study are 100% conserved (Diamond et al., 1991), consistent with the hypothesis that these amino acids are important to the proper folding and function of CFTR. Only Gly\(^{1069}\) is replaced by different residues in other species. Interestingly in murine CFTR, Gly\(^{1069}\) is replaced by Arg, a human mutation that we found to leave biosynthetic processing unaffected but to reduce channel open probability. Alignment of CFTR with a number of other traffic ATPases (Manavalan et al., 1993) indicates that Arg\(^{1066}\) is the most conserved of the 14 residues studied in CL4. All three of the mutations reported at this position result in failure of maturation. The conservation of this residue may thus be a result of its importance for the proper biosynthetic processing of these proteins.

In summary, it was found that 12 of the mutations identified in CL4 result in the CF phenotype due to misprocessing of CFTR, thus showing that point mutations in CL4 can perturb the overall structure of the molecule sufficiently to mark it for retention in the ER. Six mutations allowed the protein to mature, and in these, the CF phenotype is likely a consequence of the decreased channel open probability detected. The decrease in open probability could generally be attributed to an increase in the channel mean closed time and a decrease in the mean open time. This result together with the unaltered conductance suggests that mutations in CL4 do not affect pore properties of CFTR, but rather the mechanism of channel gating. Finally, these data provide the first functional evidence that this set of mutations probably does cause disease.

**Fig. 5. Mean kinetic properties of single CL4 channels.** Open probability, burst duration, and interburst duration were all measured as described under “Materials and Methods.” In each case, data are the mean ± S.E. from 12 patches for wild-type and four to five patches for each mutant. * represents a significant difference from wild-type (p < 0.05, two-tailed t test).

**Table I**

| Mutation     | Biosynthetic processing | Pancreatic status | Ref.          |
|--------------|-------------------------|-------------------|---------------|
| F1052V       | +                       | —                 | Mercier et al., 1993 |
| H1054D       | —                       | PS                | Ferec et al., 1993 |
| K1060T       | +                       | PS                | Casals et al., 1995 |
| G1061R       | —                       | PI                | Mercier et al., 1993 |
| L1065P       | —                       | PI                | Ghanem et al., 1994 |
| R1066C       | —                       | —                 | Fanan et al., 1992 |
| R1066H       | —                       | PI                | Ferec et al., 1992 |
| R1066L       | —                       | PI                | Mercier et al., 1993 |
| A1067T       | +                       | PI                | Ferec et al., 1992 |
| G1069R       | —                       | —                 | Savov et al., 1994 |
| R1070W       | +                       | PS                | Casals et al., 1995 |
| R1070Q       | +                       | PS/PI             | Mercier et al., 1993 |
| Q1071P       | —                       | PI                | Ghanem et al., 1994 |
| L1077P       | —                       | PS                | Bozon et al., 1994 |
| H1085R       | —                       | PS                | Mercier et al., 1993 |
| W1098R       | —                       | PI                | Zielenski et al., 1995 |
| M1101K       | —                       | PI                | Zielenski et al., 1993 |
| M1101R       | —                       | PI                | Mercier et al., 1993 |

* Not reported. PS/PI for R1070Q because of five patients analyzed, one was PS, one PI and three unspecified.

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