Disease-Associated Mutations in Cytoplasmic Loops 1 and 2 of Cystic Fibrosis Transmembrane Conductance Regulator Impede Processing or Opening of the Channel†

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ABSTRACT: Since little is known about the contribution to function of the N-terminal cytoplasmic loops (CL1, residues 139−194; CL2, residues 242−307) of cystic fibrosis transmembrane conductance regulator (CFTR), all nine point mutations identified in CLs 1 and 2 from patients with cystic fibrosis were reconstructed in the expression vector pcDNA3-CFTR and expressed transiently in COS-1 and HEK-293 cells and stably in Chinese hamster ovary (CHO) cells. Four amino acid substitutions retarded production of mature, fully glycosylated CFTR, suggesting that misprocessing of the channel causes the disease symptoms in the affected patients. Protein maturation could not be promoted by cell culture conditions of reduced temperature (26 °C). When properly processed mutants were evaluated for functional defects by the iodide efflux method, the G178R- and E193K-CFTR-expressing cell lines showed impaired anion translocation activities. Patch-clamp studies of single channels revealed that E193K variants had a significantly decreased open probability, which resulted from an increase in the mean closed time of the channels. This contrasted with a previous study of disease-associated point mutations in CL3 that mainly affected the mean open time. None of the maturation-competent CL 1 and 2 mutants had altered conductance. Thus, the N-terminal CLs appear not to contribute to the anion translocation pathway of CFTR; rather, mutations in CL1 can impede transition to the open state. Interestingly, the ability of the non-hydrolyzable ATP analogue adenylyl imidodiphosphate (AMP-PNP) to lock the channel into open bursts was abolished by the I148T and G178R amino acid substitutions.

The cystic fibrosis transmembrane conductance regulator (CFTR)[1] is a distinctive chloride channel that utilizes the structural motif of transporters to regulate anion movement across a transmembrane pore (*1*). An understanding of the structure−function relationships of this molecule is thus of interest not only to basic sciences but also to medical research since mutations in the gene coding for CFTR result in cystic fibrosis (CF) (*2*). CFTR was predicted and largely confirmed to be composed of two transmembrane domains, each consisting of six transmembrane helixes (TMs) (*1*, *3*). The C-terminus of each membrane-integrated domain ends in a cytoplasmic nucleotide binding fold (NBF) containing Walker A and Walker B motifs for interactions with ATP (*4*). Linking the two halves is the cytoplasmic R-domain, rich in consensus sequences for potential phosphorylation by the cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). Functional studies have indicated that hormonally induced phosphorylation by PKA puts CFTR into an activation-competent form and is required to allow gating of the channel through ATP binding/hydrolysis at the NBFs (*5*−*16*). Constitutive phosphorylation by PKC is necessary in order for CFTR to respond to PKA (*17*). Recent studies showed that the four cytoplasmic loops (CLs) that connect the TMs on the intracellular side of the pore-forming unit (Figure 1) also play a critical role in channel function as well as structure (*18*−*22*).

The CLs are highly conserved (*23*) and are predicted to range from 55 to 65 amino acids in length (*3*). Their significance was demonstrated by the frequent occurrence of CF-associated mutations in these segments (CF Genetic Analysis Consortium, personal communication). Recent reconstruction of CF-associated point mutations in the C-terminal CLs 3 and 4 showed that amino acid substitutions in CL3 (predicted residues 933−990) significantly modify the mean open time of CFTR channels (*21*), whereas

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[1] Abbreviations: AMP-PNP5′-adenylyl imidodiphosphate; ATP adenosine triphosphate; CF cystic fibrosis; CFTR cystic fibrosis transmembrane conductance regulator; CHO Chinese hamster ovary; CL cytoplasmic loop; ER endoplasmic reticulum; *I*−*V* current−voltage; NBF nucleotide binding fold; P_o open state probability; PAGE polyacrylamide gel electrophoresis; PKA cyclic AMP-dependent protein kinase; PKC protein kinase C; TM, transmembrane helix.

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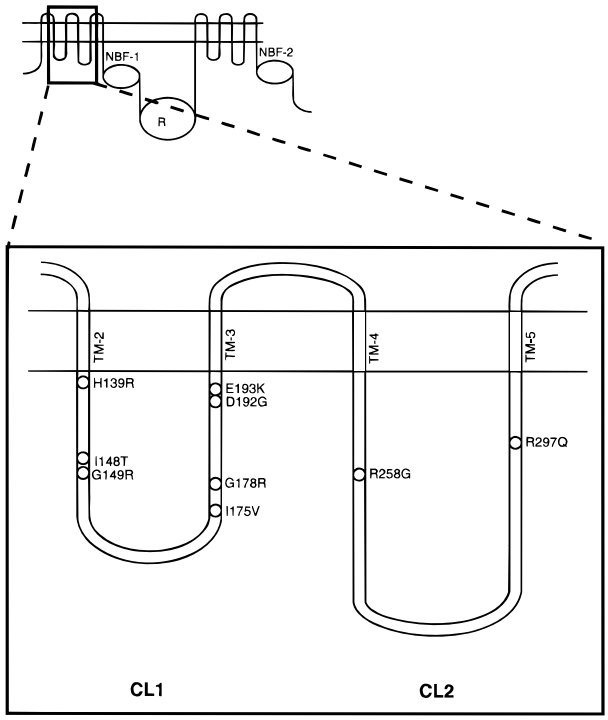

FIGURE 1: Schematic representation of CL 1 and 2 mutations. The model is based on the predictions of Riordan et al. (*3*). CL1, predicted to correspond to amino acids 139−194, and CL2, predicted to correspond to amino acids 242−307, are enlarged to depict the CF-associated point mutations identified within these regions of the protein. For each mutation the first letter corresponds to the original residue, the number gives the location within the primary sequence of CFTR, and the second letter describes the residue that results from the mutation. Original publications reporting those mutations in CF patients are listed in Table 1. CL, cytoplasmic loop; NBF, nucleotide binding fold; R, R-domain; TM, transmembrane helix.

alterations in CL4 (predicted residues: 1035−1102) exert relatively smaller effects on chloride channel activity (*20*, *22*). In view of current models for CFTR regulation (*10*−*12*, *16*), such observations would agree well with an interpretation of mutations in CL3 influencing events at the C-terminal NBF2, postulated to be crucial for channel closing, or influencing the communication from NBF2 to the pore of the channel. In addition, correct folding of the loops may be critical for achievement of the correct overall conformation of the protein since many mutations in CLs 3 and 4 result in misprocessing (*20*−*22*).

For the N-terminal CL1 (predicted residues 139−194) and CL2 (predicted residues 242−307) of CFTR, only two deletion mutants have been investigated. Both variants show aberrant processing. When reconstituted into the lipid bilayer, a 19 amino acid spontaneous deletion mutant within CL2 (265−283) exhibits channel properties that are indistinguishable from those of wild-type CFTR, except for an increased tendency to enter an intermediate subconductance state (*18*). More pronounced functional defects were reported for a 30 amino acid splice variant (164−193) of CL1. This mutant has a significantly reduced open probability and an increased likelihood of residing in a low subconductance state (*19*). Such initial studies suggest that the N-terminal CLs, especially CL1, contribute to CFTR function. Our aim was to further investigate the importance of CLs 1 and 2 by utilizing a tool provided by nature, CF-associated point mutations. These gene alterations are found in affected patients and therefore may highlight individual residues that are crucial for the biosynthetic processing of CFTR, for its chloride channel activity, and/or possibly for other functions attributed to the protein. Reconstruction of the mutations and an investigation why they cause disease would give more insight into the roles of CLs 1 and 2. Such an approach has already proved informative for CLs 3 and 4 (*20*−*22*), as well as for transmembrane helixes 1 and 6 (*24*, *25*) and the NBFs (*26*−*29*).

MATERIALS AND METHODS

Construction of Vectors and Mutants. CF-associated point mutations in CLs 1 and 2 were reconstructed in the transient expression vector pcDNA3-CFTR (*22*) by subcloning of newly synthesized polymerase chain reaction (PCR) fragments. Depending on the location of the mutation, either an *Ava*I site (nucleotide 126; *3*) in combination with a *Xba*I site (nucleotide 649) was utilized, or the *Xba*I site (nucleotide 649) in combination with a *Bam*HI site (nucleotide 1508). The mutations were then cloned into the pNUT vector (*30*) by blunt-end ligation of the entire CFTR sequence. PCR was performed as described by Higuchi (*31*). Sequences of the PCR fragments generated were verified after insertion into the pcDNA3-CFTR vector using the T7 sequencing kit (Pharmacia).

Expression of Mutants. COS-1, HEK-293, and Chinese hamster ovary (CHO) cells were grown at 37 °C (5% CO_2) in Dulbecco's modified Eagle's medium containing 10% calf serum (Gibco BRL). The cells were transfected at a subconfluent stage with 2 μg/mL of the various vector constructs using a calcium phosphate precipitation method adapted from Chen and Okayama (*32*). Subsequently, pcDNA3-transfected COS-1 and HEK-293 cells were cultured for 48 h before further analysis. For pNUT-transfected CHO cells, 50 μM methotrexate was added to the growth medium 72 h posttransfection. The cells were cultured in the selective medium for 14 days before individual surviving colonies were picked and further amplified in the selective medium.

Temperature Shift and Glycerol Exposure. Cell lines expressing misprocessed CFTR variants were shifted to 26 °C for 48 h. A control sample remained at 37 °C.

Protein Detection. Cells were lysed in 1% sodium dodecyl sulfate (SDS) containing several protease inhibitors [10 μM E-64, 12 μg/mL leupeptin, 100 μg/mL aprotinin (ICN), 50 μg/mL AEBSF, and 25 μg/mL benzamidine; the same composition was utilized whenever protease inhibitors were applied in later methods]. Total cell protein content was determined with the BCA protein assay kit (Pierce). After the lysate was diluted 1:1 with 2× sample buffer (3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 62.5 mM Tris-HCl, pH 6.8) it was subjected to SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and analyzed by Western blotting (*33*) with M3A7 as the primary antibody (1 μg/mL; generated against a fusion protein containing residues 1197−1480 of CFTR; *34*). The secondary antibody was a goat anti-mouse antibody labeled with horseradish peroxidase (Gibco), which is detected by chemiluminescence with the ECL kit (Amersham Corp.).

Endoglycosidase H Digestion. Forty-eight hours post-transfection, cells were washed with PBS (150 mM NaCl, 3 mM KCl, 10 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.5 mM KH_2PO_4)

and lysed in a denaturation solution (0.5% SDS, 1% β-mercaptoethanol, 10 mM EDTA, and protease inhibitors). DNA was removed by centrifugation. The supernatant was incubated with endo H buffer (10× stock; New England Biolabs) plus or minus endoglycosidase H (New England Biolabs) for 10 min at room temperature. After dilution in 2× sample buffer, the samples were analyzed by Western blotting (*33*) as described above.

Cell Surface Labeling. Cells were washed with PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (+CM) before a 5 min incubation with 10 mM sodium periodate in PBS + CM in the dark. The periodate solution was aspirated, and the cells were washed with 0.1 M sodium acetate + CM and overlaid with 1 mM biotin-LC-hydrazide (Pierce) dissolved in 0.1 M sodium acetate + CM. The biotin-LC-hydrazide incubation was performed for 5 min in the dark before the labeling reaction was stopped with a 5 min incubation in 0.1 M Tris-HCl, pH 7.5. To this point all steps were performed at room temperature. The cells were washed twice with PBS and solubilized in buffer I (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, and protease inhibitors) at 4 °C for 30 min under agitation. DNA was removed by centrifugation and CFTR was immunoprecipitated with 1 μg/mL M3A7 and 15 μg/mL protein G−Sepharose 4B beads (Sigma) (*35*). After elution from the beads with 2× sample buffer, the sample was split into two parts, both of which were separated by SDS−PAGE and transferred to a nitrocellulose membrane (*33*). One sample was processed as described under *Protein Detection*. Nitrocellulose with the second half of the sample was incubated with streptavidin conjugated horseradish peroxidase in 2% BSA in TBS. Both reporter molecules were detected by chemiluminescence.

Iodide Efflux Studies. The same method was used as described previously (*36*). Briefly, transiently transfected COS-1 cells were incubated in iodide loading buffer [136 mM NaI, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, and 20 mM HEPES, pH 7.4] for 1 h at room temperature. The iodide loading buffer was aspirated. For subsequent steps, cycles were performed of overlaying cells with 0.5 mL of iodide efflux buffer [136 mM $NaNO_3$, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, and 20 mM HEPES, pH 7.4], leaving the iodide efflux buffer on the cells for 60 s, and then aspirating the buffer. The aspirated buffer was discarded for the first 10 cycles, but buffer from the following three cycles was collected, representing the baseline before CFTR stimulation. The iodide efflux buffer for subsequent cycles was supplemented with 10 μM forskolin (final concentration). The amount of iodide in each sample was measured using an iodide-specific electrode (Analytical Systems).

Patch-Clamp Studies of CFTR-Expressing CHO Cells. CHO cells stably expressing wild-type or mutant CFTRs were plated at low density on glass cover slips and cultured under standard conditions for 2−4 days before use. Single-channel currents were measured in excised inside-out membrane patches. The pipette and bath solutions contained 150 mM NaCl, 2 mM $MgCl_2$, and 10 mM TES, pH 7.2. Fresh Mg·ATP (1 mM final concentration) and catalytic subunit of PKA (180 nM final concentration; prepared in the laboratory of M. P. Walsh, University of Calgary, AB) were added to the bath before each experiment. Currents were filtered at 50 Hz using an 8-pole low-pass Bessel filter and digitized at 250 Hz using pClamp (6.0.3; Axon Instruments, Inc.). Recordings were made at room temperature (20−22 °C).

Channel open probability (P_o) was determined using recordings that lasted from 10 to 15 min. The membrane potential was held at −30 mV. To ensure a reliable estimate of the number of channels in the patch, 1 mM 5′-adenylyl imidodiphosphate (AMP-PNP; Sigma) was added to the bath solution at the end of each experiment. This nonhydrolyzable ATP analogue causes CFTR channels to become locked in the open state in the presence of ATP (*10*). Mean open burst and interburst durations were calculated for patches containing more than one CFTR channel as described (*22*).

RESULTS

Biosynthetic Maturation of CL 1 and 2 Mutants. Nine different point mutations have been reported in the N-terminal CLs from CF patients, seven within CL1 and two within CL2 (Figure 1). Since a majority of amino acid substitutions in CLs 3 and 4 resulted in misprocessing of CFTR, the CL 1 and 2 gene variations were initially screened for processing defects by reconstruction in the vector pcDNA3-CFTR (*22*) and transient expression in COS-1 cells. Cell lysis and Western blotting with the monoclonal antibody M3A7 (*34*) demonstrated that all CFTR variants properly produced the core-glycosylated 150 kDa form of the molecule (band B) (Figure 2, top). However, only the mutations I148T, I175V, G178R, E193K, and R297Q allowed wild-type-like maturation of the protein to the fully glycosylated 170 kDa species (band C). The remaining amino acid substitutions significantly decreased the yield of band C, with relative amounts of "vector only" (background) < G149R-CFTR < H139R-CFTR < R258G-CFTR < D192G-CFTR ≪ wild-type CFTR (Figure 2, bottom). For patients carrying these misprocessed variants, lack of CFTR at its site of action seems a likely cause of the disease symptoms.

The four mutants that could not fully mature appeared to be defective in their ability to escape from the endoplasmic reticulum (ER) since their core-glycosylated band B was sensitive to cleavage by endoglycosidase H (data not shown). For the most common CF-causing mutation, deletion of Phe 508 (ΔF508), a similar situation of maturation arrest occurs (*34*, *37*). However, in the case of ΔF508-CFTR some molecules can be rescued to the plasma membrane by culturing the expressing cells at reduced temperatures (*38*). To investigate whether CL 1 and 2 variants demonstrated a similar temperature sensitivity, the four misprocessed proteins were stably expressed in CHO cells and incubated for 48 h at 26 °C. The treatment did not promote processing to a degree detectable by Western blotting (Figure 3); this finding was not unexpected because of all mutations in the CLs examined thus far, only the H949Y-CFTR variant could be rescued to some degree (*18*, *19*, *21*; unpublished observations). Note that the relative processing characteristics (amount of band C versus band B) of all four misprocessed mutants were similar whether expressed stably (Figure 3) or transiently (Figure 2).

Functional Evaluation of CL 1 and 2 Variants by Iodide Efflux. Four mutations in CL1 and one mutation in CL2 did not retard maturation of CFTR. Since these mutations are found in CF patients, it was anticipated that they would

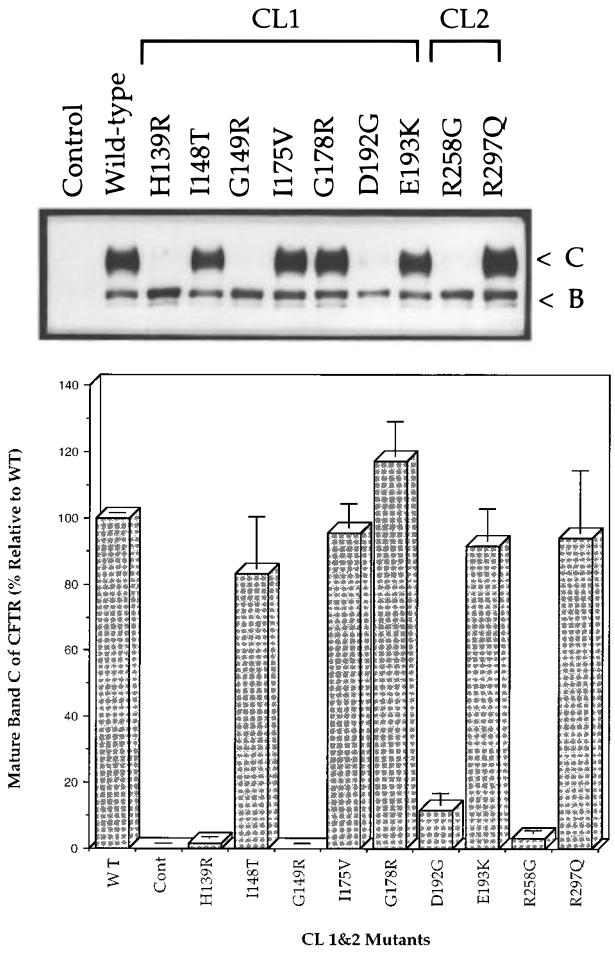

FIGURE 2: Processing characteristics of CFTR carrying CL 1 and 2 mutations. (Top) The Mutations were introduced into pcDNA3-CFTR, transiently expressed in COS-1 cells, and analyzed by Western blotting (SDS−5% PAGE, M3A7). Above the figure, the loop of origin is shown for each mutation. Positions of core-glycosylated band B and fully glycosylated band C are indicated. (Bottom) Amounts of band B and band C were analyzed by densitometry. The values were first normalized according to the amount of band B relative to wild type. The amount of band C was then calculated as a percentage relative to wild type (mean ± SE; $n = 4$). The same levels of maturation were observed when the CFTR variants were transiently expressed in HEK-293 cells (data not shown).

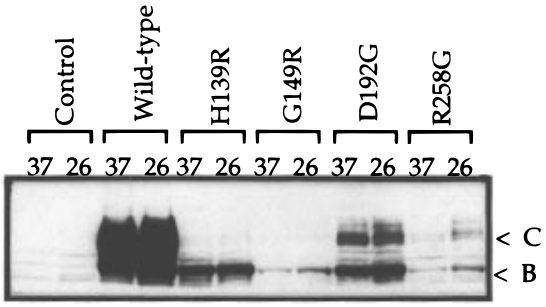

FIGURE 3: Temperature sensitivity of CL 1 and 2 CFTR variants. The misprocessed mutants were cloned into pNUT (*30*) and stably expressed in CHO cells. For each cell line, one batch was grown at 26 °C for 48 h, while a second batch remained at 37 °C for the same time period. The cells were lysed and equal amounts of total protein for each sample were separated by SDS−PAGE (5%) and analyzed by Western blotting (monoclonal antibody M3A7).

have some impact on the chloride channel activity of CFTR, an alteration of which could give important indications as to the contribution of the CLs to the function of the whole molecule. To obtain an initial evaluation of their effects on anion conduction, the CL 1 and 2 variants as well as wild-type CFTR were transiently expressed in COS-1 cells and analyzed by iodide efflux (Figure 4). Upon iodide loading and forskolin-induced elevation of intracellular cAMP levels, cells expressing wild-type CFTR demonstrated iodide fluxes that were absent in mock-transfected cells. In accordance with reduced levels of processing, the H139R, G149R, D192G, and R258G mutations significantly decreased the anion translocation capability of CFTR, whereas the properly processed I148T, I175V, and R297Q variants allowed iodide movement comparable to that of wild type. The only exceptions to this scheme were the G178R and E193K variants, which both produced activities that were lower than predicted from their wild-type-like maturation profile; in all five experiments analyzed, the decrease was more severe for G178R-CFTR-expressing cells. Western blotting confirmed that the level of expression was similar for all mutants in the applied transient system (data not shown).

Presence of band C in Western blotting generally is thought to indicate that the CFTR molecules not only escape the quality control machinery of the ER but also are properly shuttled to the plasma membrane (*39*). Still, to ensure that the decreased levels of activity of G178R-CFTR- and E193K-CFTR-expressing cells were not the result of a post-ER targeting defect but that the CFTR variants indeed reached their site of action, surface labeling was performed with the membrane-impermeant reagent biotin-LC-hydrazide (*40*−*42*). This demonstrated that all five fully glycosylated CFTR variants were properly expressed at the cell surface since for every mutant the amount of CFTR protein labeled with biotin-LC-hydrazide (Figure 5A) was proportional to the total amount of fully glycosylated band C in Western blotting with a CFTR-specific antibody (Figure 5B). The lack of labeling of band B with biotin-LC-hydrazide (Figure 5A) served as an internal control that this reagent did not have access to the cytoplasmic side of the cells.

Single-Channel Patch-Clamp Analysis of CL 1 and 2 Variants. To further characterize decreases in the anion permeation profile of G178R-CFTR and E193K-CFTR and to observe potentially small changes in the chloride channel activity of the remaining three maturation-competent mutants, the more sensitive patch-clamping method was applied. For this purpose, all processed mutations were cloned into pNUT (*30*) and stably expressed in CHO cells. When membrane patches were excised into symmetrical 152 mM NaCl solutions and 180 nM PKA and 1 mM ATP in the bath, single-channel activity was observed that was absent in untransfected cells (Figure 6A). All CFTR variants had linear current−voltage relationships and the same conductance as the wild-type channel (Figure 6B,C) and generally had indistinguishable gating properties (Figure 7). A striking exception was E193K that, in agreement with the iodide efflux data for intact cells, produced a significant decrease in the open probability (P_o) of CFTR when measured in excised patches (Figure 7A). The magnitude of this decrease was comparable to those caused by CL3 mutations (*21*). Interestingly, however, the reduction in P_o was due to an increase in the mean closed time of the E193K channels (Figure 7C), in marked contrast to the effect of CL3 mutations, which modified the mean open time of CFTR (*21*).

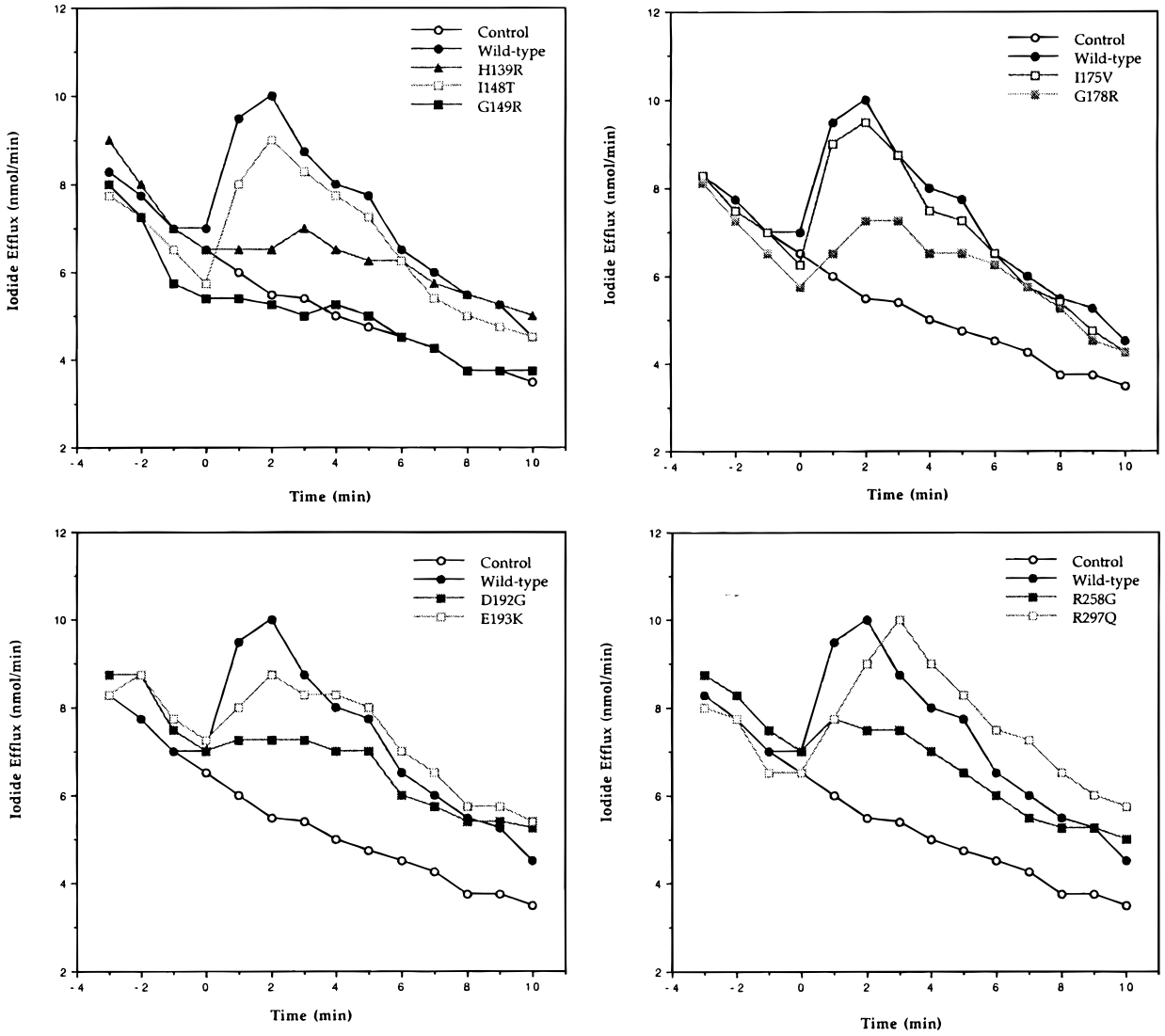

FIGURE 4: Functional evaluation of CL 1 and 2 CFTR variants by iodide efflux. Each CFTR variant was transiently expressed in COS-1 cells. Forty-eight hours posttransfection, the cells were loaded with 136 mM NaI and then stimulated with 10 μM forskolin starting at time 0. The resulting iodide efflux was determined with an iodide-sensitive electrode. The bottom right panel depicts effluxes from CL2 mutant-expressing cell lines; all other panels show results obtained from CL1 variant-expressing cell lines. These data are from representative experiments, each of which was repeated at least five times. WT, wild-type CFTR-expressing cells.

For G178R-CFTR, which had greatly reduced anion translocation capability in the iodide efflux assay, alterations in single-channel kinetics could not be identified by the patch-clamp technique. This discrepancy may originate from the additional observation that G178R-CFTR (as well as I148T-CFTR) could not be locked open by the nonhydrolyzable ATP analogue AMP-PNP. To acquire the electrophysiological data, the standard procedure involved locking open with AMP-PNP (*10*) at the end of each experiment to obtain a more accurate estimation of the number of channels per patch (*43*). Thus, the number of channels per patch may have been underestimated for I148T-CFTR- and G178R-CFTR-expressing cells, resulting in a systematic overestimation of the P_o. It is intriguing that amino acid substitutions in CL1 within the first half of the molecule can disturb locking open, which has been ascribed to a stabilizing effect of NBF2 on ADP binding at NBF1 (*44*). The results suggest that locking open is complex and may involve multiple sites as recently suggested by Mathews et al. (*43*).

DISCUSSION

Several point mutations have been reported in the N-terminal CLs of CFTR in CF patients, but thus far no information has been available regarding their inferred biochemical defect (*45*). When reconstructed in heterologous expression systems, four of the amino acid substitutions (H139R, G149R, D192G, and R258G) inhibited maturation and transport of CFTR to the cell surface, so that the protein cannot carry out its regular functions at that location. Two additional mutations, G178R and E193K, significantly reduced CFTR's anion translocation capability as observed by iodide efflux assays. In the case of the E193K variants the reduced iodide efflux was explained by the decreased P_o found in single-channel patches. This could be attributed to an increase in the mean closed time of the channels. It therefore appears that these six mutations are not only disease-associated but disease-causing. According to the scheme proposed by Welsh and Smith (*46*) the investigated gene defects fall into classes II and III of CF-causing mutations.

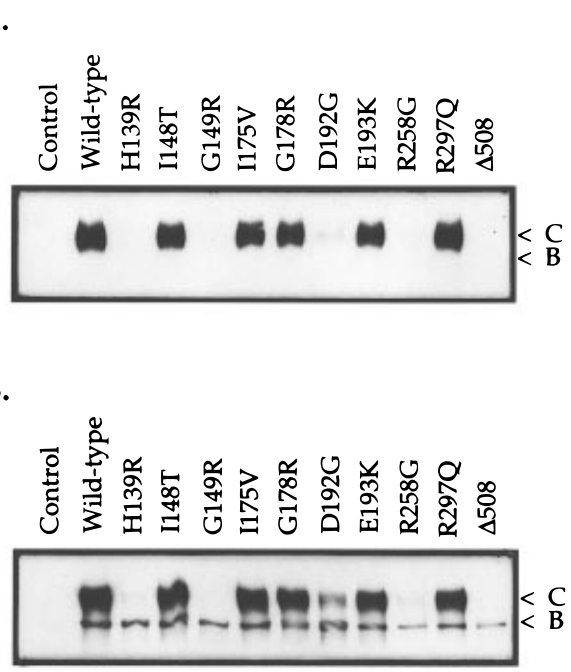

FIGURE 5: Cell surface labeling of CL 1 and 2 CFTR variants. The surface sugars of transiently transfected HEK-293 cells were oxidized and covalently linked to biotin-LC-hydrazide. After cell lysis, immunoprecipitation with M3A7, and separation by SDS−PAGE (5%), Western blotting was performed with either (A) peroxidase-labeled streptavidin to detect biotinylation or (B) M3A7 to detect CFTR.

When CFTR is studied, the molecule traditionally has been dissected into three functional units: (i) the transmembrane pore that forms the anion-conductive pathway, (ii) two cytoplasmic nucleotide binding folds that interact with ATP to open as well as to close the channel, and (iii) the cytoplasmic R-domain, phosphorylation of which enhances ATP-dependent gating by increasing the apparent affinity of CFTR for nucleotides by ~20-fold (*13*, *43*, *47*). However, there is little understanding of how the regulatory domains communicate with each other and especially how information is relayed from the regulatory domains to the pore of the channel. By analogy with bacterial relatives of CFTR, it has been suggested that the CLs could be involved in such processing of information (*20*). Bacterial permeases derive their transmembrane and regulatory domains from four different proteins, which precludes communication via the primary amino acid sequence. The structures most likely to mediate gating signals to the pore are those that protrude from the membrane, i.e., the CLs. In addition, due to their strongly hydrophilic nature (*3*), the CLs of CFTR were suggested to have the potential for intramolecular interactions with other charged portions of the molecule (*48*). The finding that the two mutations in CLs 1 and 2 with the most severe effects on the chloride channel activity of CFTR introduce a positive charge into CL1 (G178R and E193K) is consistent with an important role of electrostatic interactions in the normal functioning of the loops.

Indeed, data from this study and from earlier investigations of CLs 3 and 4 (*20*−*22*) collectively support a possible role of the CLs in communicating regulatory information. Although the loops are integral parts of the pore-forming domains of CFTR, CF-associated point mutations in the CLs do not affect the conductance of the channel in any way that would suggest a role in anion permeation. Rather, these amino acid substitutions have distinct effects on the kinetics of the CFTR channel: mutations in CL3 modulate mean open time (*21*) which, according to current models for CFTR gating (*10*−*12*, *16*), may indicate that these gene alterations affect events at NBF2 or the communication from NBF2 to the pore. In contrast, the decreased anion translocation capability caused by mutations in CL1, in the case of E193K, resulted from an increase in the mean closed time. Interpretation of this type of change in terms of potential underlying domain interactions is less descriptive, since an increase in the mean closed time was observed subsequent to mutagenic removal of phosphorylation sites in the R-domain (*43*), as well as to mutations in NBF1 (*16*, *49*, *50*). Therefore, an effect on, or an altered interaction with, either NBF1 or the R-domain could account for the increase in the mean closed time. However, by analogy with the proposed association between CL3 and NBF2, it is tempting to speculate that a parallel interaction may occur between CL1 and NBF1. Consistent with such a proposal, it was determined in the structurally related drug transporter P-glycoprotein that the N-terminal transmembrane domain can be coprecipitated with NBF1 and the C-terminal transmembrane domain can be coprecipitated with NBF2, but not vice versa (*51*).

In the study of deletion mutants within CLs 1 and 2, Xie et al. (*18*, *19*) observed an increased probability for CFTR variants to reside in low and intermediate subconductance states. Subconductance states have been described by several groups (*47*, *48*) and their prevalence seems to vary depending on the experimental system used. In the presented patch-clamp studies of CFTR in patches excised from CHO cells, subconductance states were extremely rare and did not contribute significantly to channel activity.

Four mutations retarded processing of CFTR to the fully glycosylated state. Interestingly, all four misprocessed CFTR variants of CLs 1 and 2 had altered charge. This is not an isolated finding for CFTR, as it has been observed in other proteins. For example in the anion transporter Band 3, Jarolim et al. (*52*) described three disease-causing mutations, all of which appear to result in biosynthetic arrest, and all of which involve charge changes. Thus, the preponderance of charge changes of CF-associated point mutations in the CLs may reflect a common mechanism of misprocessing rather than altered electrostatic interactions with permeant anions, such as those that occur in the vestibules of other ion channels (*53*). Note that some charge changes did allow full maturation, e.g., G178R. The fact that many mutations in addition to the most common ΔF508 defect halt biosynthesis indicates that a therapeutic strategy designed to facilitate processing could be beneficial for patients carrying various gene alterations. Such a finding is especially significant in view of a recent development in P-glycoprotein research where all processing-defective mutants examined could be promoted to the cell surface by exposure to substrates and modulators of the protein (*54*).

I148T, I175V, and R297Q did not adversely affect the processing, gating, or conductance of CFTR. Nevertheless, these residues appear to be important because their alteration is associated with the very severe pancreatic-insufficient CF phenotype (Table 1). The discrepancy between the CFTR functional data (patch-clamp and efflux studies) and pancreatic phenotype may be due to variability in clinically assessing the pancreatic status of the CF patients. The

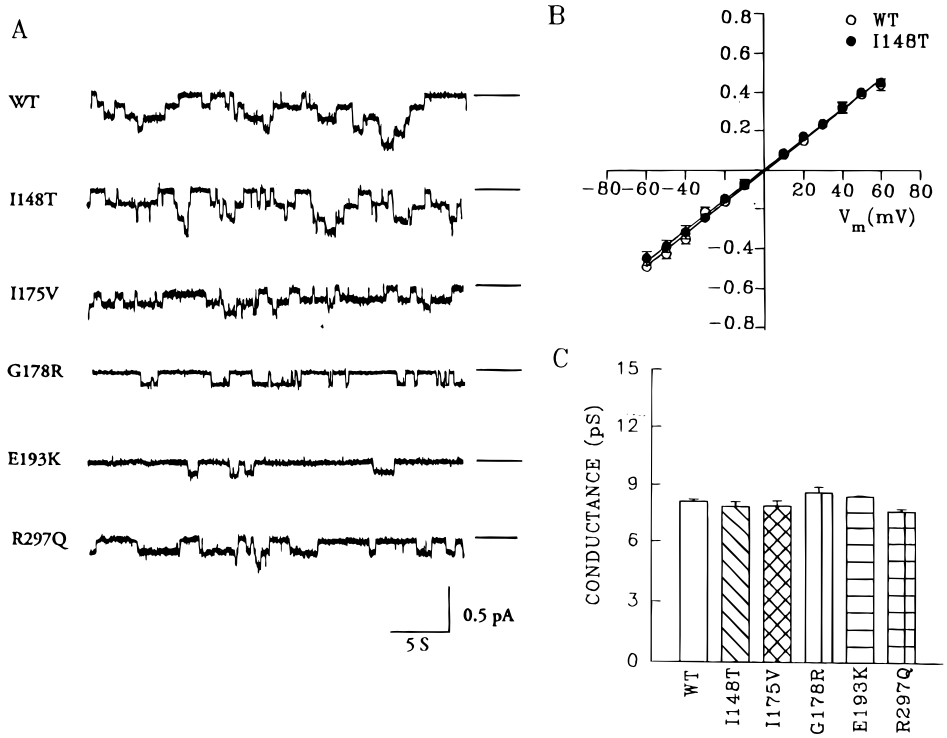

FIGURE 6: Single-channel activity associated with processed CL 1 and 2 mutants. (A) Upon excision of membrane patches from CHO cells into PKA- (180 nM) and ATP- (1 mM) containing buffer, channel activity was observed for wild-type (WT) and all maturation-competent CL 1 and 2 mutated CFTRs. Currents were recorded at a membrane potential of −30 mV. The closed state is indicated by a horizontal line on the right. (B) Mean single-channel current−voltage relationship of wild-type CFTR and I148T-CFTR as a representative CL 1 and 2 mutant. Each point represents the mean ± SE (where this is larger than the size of the symbol) of data from four (I148T) or five (WT) patches. (C) Mean single-channel conductance of those CL 1 and 2 mutants studied, showing mean data from 3−5 patches. In each case the error bar represents 1 SE. None of the conductances were significantly different from wild type ($p > 0.05$, two-tailed *t*-test).

clinical diagnosis and assessment of the severity of pancreatic dysfunction depend significantly on the criteria used by the physician. This problem is compounded by the fact that most of the CFTR mutations that have been published usually have been observed in a single or a few patients. Another problem is that the severity of pancreatic dysfunction could be due to another undetected mutation(s) in another part of the coding region or noncoding regions of the CFTR gene. The entire CFTR gene was not sequenced in patients with mutations I148T, I175V, or R297Q when these mutations were published. Mutation(s) in the noncoding region of CFTR could affect transcription. It is also possible that these mutations affect some other function of CFTR such as inhibition of a sodium channel (*61*, *62*) or activation of an outwardly rectifying anion channel (*63*, *64*); a kidney isoform that only produces the N-terminal half of CFTR reportedly can still regulate the outward rectifier (*65*).

Overall, the CLs are emerging as significant contributors to CFTR function, with site-directed mutagenesis indicating that each loop has a distinct role. Mutations in CLs 1 and 3 had drastic effects on the ability of CFTR to respond to regulatory stimuli: E193K in CL1 decreased the opening rate, in agreement with the decreased P_o of a CL1 deletion variant (*19*) and the reduced iodide efflux activity of G178R-CFTR, whereas mutations in CL3 affected the duration of the open state (21). In contrast, R297Q, the only CF-associated mutation that could be evaluated in CL2 or deletion of CL2 (*18*), apparently had little effect on the chloride channel activity of CFTR, as was shown previously for mutations in CL4 (*20*, *22*). The correct folding of the

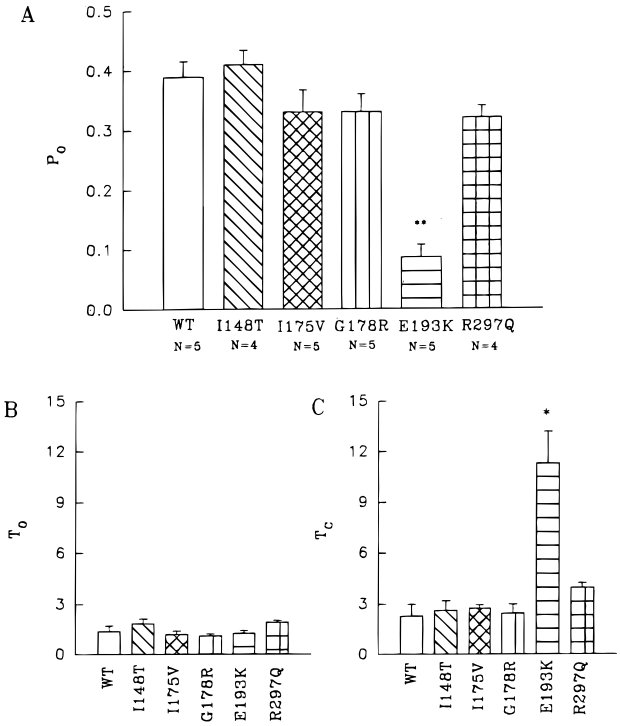

FIGURE 7: Mean kinetic properties of single CL 1 and 2 mutant channels. Open probability P_o (A), mean burst duration T_o (B), and mean interburst duration T_c (C) were calculated as described. In each case the data are the mean ± SE from 4−5 patches as indicated by the *N* value. Parameters that are significantly different from wild type (WT) are marked (*, $p < 0.005$; **, $p < 0.0001$; two-tailed *t*-test). Note that the I148T- and G178R-CFTR variants could not be locked open with AMP-PNP, so that for these mutants the number of channels in each patch may have been underestimated.

Table 1: Processing Characteristics versus Patient Phenotype

mutation	pancreatic status	ref
	Processed	
I148T	PI	*55*
I175V	PI	*56*
G178R	PI	*57*
E193K	PS	*58*
R297Q	PI	*59*
	Misprocessed	
H139R	−	*45*
G149R	PS	*58*
D192G	PI	*60*
R258G	PS	*58*

[a] The nine mutations within CLs 1 and 2 are grouped according to their impact on processing. The original paper describing each mutation is indicated. PS, pancreatic sufficient; PI, pancreatic insufficient; −, unspecified pancreatic status. The severity of pancreatic dysfunction was determined clinically as indicated in the reference.

CLs appears to be crucial for achievement of the correct overall conformation of the protein since many mutations caused misprocessing of CFTR.

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