Intracellular Loop between Transmembrane Segments IV and V of Cystic Fibrosis Transmembrane Conductance Regulator Is Involved in Regulation of Chloride Channel Conductance State*

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The cystic fibrosis transmembrane conductance regulator (CFTR) contains two membrane-spanning domains; each consists of six transmembrane segments joined by three extracellular and two intracellular loops of different length. To examine the role of intracellular loops in CFTR channel function, we studied a deletion mutant of CFTR (Δ19 CFTR) in which 19 amino acids were removed from the intracellular loop joining transmembrane segments IV and V. This mutant protein was expressed in a human embryonic kidney cell line (293 HEK). Fully mature glycosylated CFTR (~170 kDa) was immunoprecipitated from cells transfected with wild-type CFTR cDNA, while cells transfected with the mutant gene expressed only a core-glycosylated form (~100 kDa). The chloride efflux rate (measured by 6-methoxyn- (3-sulfopropyl) quinolinium SPQ fluorescence) from cells expressing wild-type CFTR increased 600% in response to forskolin. In contrast, Δ19 CFTR-expressing cells had no significant response to forskolin. Western blotting performed on subcellular membrane fractions showed that Δ19 CFTR was located in the same fractions as ΔF508 CFTR, a processing mutant of CFTR. These results suggest that Δ19 CFTR is located in the intracellular membranes, without reaching the cell surface. Upon reconstitution into lipid bilayer membranes, Δ19 CFTR formed a functional Cl⁻ channel with gating properties nearly identical to those of the wild-type CFTR channel. However, Δ19 CFTR channels exhibited frequent transitions to a Δ6-picosiemens subconductance state, whereas wild-type CFTR channels rarely exist in this subconductance state. These data suggest that the intracellular loop is involved in stabilizing the full conductance state of the CFTR Cl⁻ channel.

The cystic fibrosis transmembrane conductance regulator (CFTR) consists of five distinct regions, with two putative membrane-spanning domains, two nucleotide-binding folds, and a regulatory domain (1). CFTR forms a Cl⁻ channel of linear conductance (2,3), which is regulated by cAMP-dependent protein kinase phosphorylation (2,4–6) at multiple sites in the regulatory domain and by binding and hydrolysis of ATP by the nucleotide-binding folds (7–10). The structure and function of these five domains of CFTR have been extensively studied (11–14). In contrast, little is known about the role of intracellular loops and their contribution to the function of the CFTR Cl⁻ channel.

The intracellular loops of other channel proteins appear to participate in channel function. For instance, mutations in the second intracellular loop of the Shaker K⁺ channel affect channel inactivation (15). Deletion of a portion of the putative cytosolic loops between two transmembrane repeats of the Na⁺ channel slows the rate of channel inactivation (16). To investigate the role of the intracellular loops of CFTR in Cl⁻ channel function, we studied a deletion mutant of CFTR (Δ19 CFTR) in which 19 amino acids were removed from the intracellular loop joining transmembrane segments IV and V in the first membrane-spanning domain. This is the largest and most hydrophilic loop in the first membrane-spanning domain, as one-third of the residues in this loop are charged. These features make this loop a candidate for electrostatic or allosteric interactions with other cytosolic domains of CFTR (nucleotide-binding folds, the regulatory domain, or other intracellular loops) and cellular proteins to contribute to channel function.

EXPERIMENTAL PROCEDURES

Subcloning of CFTR Gene—The wild-type and ΔF508 CFTR cDNAs (17) were subcloned into an Epstein-Barr virus-based episomal eukaryotic expression vector, pCEP4 (Invitrogen, San Diego, CA), between the Nhel and Xhol restriction sites. The mutant gene, which lacks 57 nucleotides between nucleotides 930 and 987, was shuttled from pBlue-script into pCEP4 by substituting the corresponding fragment in pCEP4(WT) (where WT is wild type) with the mutant one between the KpnI and Alfl restriction sites. The mutant clone (pCEP4(A19)) was confirmed by restriction enzyme digestion and DNA sequencing of the shuttled fragment from nucleotides 874 to 1040.

Cell Culture—A human embryonic kidney cell line (293-EBNA, Invitrogen) was used for transfection and expression of wild-type and mutant CFTR proteins. This cell line contains a pCMV-EBNA vector, which constitutively expresses the Epstein-Barr virus EBNA-1 gene product and increases the transfection efficiency of Epstein-Barr virus-based vectors. The cell line exhibited high transfection efficiency (up to 80%) with Lipofectin reagent (Life Technologies, Inc.) following the manufacturer’s instructions. The cells were maintained in Dulbecco’s modified Eagle’s medium (Biofluids, Inc., Rockville, MD) containing 10% fetal bovine serum (Life Technologies, Inc.) and 1% glutamine. Geneticin (G418 sulfate, 250 μg/ml; Life Technologies, Inc.) was added to cell culture medium for the continuous selection of cells containing the pCMV-EBNA vector. The parent cell line was grown to confluence in a 37 °C incubator with 5% CO₂ and passed 1-2 days before the gene manufacturer’s instructions. The cells were maintained in Dulbecco’s modified Eagle’s medium (Biofluids, Inc., Rockville, MD) containing 10% fetal bovine serum (Life Technologies, Inc.) and 1% glutamine. Geneticin (G418 sulfate, 250 μg/ml; Life Technologies, Inc.) was added to cell culture medium for the continuous selection of cells containing the pCMV-EBNA vector. The parent cell line was grown to confluence in a 37 °C incubator with 5% CO₂ and passed 1-2 days before the gene

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‡ The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; p5, picomoles; SPQ, 6-methoxy-N-(3-sulfopropyl) quinolinium.
Fig. 1. Predicted topology of CFTR. The predicted membrane topology of CFTR is shown, with the deleted 19 amino acids indicated. L, loop; TM, transmembrane segment; NBF, nucleotide-binding fold; R, regulatory.

**RESULTS**

Subcloning of CFTR Mutant Gene into Eukaryotic Expression Vector—The CFTR mutant studied in this paper was a

immunoprecipitation/Western Blot Assay of CFTR—293 HEK cells transfected with pCEP4(WT), pCEP4(ΔF508), or pCEP4(Δ119) were cultured to confluence in 162-cm² flasks (Costar, Cambridge, MA). Cells were washed three times with ice-cold phosphate-buffered saline (137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, 5 mM diisopropyl fluorophosphate, pH 7.4), lysed with 1 ml of ice-cold radiolabeled membrane protein extraction assay solution (150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) in the presence of protease inhibitors (10 μg/ml EDTA, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 9.6 mg/ml benzamidine) prior to lysis by 10 strokes in tight-fitting Dounce homogenizer, followed by 15 strokes after addition of an equal volume of sucrose buffer (500 mM sucrose, 1 mM EDTA, 10 mM Heps, pH 7.2) for 30 min in glass cover slips mounted internally in a host 386 computer and processed with Image-1 (Imaging Technology Inc., Cambridge, MA). The images were quantified with a microchannel plate image intensifier (Model KS-1381) in a swinging bucket rotor (SW28). Each fraction (I–V) was collected at the interface of the two sucrose gradients. Subsequently, an abbreviated fractionation scheme was used that separated ΔF508 CFTR from the wild type and “intracellular membrane” from “plasma membrane.” In this experiment, cells were washed and lysed in buffer as before, except that diisopropyl fluorophosphate was not included. The post-mitochondrial supernatant was spun at 100,000 g for 45 min to give plasma membrane (PM).

Detection of CFTR by Western Blotting—The membrane fractions collected from the above procedure were washed and resuspended in isotonic buffer, 50 μg of protein from each fraction were denatured with 6× gel sample buffer (300 mM Tris-Cl, pH 6.8, 600 mM dithiothreitol, 12% SDS, 0.6% bromphenol blue) and loaded onto a 5% SDS-polyacrylamide gel. The proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad) and probed with monoclonal antibody 13-1 (0.8 μg/ml). A secondary peroxidase-conjugated affinity-purified goat antibody to mouse IgG (Organon Teknika Corp., West Chester, PA) was added to allow visualization of the CFTR-antibody complex. The proteins were detected by chemiluminescence according to the manufacturer’s recommendations (ECL kit, Amersham Corp.).

SPQ Assay of CFTR Transport—Cells were transferred to glass cover slips, cultured for 2–3 days, and then loaded with SPQ fluorescent dye (600 μg/ml aprotinin, 9.6 mg/ml benzamidine, 5 mM diisopropyl fluorophosphate, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 9.6 mg/ml benzamidine) prior to incubation at 4 °C for 30 min in the presence of a solution that quenched all maximum fluorescence and in the presence of a solution that quenched all maximum fluorescence. SPQ fluorescence was calibrated by subtracting a straight line that connects the two points at the beginning of the experiment and before KSCN addition was corrected by subtracting a straight line that connects the two points at the beginning of the experiment and before KSCN addition at a ratio of 6:6:1; the lipids are dissolved in decane at a concentration of 40 mg/ml (20). The recording solutions contained the following: cis-side solution (extracellular), 50 mM KCl, 50 mM Hepes, pH 7.2, and trans-side solution (extracellular), 50 mM KCl, 10 mM Hepes/Tris, pH 7.4. Microsomal vesicles were collected from the above procedure were washed and resuspended with 20 μl of protein G-agarose beads (Boehringer) by incubation at 4 °C for 30 min on a rocker. The beads were washed with radiolabeled membrane protein extraction assay solution three times, and the bound proteins were solubilized with 20 μl of gel sample buffer (200 mM Tris-Cl, pH 6.7, 9% SDS, 6% β-mercaptoethanol, 15% glycerol, 0.01% bromphenol blue) and loaded onto a 5% SDS-polyacrylamide gel. The proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad) and probed with monoclonal antibody 13-1 (0.8 μg/ml). A secondary peroxidase-conjugated affinity-purified goat antibody to mouse IgG (Organon Teknika Corp., West Chester, PA) was added to allow visualization of the CFTR-antibody complex. The proteins were detected by chemiluminescence according to the manufacturer’s recommendations (ECL kit, Amersham Corp.).

SPQ Assay of CFTR Transport—Cells were transferred to glass cover slips, cultured for 2–3 days, and then loaded with SPQ fluorescent dye by hypotonic loading as described previously (18). Briefly, cells were incubated at room temperature for 4 min with a 1:1 mixture of Cl⁻ buffer (126 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 20 mM Hepes, 0.1% bovine serum albumin, 0.1% d-glucose, pH 7.2) and distilled H₂O containing 5 mM SPQ. The glass coverslip was mounted in a chamber on a stage heated to 37 °C, which was arranged for continuous flow of warmed buffer solutions and studied one-by-one using an upright Zeiss epifluorescence microscope. Fluorescence was excited at 355 nm (Omega Optical Inc., Brattleboro, VT) by a 75-watt xenon lamp. Emission light was reflected by a 400-nm fused silica dichroic mirror (Omega Optical Inc.) and illuminated through a 20× objective (numerical aperture of 0.75, working distance of 0.66 mm; Nikon Inc., Garden City, NY). Emitted light was filtered by a 450-nm lens and detected by a microchannel plate image intensifier (Model KS-1381). Single channel data generation were performed with a 486 computer and a 1200 Digidata (Axon Instruments, Inc., Foster City, CA). Data acquisition and pulse photobleaching. Cells that exhibited >15% photobleaching or dye leak-
age were not used for analysis.

Subcellular Fractionation of Membrane Vesicles—The protocol used was a modified version of that of Gunderson and Kopito (19) (see Fig. 5A). Briefly, 12 75-cm² flasks of 293 HEK cells transfected with the pCEP4(WT), pCEP4(ΔF508), or pCEP4(Δ119) vector were harvested by scraping from the flask bottom following three washes with ice-cold phosphate-buffered saline. The cell pellet (600 × g, 5 min, 4 °C) was then resuspended by incubation for 10 min in ice-cold hypotonic lysis buffer (10 mM Hepes, pH 7.2, 1 mM EDTA, 5 μM diisopropyl fluorophosphate, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 9.6 mg/ml benzamidine) prior to lysis by 10 strokes in tight-fitting Dounce homogenizer, followed by 15 strokes after addition of an equal volume of sucrose buffer (500 mM sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.2) for 30 min in glass cover slips mounted internally in a host 386 computer and processed with Image-1 (Imaging Technology Inc., Cambridge, MA). The images were quantified with a microchannel plate image intensifier (Model KS-1381) in a swinging bucket rotor (SW28). Each fraction (I–V) was collected at the interface of the two sucrose gradients. Subsequently, an abbreviated fractionation scheme was used that separated ΔF508 CFTR from the wild type and “intracellular membrane” from “plasma membrane.” In this experiment, cells were washed and lysed in buffer as before, except that diisopropyl fluorophosphate was not included. The post-mitochondrial supernatant was spun at 12,000 × g for 30 min to give intracellular membrane (Fig. 5C, IM), and the supernatant was spun at 100,000 × g for 45 min to give plasma membrane (PM).

Detection of CFTR by Western Blotting—The membrane fractions collected from the above procedure were washed and resuspended in isotonic buffer, 50 μg of protein from each fraction were denatured with 6× gel sample buffer (300 mM Tris-Cl, pH 6.8, 600 mM dithiothreitol, 12% SDS, 0.6% bromphenol blue) and loaded onto a 5% SDS-polyacrylamide gel. The proteins were then transferred to a polyvinylidene difluoride membrane and detected as described under “Immunoprecipitation/Western Blot Assay of CFTR.”

Reconstitution of CFTR Cl⁻ Channel—Lipid bilayer membranes were formed across an aperture of ~200-μm diameter with a lipid mixture of phosphatidylethanolamine/phosphatidylserine/cholesterol at a ratio of 6:6:1; the lipids are dissolved in decane at a concentration of 40 mg/ml (20). The recording solutions contained the following: cis-side solution (intracellular), 200 mM KCl, 2 mM MgATP, and 10 mM Hepes/Tris, pH 7.4; and trans-side solution (extracellular), 50 mM KCl, 10 mM Hepes/Tris, pH 7.4. Microsomal vesicles (3–6 μl) containing the wild-type or Δ119 CFTR protein were added to the cis-side solution, 50 μl of cisAMP-dependent protein kinase catalytic subunit (Promega, Madison, WI) was always present in the cis-side solution. Single channel currents were recorded with an Axopatch 200A patch clamp unit (Axon Instruments, Inc., Foster City, CA). Data acquisition and pulse generation were performed with a 486 computer and a 1200 Digidata A/D-D/A converter (Axon Instruments, Inc.). The currents were sampled at 1–2.5 ms/point and filtered at 100 Hz. Single channel data analyses were performed with pClamp software (Axon Instruments, Inc.).
spontaneous deletion mutation in the CFTR cDNA in which 57 nucleotides corresponding to 19 amino acids (MIENIQSVKAY-CWEEAMEK, residues 266–284) were deleted. These residues are located in the intracellular loop joining transmembrane segments IV and V in the first membrane-spanning domain (Fig. 1). The mutant gene was shuttled from pBluescript into pCEP4 for eukaryotic cell expression. The pCEP4 vector contains the cytomegalovirus promoter, which drives the transcription of the donned gene.

Expression of Wild-type and Mutant CFTR Proteins—The CFTR protein was expressed in the eukaryotic cells by transfecting pCEP4(WT), pCEP4(Δ508), or pCEP4(Δ19) into 293 HEK cells using the Lipofectin reagent (21). To confirm CFTR expression, the immunoprecipitation/Western blot assay was performed on the transfected cells. Cells transfected with pCEP4(WT) expressed a readily detectable amount of a fully glycosylated form of the CFTR protein with a molecular mass of ~170 kDa, while the untransfected cells did not (Fig. 2). Cells transfected with either pCEP4(Δ508) or pCEP4(Δ19) showed a dominant band at ~140 kDa (Fig. 2), which probably corresponds to the core-glycosylated form of CFTR. Consistent with other studies (22, 23), Δ508 CFTR, which is incompletely glycosylated at 37 °C, displayed increased glycosylation when the culture temperature was lowered to 26 °C (Fig. 2, lane 5). However, incubation of pCEP4(Δ19)-transfected cells at 26 °C for 2 days did not increase the amount of Δ19 CFTR protein in the fully glycosylated form (Fig. 2, lane 7). The amount of 140-kDa protein associated with both Δ508 CFTR and Δ19 CFTR increased significantly following the lower temperature incubation (Fig. 2, lanes 5 and 7), probably due to the slower degradation of the abnormal protein in the intracellular organelles.

SPQ Assay of Cl⁻ Transport—The immunoprecipitation/Western blot assay detected no fully glycosylated Δ19 CFTR protein. However, other studies have shown that some CFTR mutants produce the fully glycosylated form in quantities too small to be detected by the above assay, but can nevertheless result in functional channels at the cell surface (23, 24). To test the surface function of Δ19 CFTR, the SPQ assay was performed on pCEP4(Δ19)-transfected cells using pCEP4(WT)-transfected cells as a positive control and untransfected cells as a negative control. Representative traces of chloride movement for five to six cells of each cell line in the absence and presence of 10 μM forskolin are depicted in Fig. 3. A decrease in SPQ fluorescence indicates Cl⁻ influx, and an increase in fluorescence indicates Cl⁻ efflux. Forskolin failed to elicit an increase in the rate of Cl⁻ efflux in untransfected 293 HEK cells (Fig. 3A). Wild-type CFTR-transfected cells, on the other hand, exhibited a significant increase in the rate of Cl⁻ efflux upon forskolin stimulation (Fig. 3B). In cells transfected with Δ19 CFTR, stimulation of the rate of Cl⁻ efflux by forskolin was not statistically different from that of untransfected cells (Fig. 3C).
Role of Intracellular Loops in CFTR Function

Data from multiple experiments are summarized in Fig. 4. Incubation of the Δ19 CFTR-expressing cells at 26 °C for 15–48 h did not increase the rate of Cl⁻ efflux following forskolin stimulation (Fig. 4). This result is consistent with the immunoprecipitation/Western blot studies (Fig. 2), which indicate that little or no Δ19 CFTR reaches the Golgi apparatus for final glycosylation and presumably does not transport to the plasma membrane.

Localization of Δ19 CFTR Protein—Subcellular membrane fractionation was performed with 293 HEK cells transfected with CFTR cDNA to localize the Δ19 CFTR protein. Intracellular membranes were separated from the plasma membrane using a discontinuous sucrose density gradient (Fig. 5A). Wild-type CFTR was used as a marker for the plasma membrane, and ΔF508 CFTR, a known CFTR processing mutant, as a marker for the intracellular membranes. Western blotting showed that the wild-type CFTR protein (~170 kDa) was detected mainly in fractions II and III, while the ΔF508 CFTR protein (~140 kDa) was detected mainly in fraction IV of the sucrose density gradient (Fig. 5B). Intracellular membrane fractions isolated from Δ19 CFTR-expressing cells contained a 140-kDa CFTR protein, which colocalized with the ΔF508 CFTR protein (Fig. 5C). Together with the immunoprecipitation/Western blot and SPQ assays, these data suggest that Δ19 CFTR is a processing mutant.

Functional Characterization of Wild-type and Δ19 CFTR Cl⁻ Channels—To further study the function of Δ19 CFTR, microsomal vesicles, which contain mixtures of plasma membrane, endoplasmic reticulum, and Golgi membranes, were incorporated into planar lipid bilayers. Its function was compared with that of wild-type CFTR, incorporated into planar lipid bilayers from plasma membrane vesicles. Consistent with previous studies (19), wild-type CFTR showed a cAMP-dependent protein kinase phosphorylation-dependent Cl⁻ channel with linear conductance of 8.2 ± 0.6 pS (Fig. 6). The extrapolated reversal potential was +22.2 ± 1.4 mV in a KCl gradient of 200 mM (cis) and 50 mM (trans), consistent with a chloride-selective channel. Without cAMP-dependent protein kinase, this 8-pS Cl⁻ channel was never observed in vesicles isolated from 293 HEK cells with (n > 30) or without (n > 20) transfection with CFTR cDNA.

The reconstituted CFTR channels had slow kinetics of gate-
The open lifetime histogram contained at least two exponentials, with mean open lifetimes of $t_{O1} = 23.6$ ms and $t_{O2} = 111.9$ ms (Fig. 7A). The relative occurrence of $t_{O1}$ was $y_{O1}/(y_{O1} + y_{O2}) = 0.38$. An average open probability ($p_O$) of $0.318 \pm 0.028$ was measured at $-80$ mV. In separate studies, we found that the wild-type CFTR channel contained two distinct subconductance states with conductances of 6 pS (O2) and 2.7 pS, in addition to the full conductance state (8 pS, O1). The occurrence of O2, however, was low for wild-type CFTR and accounted for $<10\%$ of the open events (see Fig. 10A).

When Δ19 CFTR was incorporated into the lipid bilayer membrane, functional Cl⁻ channel activity was identified. The full conductance state (O1) of the Δ19 CFTR Cl⁻ channel was identical to that of the wild-type CFTR channel (Figs. 6C and 8). Similar to the wild-type CFTR channel, the Δ19 CFTR channel had an average open probability of $0.308 \pm 0.043$ and mean open lifetimes of $t_{O1} = 30.6$ ms and $t_{O2} = 115.8$ ms at $-80$ mV (Fig. 7B). The relative occurrence of $t_{O1}$ was $y_{O1}/(y_{O1} + y_{O2}) = 0.48$. Thus, gating of the Δ19 CFTR channel is not significantly different from that of the wild-type CFTR channel at a time resolution of 100-Hz cutoff frequency in the bilayer system.

A significant difference between Δ19 CFTR and wild-type CFTR was found in the distribution of channel subconductance states. Unlike the wild-type CFTR channel, the Δ19 CFTR channel displayed a prominent subconductance state (O2; Fig. 8). Fig. 9 shows the amplitude histogram of two separate experiments with the wild-type and Δ19 CFTR channels. The histograms were different from each other, particularly in the first open level. The frequent occurrence of subconductance state resulted in an asymmetric distribution of the first open...
level of the \( D^{19} \) CFTR channel (indicated by O1 and O2). Similar phenomena were observed in 13 other experiments with \( D^{19} \) CFTR channels. There was a relative paucity of instances of two channels in these recordings, especially for the \( D^{19} \) mutant, in records in which two channels are clearly present (e.g. Figs. 8 and 9). The reasons for this are unclear, and detailed analysis is beyond the scope of this paper.

Fig. 10 shows the amplitude histograms of open channel current pooled from multiple experiments in which a single CFTR channel was incorporated into the bilayer membrane. Clearly, the \( D^{19} \) CFTR channel contained more openings to the O2 state than the wild-type CFTR channel (Fig. 10, compare A with B). The histograms could be fitted with the sum of two gaussian distribution functions, with mean currents of \(-0.65\) pA (O1) and \(-0.46\) pA (O2), at \(-80\) mV. The relative occurrence of the O2 state was estimated at 9% for the wild-type CFTR channel and at 23% for the \( D^{19} \) CFTR channel.

**DISCUSSION**

In this study, we examined the function of a CFTR deletion mutant, \( D^{19} \) CFTR. This deletion mutation results in incomplete glycosylation and intracellular retention of CFTR based on the following observations. (i) An immunoprecipitation/Western blot assay identified a core-glycosylated form of \( D^{19} \) CFTR (\( \approx 140 \) kDa), different from the fully mature glycosylated protein (\( \approx 170 \) kDa); (ii) subcellular fractionation showed the \( D^{19} \) CFTR protein localized predominantly in the intracellular membranes; and (iii) an SPQ assay of cells expressing \( D^{19} \) CFTR showed no forskolin-stimulated \( Cl^- \) transport. However, this processing mutant maintained functional \( Cl^- \) channel activity, presumably in the intracellular organelles, when reconstituted into lipid bilayer membranes. The most notable difference between wild-type and \( D^{19} \) CFTR channels lies in the distribution of conductance states. The deletion mutation caused frequent occurrence of a subconductance state within the \( Cl^- \) channel.

The most common disease-causing mutation of CFTR is the deletion of a single phenylalanine residue at position 508 (\( \Delta F^{508} \) CFTR). Most of the \( \Delta F^{508} \) CFTR protein is retained in the endoplasmic reticulum and fails to reach its intended site of action in the plasma membrane. With regard to processing at 37 °C, \( \Delta F^{19} \) CFTR appears to be similar to \( \Delta F^{508} \) CFTR. However, \( \Delta F^{508} \) CFTR appears in the fully mature glycosylated form and can traffic to the plasma membrane following incubation at lower temperature (26–30 °C) (Fig. 2) (22, 23). Unlike \( \Delta F^{508} \) CFTR, the misprocessing of \( \Delta F^{19} \) CFTR is temperature-insensitive: the protein remained in the core-glycosylated state in intracellular organelles even at 26 °C. The \( \Delta F^{508} \) CFTR proteins, once at the plasma membrane, give rise to functional \( Cl^- \) channels similar to those of wild-type CFTR in terms of open probability (26) and conductance state (24, 26).

A cardiac-specific isoform of CFTR lacks 30 amino acids in the intracellular loop between transmembrane segments II and III. It is caused by alternative splicing of exon 5 of the CFTR mRNA (27). Cardiac CFTR functions as a cAMP-dependent
protein kinase-regulated Cl\(^-\) channel with conduction properties similar to those of epithelial CFTR (9, 28). However, when expressed in HeLa cells, human CFTR lacking exon 5 failed to generate a cAMP-mediated chloride transport by the SPQ assay, apparently due to defective intracellular processing (29). The strategy applied here to the study of CFTR channel function can be used to study even misprocessed or mislocalized channels and therefore ought to be applicable to the study of the effects of deleting exon 5 on CFTR channel function as well.

The 19 amino acids deleted in Δ19 CFTR span a major part of the intracellular loop joining transmembrane segments IV and V. This segment is highly hydrophilic, with about one-third of the residues being charged (two lysines and four glutamates). Several factors could account for the subconductance state associated with the Δ19 CFTR channel.

One possibility is that retention of the Δ19 CFTR protein in intracellular membranes alters the function of the Cl\(^-\) channel. Maturation of CFTR from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane involves several processing and post-translational modifications (e.g. folding and glycosylation). The endoplasmic reticulum membrane contains other proteins, including various types of chaperons, that could interact with CFTR and alter the function of the Cl\(^-\) channel. However, the gating kinetics and open probability of the Δ19 CFTR channel in the intracellular membrane are similar to those of the wild-type CFTR channel in the plasma membrane, so the deletion of these 19 amino acids did not alter the essential function of the CFTR Cl\(^-\) channel. Moreover, the wild-type CFTR channel occasionally enters the O2 subconductance state characteristic of the Δ19 CFTR channel. These observations suggest that the subconductance state associated with the Δ19 CFTR Cl\(^-\) channel is probably an intrinsic property of the CFTR protein, but the deletion affects CFTR in such a way as to favor the O2 conductance state.

A second possibility is that this loop interacts with other domains (nucleotide-binding folds, the regulatory domain, and other intracellular loops) in CFTR or with other cellular proteins that are involved in the conductance state transition. Deletion of part of this loop might then affect the channel structure allosterically by changing these inter- or intramolecular interactions and thus induce a quasi-stable open configuration in addition to the full open state. Understanding the distribution of subconductance states associated with the single Cl\(^-\) channel should provide valuable information about structure-function relationships in the CFTR Cl\(^-\) channel.

The approach reported here allows functional characterization of CFTR in any intracellular membranes besides the endoplasmic reticulum membrane (25). By preparing vesicles from intracellular membranes separated from the plasma membrane, in principle, one can study any processing mutant in the lipid bilayer system. Further experiments are necessary to test the role of specific amino acids of the intracellular loops (e.g. positively and negatively charged and hydrophobic amino acids) in the regulation of the conductance state of the CFTR Cl\(^-\) channel.

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Intracellular Loop between Transmembrane Segments IV and V of Cystic Fibrosis Transmembrane Conductance Regulator Is Involved in Regulation of Chloride Channel Conductance State

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