Contribution of Proline Residues in the Membrane-spanning Domains of Cystic Fibrosis Transmembrane Conductance Regulator to Chloride Channel Function*

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David N. Sheppard, Sue M. Travis, Hiroshi Ishihara, and Michael J. Welsh

From the Howard Hughes Medical Institute, Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

Proline residues located in membrane-spanning domains of transport proteins are thought to play an important structural role. In the cystic fibrosis transmembrane conductance regulator (CFTR), the predicted transmembrane segments contain four prolines: Pro99, Pro324, Pro1021, and Pro205. These residues are conserved across species, and mutations of two (P99L and P205S) are associated with cystic fibrosis. To evaluate the contribution of these prolines to CFTR Cl⁻ channel function, we mutated each residue individually to either alanine or glycine or mutated all four simultaneously to alanine (P-Quad-A). We also constructed the two cystic fibrosis-associated mutations. cAMP agonists stimulated whole cell Cl⁻ currents in HeLa cells expressing the individual constructs that resembled those produced by wild-type CFTR. However, the amount of current was decreased in the rank order: wild-type CFTR = Pro³²⁴ > Pro¹⁰²¹ > Pro⁹⁹ > Pro²⁰⁵. The anion selectivity sequence of the mutants (Br⁻ > CI⁻ > I⁻) resembled wild-type except for P99L (Br⁻ > CI⁻ > I⁻). Although the Pro９⁹, Pro³２⁴, and Pro¹⁰²¹ mutants produced mature protein, the amount of mature protein was much reduced with the Pro²⁰⁵ mutants, and the P-Quad-A made none. Because the Pro９⁹ constructs produced mature protein but had altered whole cell currents, we investigated their single-channel properties. Mutant channels were regulated like wild-type CFTR; however, single-channel conductance was decreased in the rank order: wild-type CFTR > P99G > P99L > P99A. These results suggest that proline residues in the transmembrane segments are important for CFTR function, Pro⁹⁹ may be critical for correct protein processing, and Provisions may contribute either directly or indirectly to the Cl⁻ channel pore.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a regulated Cl⁻ channel (for reviews see Refs. 2

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† Present address: Depts. of Medicine and Biochemistry, University of Edinburgh, Edinburgh, UK.

§ Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Institute, 500 EMRB, University of Iowa College of Medicine, Iowa City, IA 52242. Tel.: 319-335-7619; Fax: 319-335-7623; E-mail: mjwelsh@blue.weeg.uiowa.edu.

1 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; I-V, current-voltage; M, transmembrane segment; MSD, membrane-spanning domain; TES, 9-N-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid).
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Type Culture Collection (Rockville, MD). cAMP-dependent protein kinase was from Promega (Madison, WI) and [γ-32P]ATP was from Du-Pont NEN. MgATP, Na2ATP, 8-(4-chlorophenylthio)-cAMP sodium salt, forskolin, and 3-isobutyl-1-methylxanthine were from Sigma. All other chemicals were of reagent grade.

Statistics—Results are expressed as the means ± S.E. of n observations. To compare mean values, we used Student’s t test. Differences were considered statistically significant when the p value was <0.05.

RESULTS

Expression of Proline Mutants Generates cAMP-activated Cl− Currents—To examine the contribution of prolines in the MSDs to CFTR Cl− channel function, we mutated each proline individually to either alanine or glycine. We chose alanine and glycine because they might be expected to have different effects (10). Alanine has a small side chain and is prevalent in transmembrane α-helices. Because it lacks a side chain, glycine has significant conformational freedom and is frequently found in hinge regions. Like proline, glycine is not favored in transmembrane α-helices.

We expressed the mutants in HEK293 cells and studied their function using the whole cell patch-clamp technique. Cyclic AMP agonists activated whole cell currents in cells expressing each of the individual proline to alanine or glycine mutants and in cells expressing the CF-associated mutations, P99L and P205S. As an example, Fig. 2 shows data from studies of P99A, P99G, and P99L; qualitatively similar results were obtained with the Pro205, Pro204, and Pro1022 mutants (data not shown).

All of the proline mutants had whole cell properties that resembled those of wild-type CFTR (2, 3). Under basal conditions there was little or no whole cell current. Whole cell currents were reversibly activated by cAMP agonists, were time- and voltage-independent (Fig. 2A), had linear I-V relationships, and were selective for anions over cations (Fig. 2B).

Because the MSDs of CFTR contribute to the Cl−-conducting pore and control anion selectivity and because proline residues may have an important structural role, we speculated that these proline residues might contribute directly or indirectly to the anion selectivity filter. We tested this hypothesis by examining the anion permeability and conductance sequence of cAMP-stimulated whole cell currents. The anion selectivity sequence of whole cell currents in wild-type CFTR is Br− > Cl− > I− (Table I and Ref. 4). Most proline mutants had anion selectivity sequences qualitatively similar to that of wild-type CFTR (Figs. 3 and Table I). However, the CF-associated mutation P99L had an altered anion selectivity sequence: Br− > Cl− > I− (Figs. 3 and Table I). Despite the change in anion selectivity, all of the mutant channels retained their selectivity for anions over Na+ (for example see Figs. 2B and 3).

Because many mutations in CFTR have reduced cAMP-stimulated Cl− currents compared with wild type (5, 23), we measured the change in whole cell Cl− current that was stimulated by cAMP agonists. Fig. 4 shows that the Pro99 and Pro205 mutants generated <30% of wild-type Cl− current. Moreover, the CF-associated mutants retained <15% of wild-type Cl− current. The amount of Cl− current generated by the Pro204 mutants was indistinguishable from that of wild type, whereas the Pro1022 mutants retained intermediate amounts of Cl− current (Fig. 4). Uninfected HEK293 cells and cells infected with vTF7–3 as a control did not generate cAMP-activated Cl− currents (Fig. 4). In cells expressing P-Quad-A, no increase in current was observed under either basal or cAMP-stimulated conditions (Fig. 4). This suggests that either P-Quad-A does not form a functional Cl− channel, or it is severely misprocessed.

Analysis of the Processing of Proline Mutants—To determine why the proline mutants tended to generate less Cl− current, we studied the production of mature CFTR in HEK293 cells by analyzing the glycosylation status of CFTR protein (14). Wild-
type CFTR produces substantial amounts of mature, fully glycosylated protein (band C), whereas the CF-associated mutant ΔF508 produces little mature protein. Fig. 5A shows that like wild-type CFTR, the Pro99, Pro324, and Pro1021 mutants produced the band C form of CFTR. However, band C production was much reduced with the Pro205 mutants and the P-Quad-A mutant. Fig. 5B shows the relative amount of CFTR present in the mature form at 16 h after transfection. Production of mature protein was reduced in the rank order: wild-type CFTR > Pro1021 mutants > Pro324 mutants > Pro99 mutants > Pro205 mutants ≥ P-Quad-A = ΔF508.

**Single-channel Properties of the Pro99 Mutants**—Because the Pro99 mutants produced some mature protein, yet had altered whole cell properties, we examined their single-channel properties using excised, inside-out membrane patches. Like wild-type CFTR, mutant Cl⁻ channels were reversibly activated by phosphorylation with cAMP-dependent protein kinase and required intracellular MgATP to open (Fig. 6A) (2, 3). However, the single-channel current amplitudes of the Pro99 mutants were decreased compared with wild-type CFTR (Fig. 6B).
though for P99G the reduction was small, for P99A and P99L the effect was marked. To quantitate the reduction, we analyzed single-channel I-V relationships (Fig. 6C); we used a Cl\textsuperscript{2} concentration gradient (external [Cl\textsuperscript{2}], 10 mM; internal [Cl\textsuperscript{2}], 147 mM) to magnify the small current amplitude. We have previously shown that under these conditions, wild-type CFTR has a rectifying I-V relationship with a reversal potential at about 160 mV, consistent with Cl\textsuperscript{2} selectivity (see Fig. 4B in Ref. 5). At negative voltages, where the I-V relationship was linear, wild-type CFTR had a slope conductance of 7.72 ± 0.22 pS (n = 4). The conductance for P99G was 7.31 ± 0.24 pS (n = 5), not significantly different from wild type (p = 0.26). In contrast, the conductances of P99A and P99L were significantly decreased at 4.66 ± 0.25 pS (n = 5, p < 0.0001) and 4.97 ± 0.24 pS (n = 5, p < 0.0001), respectively. We could not determine the reversal potential of the mutant channels because of their small current amplitudes.

Because the single-channel current amplitudes of P99A and P99L were much reduced and because in most cases the patches of membrane contained large numbers of channels, we could not accurately measure single-channel kinetics. However, visual inspection suggested that the gating behavior of the Pro\textsuperscript{99} mutants was not dramatically different from wild-type CFTR with bursts of activity containing brief flickery closures separated by longer closures between bursts (Fig. 6B).

**DISCUSSION**

Our data indicate that proline residues located in the predicted transmembrane segments of the MSDs are important for CFTR Cl\textsuperscript{-} channel function. Pro\textsuperscript{205}, which lies in the middle of a putative \alpha-helix, is critical for correct protein processing. Pro\textsuperscript{99}, which is near the external surface of CFTR, may contribute either directly or indirectly to the Cl\textsuperscript{-} channel pore.

Proline residues in the transmembrane segments could affect the selectivity of the Cl\textsuperscript{-} channel, as indicated by the anion selectivity experiments (Fig. 3). The data are I-V relationships of cAMP-activated whole cell currents recorded from HeLa cells transiently expressing the indicated Pro\textsuperscript{99} mutants. I-V relationships (recorded as described in Fig. 2) were made in the presence of 140 mM Cl\textsuperscript{-}, Br\textsuperscript{-}, or I\textsuperscript{-} in the extracellular (bath) solution.

**FIG. 3.** Anion selectivity of Pro\textsuperscript{99} mutants. The data are I-V relationships of cAMP-activated whole cell currents recorded from HeLa cells transiently expressing the indicated Pro\textsuperscript{99} mutants. I-V relationships (recorded as described in Fig. 2) were made in the presence of 140 mM Cl\textsuperscript{-}, Br\textsuperscript{-}, or I\textsuperscript{-} in the extracellular (bath) solution.
fect structure in at least two ways. First, proline residues can form cis peptide bonds, and the energy barrier to cis-trans isomerization about the peptide bond preceding proline is reduced compared with other residues (10). Cis-trans isomerization of peptide bonds could produce conformational changes and thereby regulate channel activity (9). However, because in CFTR the proline mutants showed no dramatic changes in gating, cis-trans isomerization of peptide bonds involving prolines in the MSDs may not be an important determinant of CFTR gating behavior.

Second, as mentioned above, when proline occurs within the interior of a transmembrane α-helix, it disrupts the pattern of hydrogen bonding causing the α-helix to kink (10). The packing together of proline-kinked α-helices may contribute to the formation of either a channel vestibule or ion binding site(s) (11). For example, the pore of melittin is built from four proline-kinked α-helices (24).

Effect of Proline Mutations on CFTR Biosynthesis—None of the proline mutants were processed as efficiently as wild-type CFTR. We saw no clear correlation between the amount of band C produced by mutation of individual prolines and the propensity of the amino acids to occur in α-helices (A > L > G > P; Ref. 10). Pro205 and Pro224 appear to be less critical for correct protein processing because other amino acids could be accommodated at these positions. In contrast, Pro205, which is predicted to lie in the middle of M3, is crucial for correct protein folding and processing. All Pro205 mutants tested showed significant processing defects. Thus, Pro205 mutants may pro-

**Fig. 6.** Single-channel properties of Pro⁹⁹ mutants. A, regulation of P99A by phosphorylation with the catalytic subunit of cAMP-dependent protein kinase (75 nM) and intracellular MgATP (0.88 mM). Representative recording are from an excised inside-out membrane patch from a HeLa cell transiently expressing P99A. Dashed lines indicate the closed channel state and downward deflections correspond to channel openings. Voltage was −80 mV. The data traces were filtered at 0.5 kHz, digitized at 5 kHz and substitute averaged 10× using pClamp software. Similar results were observed with P99G and P99L; n > 5 for each mutant. B, representative single-channel recordings are from excised inside-out membrane patches from HeLa cells transiently expressing wild-type CFTR, P99A, P99G, and P99L. Recordings were made after channel activation by cAMP-dependent protein kinase-dependent phosphorylation. Voltage was −100 mV. For illustration purposes, traces have been digitally filtered at 0.3 kHz. C, single-channel I-V relationships of CFTR (circles), P99A (squares), P99G (triangles), and P99L (inverted triangles). The data points are the means ± S.E. of 2–5 values at each voltage. In many cases error bars are obscured by symbols. The lines are the mean slope conductance calculated from the slope conductance of individual experiments. No channel activity was observed in recordings from excised inside-out membrane patches from control virus-infected cells made under similar conditions (5).
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foundly disrupt the normal association and packing of transmembrane segments that would disrupt the processing of CFTR and its delivery to the cell membrane. The defect in processing explains why the P-Quad-A failed to generate an increase in whole cell current under either basal or cAMP-stimulated conditions.

Prior to this study, all CF-associated mutations known to manifest a processing defect were located within the nucleotide-binding domains (14, 23, 25). The finding that a mutation in a transmembrane sequence can disrupt processing has a precedent in the T cell antigen receptor and viral glycoproteins (26, 27). Moreover, in the related transport protein, human P-glycoprotein, mutations in M7, including P709 located at the intracellular end of M7, disrupt protein processing (28, 29).

Effect of Proline Mutations on Cl− Channel Function—The whole cell properties of the individual proline mutants resembled those of wild-type CFTR. Thus, although the proline mutations affected biosynthesis, they did not produce sufficient disruption of structure to abolish Cl− channel function. In this regard they are similar to the CF mutations A455E and P574H that disrupt processing but generate channels that retain significant activity (23). Nevertheless, some changes in the properties of the mutant channels were observed. When Pro99 was mutated to leucine, the channel lost its ability to discriminate between Cl− and I−. Interestingly, substitution of alanine and glycine at Pro99 did not alter anion selectivity. We speculate that leucine, with its bulky side chain, may directly or indirectly disrupt the conformation of an anion binding site located near Pro99 and alter anion selectivity. In contrast, alanine and glycine with their shorter side chains may be more easily accommodated at this position and do not significantly affect anion selectivity.

Measurements of single-channel conductance also suggest that Pro99 contributes directly or indirectly to the formation of the Cl− channel pore. Substitution of alanine, glycine, and leucine at Pro99 decreased single-channel conductance in the rank order: wild-type CFTR > P99G > P99L ≥ P99A. Interestingly, the propensity for these amino acids to occur in α-helices follows the reverse order (A > L > G ≥ P; Ref. 10). Based on these observations, we speculate that Pro99 kinks M1 to form part of the channel structure. Glycine, which is found in hinge regions and like proline is not favored in α-helices, can substitute for proline at this residue because the single-channel conductance of P99G does not differ from wild type. In contrast, we speculate that alanine and leucine, residues that are prevalent in α-helices, eliminate the kink in M1 caused by Pro99, thereby reducing the access of permeant ions into the channel pore and decreasing single-channel conductance. Because P99C did not react with sulfhydryl-specific reagents, Akabas and collaborators concluded that Pro99 does not line the channel pore (7). Our data and their data are compatible if Pro99 contributes to pore architecture without itself lining the pore where it would be accessible to the hydrophilic sulfhydryl blockers.

Implications for Cystic Fibrosis—P99L and P205S are CF mutations located in MSD1 that are associated with a milder (pancreatic sufficiency) clinical phenotype (12, 13). Our studies of the processing and function of P99L and P205S explain why these mutants generate less Cl− current than wild-type CFTR. Loss of Cl− channel function caused by P205S was predominately a result of defective protein processing; whereas that caused by P99L was a consequence of both defective protein processing and altered Cl− channel function. Our results suggest that these mutations are associated with a milder (pancreatic sufficiency) clinical phenotype because a small amount of mutant protein is processed correctly and generates cAMP-activated CFTR Cl− currents. When we have studied the ΔF508 mutant (associated with a severe clinical phenotype) under similar conditions we found no Cl− current. The mutant ΔF508 is defectively processed in both native epithelia and heterologous cells (14, 22). This suggests that the defective processing of the P205S mutant observed in HeLa cells likely accounts for the loss of Cl− channel function in patients bearing this mutation. We previously speculated that pharmacological therapies designed to increase the activity of mutant channels in the plasma membrane might be useful for treating patients bearing these mutations associated with a milder clinical phenotype (5). Potential pharmacological therapies may include phosphatase inhibitors such as bromotetramisole and novel Cl− channel openers such as the substituted benzimidazole NS004 that have been demonstrated to activate mutant Cl− channels in recombinant cells (30, 31).

The present results complement and extend our previous study of mild CF mutants located in MSD1 (R117H, R334W, and R347P). We showed that these mutants form Cl− channels with altered permeation properties but are processed normally (5). The mechanism of dysfunction of P205S resembles that of the nucleotide-binding domain 1 pancreatic sufficiency mutants A455E and P574H, which are misprocessed (23). Interestingly, P99L forms a Cl− channel with altered pore properties and is also misprocessed. Thus, these results demonstrate that the mechanisms by which CF mutations produce defective Cl− channels are complex and that it is not possible to predict the mechanism of dysfunction of CFTR based solely on the site of mutation.

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