Destabilization of the Transmembrane Domain Induces Misfolding in a Phenotypic Mutant of Cystic Fibrosis Transmembrane Conductance Regulator*

Received for publication, September 1, 2004, and in revised form, November 8, 2004
Published, JBC Papers in Press, November 10, 2004, DOI 10.1074/jbc.M410069200

Mei Y. Choi‡§§, Anthony W. Partridge‡§§, Craig Daniels***‡‡, Kai Du****, Gergely L. Lukacs*****‡‡, and Charles M. Deber‡§§

From the **Division of Structural Biology and Biochemistry and ***Program in Cell and Lung Biology, Research Institute, Hospital for Sick Children, Toronto, Ontario M5G 1X8 and the Departments of §Biochemistry and ‡‡Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Two phenotypic missense mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) channel pore (L346P and R347P in transmembrane (TM) segment 6) involve gain of a proline residue, but only L346P represents a significant loss of segment hydrophathy. We show here that, for synthetic peptides corresponding to sequences of CFTR TM6 segments, circular dichroism spectra of wild type and R347P TM6 in membrane mimetic environments are virtually identical, but L346P loses ~50% helicity, implying a membrane insertion defect in the latter mutant. A similar defect was observed in the corresponding double-spanning (“hairpin”) TM5/6-L346P synthetic peptide. Examination of the biogenesis of CFTR revealed that the full-length protein harboring the L346P mutation is rapidly degraded at the endoplasmic reticulum (ER), whereas the wild type and the R347P protein process normally. Furthermore, a second site mutation (R347T) that restores chloride channel function of full-length L346P CFTR. The correlated in vitro membrane insertion and folding of the TM5/6-L346P peptide also rescues the folding and cell surface membrane insertion and folding of the TM5/6-L346P peptide also rescues the folding and cell surface chloride channel function of full-length L346P CFTR.

Polytopic membrane proteins may be particularly susceptible to folding defects when non-native residues of high hydrophilicity are introduced into transmembrane positions (1). These phenomena are epitomized by the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel protein that becomes dysfunctional in cystic fibrosis, the most common lethal autosomal recessive genetic disease in the Caucasian population (2). CFTR belongs to the ATP-binding cassette membrane transporter gene superfamily (3). Established as the CF gene product (4), CFTR contains 1480 amino acids and consists of two homologous halves (NBD1 and NBD2) and a predicted six-transmembrane (TM) domain (TM1–TM6). The two halves are linked by a cytoplasmic regulatory R-domain. Deletion of Phe-508 (∆F508) in NBD1, the most common mutation among CF patients (67% among all the CF patients) (6), causes CFTR misfolding in the endoplasmic reticulum that gives rise to degradation of the protein and the severe clinical phenotype (5). However, of the more than 1000 CF-phenotypic mutations in CFTR that have now been reported, over 100 occur in membrane-spanning regions.

Critical components of the channel pore have been identified in CFTR TM helices 5 and 6 (TM5/6, residues 308–350). TM6 has been shown to have a role in the determination of the permeation properties of CFTR (7); several residues in TM6 have been proposed to contribute to the anion binding sites, including Arg-334, Lys-335, Phe-337, Thr-338, Ser-341, Arg-347, and Arg-352, whereas the central region of TM6 has been localized as a main determinant of both anion binding and anion selectivity in CFTR (6–9). A number of mutations occurring in TM5/6 have been found to cause mild (usually pancreatic sufficient) forms of CF, two of which involve introduction of a proline residue: L346P, a mutation that was identified in two unrelated Cypriot patients in 1994 (10); and a second sequentially adjacent CF-phenotypic mutant, R347P (11). It has been suggested that Arg-347 in TM6 forms a salt bridge with an aspartate (∆F-979) located in TM9 (12), an interaction that would be abrogated by the loss of the native TM6 Arg residue. Arg-347 has also been proposed to contribute to the pore of the CFTR Cl− channel and anion conduction (8), and the pore properties of the channel have been observed to be altered in various Arg-347 mutants (6, 8). Pro residues have typically been implicated in channel gating when they occur in pore-forming helices (13, 14).

Gain of Pro in a TM helix can have several consequences. The Pro pyrrolidine ring is bulky, which causes steric constraints on the conformation of the preceding residue in the helix (15). As well, Pro residues may introduce a kink in the helix structure (16, 17). And, as an imino acid, Pro lacks an amide proton on the H-bonds (18). Forming helices (13, 14).

Critical components of the channel pore have been identified in CFTR TM helices 5 and 6 (TM5/6, residues 308–350). TM6 has been shown to have a role in the determination of the permeation properties of CFTR (7); several residues in TM6 have been proposed to contribute to the anion binding sites, including Arg-334, Lys-335, Phe-337, Thr-338, Ser-341, Arg-347, and Arg-352, whereas the central region of TM6 has been localized as a main determinant of both anion binding and anion selectivity in CFTR (6–9). A number of mutations occurring in TM5/6 have been found to cause mild (usually pancreatic sufficient) forms of CF, two of which involve introduction of a proline residue: L346P, a mutation that was identified in two unrelated Cypriot patients in 1994 (10); and a second sequentially adjacent CF-phenotypic mutant, R347P (11). It has been suggested that Arg-347 in TM6 forms a salt bridge with an aspartate (∆F-979) located in TM9 (12), an interaction that would be abrogated by the loss of the native TM6 Arg residue. Arg-347 has also been proposed to contribute to the pore of the CFTR Cl− channel and anion conduction (8), and the pore properties of the channel have been observed to be altered in various Arg-347 mutants (6, 8). Pro residues have typically been implicated in channel gating when they occur in pore-forming helices (13, 14).

Gain of Pro in a TM helix can have several consequences. The Pro pyrrolidine ring is bulky, which causes steric constraints on the conformation of the preceding residue in the helix (15). As well, Pro residues may introduce a kink in the helix structure (16, 17). And, as an imino acid, Pro lacks an amide proton on the H-bonds (18). As such, the introduction of Pro into a TM helix phosphocholine; TM, transmembrane; Tris, tris(hydroxymethyl)amino- methane; WT, wild type; HA, hemagglutinin; Ab, antibody.

* This work was supported, in part, by grants (to G. L. L. and C. M. D.) from the Canadian Cystic Fibrosis Foundation, the Canadian Institutes of Health Research (CIHR), and the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by an award from the Hospital for Sick Children Research Training Committee.

§§ To whom correspondence should be addressed. Tel.: 416-813-5924; Fax: 416-813-5005; E-mail: deber@sickkids.ca (to C. M. D.) or glukacs@sickkids.ca (to G. L. L.).

1 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; BHK, baby hamster kidney; CD, circular dichroism; CF, cystic fibrosis; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; LPC, 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine; TM, transmembrane; Tris, tris(hydroxymethyl)amino- methane; WT, wild type; HA, hemagglutinin; Ab, antibody.
increases the net hydrophilicity of the segment, because it results in a non-H-bonded backbone carbonyl group in the preceding turn of the helix. Furthermore, in CFTR mutant L346P, the loss of Leu significantly increases the local hydrophilicity of this TM segment, i.e. on the Liu-Deber hydrophathy index where values are scaled between +5 and −5, Leu ranks third (+4.76), whereas Pro ranks 19th (−4.92) out of the 20 commonly occurring amino acids (19). In the case of R347P, the Arg positive charge is lost, but this mutation exchanges a polar residue (Arg ranks 14th, at −2.77) with one of comparable hydrophilicity.

In the present work, we have used solid-phase peptide synthesis to prepare sequences corresponding to TM6 segments of wild type (WT) and mutants L346P and R347P of CFTR, along with some corresponding double-spanning TM5/6 peptides for comparative structural analyses. In parallel, we examined the relative effects of L346P versus R347P on cellular processing of full-length CFTR. The results provide striking in vivo/in vitro correlates of the consequences of a missense mutation located in the predicted TM6 segment and demonstrate that destabilizing local hydrophobic character may represent a sufficient signal for recognizing CFTR as a non-native protein by the ER quality control.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Purification**—Peptides were synthesized using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a PerSeptive Biosystems Pioneer peptide synthesizer (20). Synthesis employed the use of the Pioneer’s extended cycle for TM5/6 peptides or single-spanning TM6 peptides. In a typical synthesis, 0.22 mmol/g PAL-PEG-PS resin (Applied Biosystems) was used to produce amidated C terminus. The peptides were then cleaved with a mixture of 88% trifluoroacetic acid/5% phenol/5% ultrapure water/2% triisopropylsilane. The cleaved peptides were precipitated with ice-cold diethyl ether. Centrifuged pellets were redissolved in 5% acetonitrile and lyophilized.

**Circular Dichroism Spectroscopy**—CD spectra were collected using a Jasco J-720 spectropolarimeter. Lyophilized peptide was added to a buffer containing 50 mM LPC and 25 mM Tris at pH 8.0, and the mixture was vortexed to ensure complete peptide solubilization. Samples were measured at peptide concentrations between 25 and 50 μM. Measurements were taken using a quartz cuvette with a path length of 0.1 mm at room temperature. Spectral scans were performed from 250 to 190 nm with a step resolution of 0.4 nm, a speed of 20 nm/min, and a bandwidth of 1.0 nm. Spectra were measured at room temperature in the predicted TM6 segment and demonstrate that destabilizing local hydrophobic character may represent a sufficient signal for recognizing CFTR as a non-native protein by the ER quality control.

**Construction and Expression of CFTR Variants in Mammalian Cells**—The L346P, R347P, and R347H CFTR mutants were constructed by site-directed mutagenesis using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a PerSeptive Biosystems Pioneer peptide synthesizer (20). Synthesis employed the use of the Pioneer’s extended cycle for TM5/6 peptides or single-spanning TM6 peptides. In a typical synthesis, 0.22 mmol/g PAL-PEG-PS resin (Applied Biosystems) was used to produce amidated C terminus. The peptides were then cleaved with a mixture of 88% trifluoroacetic acid/5% phenol/5% ultrapure water/2% triisopropylsilane. The cleaved peptides were precipitated with ice-cold diethyl ether. Centrifuged pellets were redissolved in 5% acetonitrile and lyophilized. The crude peptide powder was dissolved in 30% acetonitrile, and 10 mg of the crude peptide was loaded onto a C4 preparative reversed phase-high performance liquid chromatography column and then eluted with a water/acetonitrile gradient (30–75% acetonitrile over 60 min). The major peak was collected and lyophilized. Mass spectrometry was used to confirm the molecular weight of the purified peptide.

**Fluorescence Resonance Energy Transfer Analysis**—FRET measurements were performed using peptides labeled with dansyl chloride as the acceptor fluorophore at the N terminus, with the Trp residue in the predicted TM6 segment and demonstrate that destabilizing local hydrophobic character may represent a sufficient signal for recognizing CFTR as a non-native protein by the ER quality control.

**TABLE I**

**Table I**

**Sequences of CFTR TM6 and TM5/6 peptides prepared in this work**

| Name            | Sequences                                      | Molecular weight (Da) |
|-----------------|------------------------------------------------|-----------------------|
| TM6-WT          | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 3664                  |
| TM6-L346P       | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 3648                  |
| TM6-R347P       | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 3605                  |
| TM6-WT(F342W)   | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 3703                  |
| TM6-L346P(F342W)| KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 3687                  |
| TM5/6-WT        | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 7474                  |
| TM5/6-L346P     | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 7489                  |
| TM5/6-WT(W)     | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 7758                  |
| TM5/6-L346P(W)  | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 7742                  |
| TM5/6-R347I     | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW         | 3605                  |
| TM5/6-L346P/R347I | KKKKGILKRKFTTFSSSTPHAVLMATVRQFVKKKW     | 7699                  |
by overlapping PCR using the appropriate mutagenic primers. The PCR products were subcloned into the BspEI/Bst1107I sites of CFTR. Baby hamster kidney (BHK) cells were stably transfected with the pNUT expression plasmids, containing the wild type (WT), L346P, or R347P CFTR, harboring an HA-epitope in the C-terminal tail of CFTR (CFTR-C<sub>terminal</sub>-HA) (21). Following clonal selection in the presence of methotrexate (500 μM), 50–100 individual colonies were pooled and expanded for experiments. Transient expression of COS-1 cells was carried out as described previously (22).

Immunoblotting and Metabolic Pulse-chase Studies—CFTR immunoblotting was performed with the mouse monoclonal anti-HA Ab (Covance) using enhanced chemiluminescence (ECL) detection, and immunoblots were quantified with densitometry, as described previously (23). The Na<sup>+</sup>K<sup>-</sup>-ATPase was visualized by the a6F Ab (Developmental Studies Hybridoma Bank, University of Iowa). The stability of CFTR variants was monitored by the pulse-chase technique. First, the cellular methionine and cysteine content was depleted in Met- and Cys-free medium (37 °C, 30 min) and then pulse-labeled for 15 min in the presence of 0.1 nM of [35S]methionine and [35S]cysteine (Amersham Biosciences) at 37 °C. Following the indicated chase period in complete medium, membrane proteins were solubilized in 1 ml radioimmune precipitation assay buffer (150 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate, pH 8.0) supplemented with protease inhibitors (10 μg/ml leupeptin and pepstatin, 10 μM phenylmethylsulfonyl fluoride, and 10 μM iodoacetamide). Immunoprecipitates, obtained with anti-HA Ab, were analyzed by SDS-PAGE and fluorography. The radioactivity incorporated into CFTR was quantified using a PhosphorImager (Amersham Biosciences) with the ImageQuaNT software (Molecular Dynamics) as described (24).

Iodide Efflux Assay—The plasma membrane cAMP-dependent chloride conductance of stably transfected BHK cells was determined by the iodide efflux assay (25). Iodide efflux was initiated by replacing the loading buffer with efflux medium (composed of 136 mM nitrate in place of iodide). The extracellular medium was replaced every minute with efflux medium (1 ml). After a steady state was reached, the intracellular cAMP level was raised by agonists (10 μM forskolin, 0.2 μM CPT-cAMP, and 0.2 mM isobutylmethyl xanthane) to achieve maximal phosphorylation of CFTR. The collection of the efflux medium was resumed for an additional 6–9 min. The amount of iodide in each sample was determined with an iodide selective electrode (Orion).

RESULTS

Hydrophobicity Threshold of CF-phenotypic Mutant TM Segments—Although both L346P and R347P represent a gain of a Pro residue in CFTR TM6, the resulting local 346/347 diads (PR and LP, respectively) differ significantly in hydrophobic character. This situation is highlighted using TM Finder, a web-based program that reliably predicts whether a stretch of amino acids has sufficient hydrophobicity to exist as a stably inserted transmembrane helix (26). The outputs for the wild type (WT) and L346P CFTR sequences support an initial hypothesis that the L346P mutation decreases the average net hydrophobicity of the original TM6 segment sufficiently to prevent the proper membrane insertion of the full TM6 segment (Fig. 1, A and B). Specifically, the program identifies a 20-amino acid stretch (residues 330–349) in the WT CFTR sequence that likely corresponds to the membrane-embedded residues of TM6. In contrast, the output for the L346P mutant sequence (residues 330–341) fails to predict a sufficiently long stretch of amino acids that are above the threshold hydrophobicity required for a TM helix (27, 28). However, unlike L346P, the R347P mutation doesn’t involve a significant change in hydrophobicity, and TM Finder predicts that the R347P mutant has the same membrane-inserted amino acid stretch (residues 330–349) as the WT TM6 sequence (Fig. 1C). It may be noted that programs such as TM Finder may underestimate the actual total of membrane-inserted residues in a given TM segment, because interfacial residues at both termini of the segment tend to be rich in hydrophilic residues, such as Lys and Arg, which strongly drive the local average hydrophathy down (26).

Synthesis and Circular Dichroism Spectra of CFTR TM6 Peptides—To experimentally test the TM-Finder prediction, we
synthesized Lys-tagged versions of the TM6-WT and the two mutant (L346P and R347P) sequences (Table I). The Lys-tagged methodology involves adding several Lys residues (in the present case, three Lys) to both the N and C termini, a strategy that greatly facilitates peptide purification and characterization while maintaining the native-like structural properties of the TM segment they flank (20). The circular dichroism (CD) spectra of these two peptides are shown in Fig. 2A. Although the TM6-WT peptide adopted an \( /H\) helical structure in the presence of LPC micelles, the TM6-L346P peptide displays only \( /H\) 50% of the helicity observed for the TM6-WT sequence. In contrast, the CD spectra of TM6-R347P and the TM6-WT peptides are virtually superimposable.

Because the L346 locus appeared to be most affected by the Pro mutation, we further examined the effect of the L346P mutation in an expanded context by synthesizing the helix-loop-helix TM5/6-WT and TM5/6-L346P peptides. As “helical hairpins” when folded, these constructs constitute the minimal tertiary contact systems representative of the CFTR TM domain (29). CD spectra indicate that the TM5/6-L346P construct exhibits a 25% decrease in helicity when compared with the TM5/6-WT construct in SDS micelles, consistent with a 50% decrease in one TM helix (Fig. 2B).

Fluorescence Studies of CFTR Single TM6 and Double TM5/6 Peptides—The proposition that the L346P TM segment is only partially inserted into micellar membranes was further examined by fluorescence experiments. To this end, we synthesized two additional peptides containing a TM-embedded fluorescent probe introduced through the conservative F342W mutation (TM6-WT(F342W) and TM6-L346P(F342W), respectively) (Table I). Characteristic Trp fluorescence spectra of these two peptides in detergent micelles are presented in Fig. 2C. Noting that a membrane-embedded Trp residue will typically display increased fluorescence intensity with a blue-shifted position versus an aqueous-located counterpart, the data indicate that the Trp residue in the WT species resides in an apolar environment (maximum near 320 nm) whereas the Trp in the L346P peptide is largely aqueous exposed (shoulder near 340 nm), supporting the notion that the apolar-to-polar mutation prevents proper TM6 insertion.

Fluorescence resonance energy transfer (FRET), the transfer of the excited-state energy from the initially excited donor to an acceptor, can provide further information as to the proximity of donor and acceptor chromophores (30). We exploited this phenomenon to detect differences in the distance between the N and C termini in TM5/6 peptides. To perform this analysis, we modified the sequences of the peptides through inser-
Disruption of CFTR Folding by a CF-phenotypic Mutant

A Second Site Mutation in TM6 Restores Biosynthetic Processing of CFTR—To test the assumption that destabilization of local TM5/6 hairpin formation may inhibit post-translational folding in the context of full-length CFTR, we searched for a second site mutation that could re-establish the stability of the L346P TM5/6. Consideration of amino acid replacements in the vicinity of the L346P mutation identified a second site mutation (R347I) that restored the hydrophobicity of the TM6 L346P-containing segment to the threshold level that ensured membrane insertion according to TM Finder (Fig. 1D) (26). The L346P/R347I single spanning TM6 peptide was first synthesized, and analysis of its CD spectrum confirmed that this mutation restored the α-helical content of this TM6 double mutant to its WT counterpart (Fig. 4A).

We then assessed whether the R347I mutation could restore hairpin formation of the TM5/6-L346P polypeptide by the FRET assay. Using a TM5/6-L346P/R347I peptide in which a Trp residue was inserted near the C terminus (Table I), and in which the N terminus was labeled with a dansyl group, we found that the donor fluorescence quench in the double mutant was now similar to that of the WT TM5/6 (Fig. 4B).

If destabilization of the TM5/6 hairpin accounts for the processing defect of the L346P CFTR, introducing the second site mutation should correspondingly restore the folding and biosynthetic processing of full-length CFTR. This was indeed the case. Immunoblot analysis demonstrated the appearance of the complex-glycosylated L346P/R347I CFTR in both transiently transfected COS-1 and stably transfected BHK cells, whereas no detectable amount of L346P CFTR was present (Figs. 3B and 5A, respectively). Functional assessment of the plasma membrane protein kinase A-activated halide conductance confirming the partial reversal of the processing defect by demonstrating that the cAMP-stimulated iodide release of the L346P CFTR (6.3 ± 0.2 nmol/min) was increased by 3-fold in the presence of the second site mutation (18.4 ± 0.3 nmol/min) (Fig. 5B). The detection of L346P CFTR by functional assay, but not by immunoblotting, is conceivable due to the higher sensitivity of the iodide efflux assay.

DISCUSSION

Membrane Insertion Defects in CF-phenotypic Mutants—Misfolding of the membrane domains of polytopic proteins arising from genetic mutations can account for the molecular basis of human disease. In the present work, we have performed parallel in vitro and in vivo studies on selected CF-phenotypic TM-based mutations of CFTR of which both involve a gain of proline, with the outcome that the introduction of a Pro residue per se is not the determinant of folding. Rather, the context of

FIG. 4. Circular dichroism and fluorescence spectra for CFTR TM6 peptides. A, CD spectra of TM6-WT and TM6-L346P/R347I in LPC micelles. B, Trp fluorescence spectra for the unlabeled and labeled TM5/6-L346P/W peptide as compared with its WT counterpart (Fig. 2A). Note that the donor quench in case of mutant TM5/6-L346P/W peptide is now similar to that of the WT TM5/6 (Fig. 4B).

The L346P Mutation Impairs the Folding of CFTR in Vivo—ER-retained, core-glycosylated (or incompletely folded) CFTR can be readily distinguished from the mature, complex-glycosylated (or folded) CFTR by immunoblot analysis, based on the faster electrophoretic mobility of the core-glycosylated form compared with the complex-glycosylated CFTR. Because impaired post-translational folding of the CFTR usually causes its biosynthetic processing arrest, we examined the processing of full-length L346P- and R347P-CFTR by immunoblotting and pulse-chase analysis of BHK cells, which stably express CFTR. To facilitate the detection of CFTR, an HA-epitope tag was inserted at the C terminus of the channel (CFTR-CmHA) (21). As shown in Fig. 3A, immunoblot analysis of equal amounts of cell extracts demonstrated that the L346P, but not the R347P, mutation, prevented the expression of the complex-glycosylated CFTR. The R347H missense mutation, associated with a mild functional defect of CFTR channel activity, was also expressed at the same level as the WT CFTR, confirming previous reports (31). Similar results were obtained in transiently transfected COS-1 cells, indicating that the cellular phenotype of the L346P CFTR is independent of the expression system used (Fig. 3B).

Impaired steady-state expression of L346P-CFTR could be a consequence of its rapid degradation at the ER and/or of accelerated removal of the channel from post-Golgi compartments (32). To distinguish between these scenarios, the biogenesis of L346P CFTR was monitored in BHK cells by the metabolic pulse-chase technique. Although the accumulation of the complex-glycosylated WT-CFTR was obvious after 2 h of chase, the L346P mutation prevented the appearance of the complex-glycosylated form as shown by autoradiography (Fig. 3C). These results suggest that the L346P mutation imposes a folding defect on CFTR, leading to the retention and degradation of the mutant at the ER. On the other hand, the L346P mutation does not appear to cause premature translational termination or failure of the TM5–6 or TM7–8 segments to insert into the ER, because similar amounts of radioactively labeled core-glycosylated WT and L346P CFTR were accumulated during a 10-min radioactive labeling (Fig. 3D).
Given that, in intact CFTR, structural effects within TM6 likely are influenced by the sequentially vicinal TM5 segment, it is conceivable that interactions with such neighboring helices could stabilize the inserted state of a mutant TM6 sequence. Previous authors have speculated that in certain cases, the insertion of TM helices can be aided by interactions with neighboring TM segments (33, 34). To address this situation, we synthesized and compared helix-loop-helix peptides corresponding to the CFTR TM5/6-WT and the TM5/6-L346P sequence. However, we observed a corresponding decrease in the helical content for the mutant sequence (in this experiment, ~25% of total helical content), supporting the findings with the single-spanning species (Fig. 2B) that approximately half of TM6 becomes aqueous-exposed. Fluorescence resonance energy transfer experiments on labeled TM5/6 constructs further suggested that L346P prevents formation of proper TM6 topology, because FRET effects were significantly reduced in the TM5/6-L346P peptide versus the corresponding WT construct.

The physiological role of the TM hairpins, which represent the basic tertiary unit in the topogenesis and folding of multi-spanning membrane proteins, is not fully understood. Although the formation of helix-helix interactions in TM hairpins is likely to be required for co- or post-translational integration (35), the cytosolic surface of TM domains may serve as a platform for the binding of the large cytosolic domains (including the NBD domains), as indicated by the crystal structure of MsbA and BtuD (35, 36). Therefore, disrupting or destabilizing the topology of the TM5–TM6 may impair the global conformation of CFTR by perturbing interdomain interactions in vivo. The biogenesis of wild type CFTR itself is inefficient. Depending on the expression system used, only 20–60% of newly synthesized WT is converted into the fully processed complex-glycosylated form (24, 37). This inefficient maturation of wild type CFTR has been attributed to the fact that the polar-residue-rich TM6 fails to behave as a proper membrane anchor (11). Thus, a mutation in TM6 such as L346P may further reduce the efficiency of proper membrane anchoring of the protein.

Consistent with these considerations, we found that full-length CFTR protein harboring the L346P mutation is subjected to core glycosylation but was unable to fold and was rapidly degraded in vivo. There are two suggested mechanisms to counteract this anchoring deficiency: the ribosome and the ER translocon co-operate to prevent TM6 from passing through the membrane co-translationally and/or cytosolic domains of the ion channel post-translationally maintain TM6 in a membrane-spanning topology (11). Because the full-length L346P protein is indeed synthesized, an additional possibility is that the protein is able to compensate, at least in part, for the topological defect at the TM5/6 locus via TM-packing interactions with the second (TM7–12) CFTR TM domain. Based on this study, it appears that L346P may affect local CFTR TM5/6 structure to such an extent that the ER-associated quality-control mechanism recognizes the mutant as non-native and marks it for degradation. As a result, escape from the ER and cell surface delivery of L346P CFTR is severely compromised.
Disruption of CFTR Folding by a CF-phenotypic Mutant

However, loss of an Arg residue at the adjacent position aliphic Pro residue drops TM6 below its threshold residue concomitant with the introduction of the hydrophobicity must be weighed against the properties/function(s) attributability/function, as any (new) properties the Pro residue imparts Pro-347 per se character (43–46), rather than preventing post-translational changes in the selectivity or effectiveness of the channel pore of CFTR stemming from loss of side-chain positive character (43–46), rather than preventing post-translational folding. Our work provides insight into the diversity of local phenomena that can produce dysfunctional forms of CFTR and of membrane proteins generally and suggests that similar circumstances will contribute to molecular defects that underlie human diseases.

Acknowledgments—M. Y. C. is grateful for an award from the Hospital for Sick Children Research Training Committee. We are indebted to Dr. N. Kartner for providing M3A7 and L12B anti-CFTR antibodies.

REFERENCES

1. Zhou, F. X., Cocco, M. J., Russ, W., Brugner, A. T., and Engelman, D. M. (2000) Nat. Struct. Biol. 7, 91–94
2. Ramsey, B. W. (1996) N. Engl. J. Med. 335, 179–188
3. Tan, A. L., Ong, S. A., and Venkatesh, B. (2003) Arch. Biochem. Biophys. 401, 215–222
4. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenkiewicz, J., Sek, S., Plavnik, N., Chou, J. L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L.-C. (1989) Science 245, 1066–1073
5. Akabas, M. H. (2000) J. Biol. Chem. 275, 3729–3732
6. Sheppard, D. N., Rich, D. P., Ostegdaard, L. S., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1995) Nature 372, 160–164
7. Gouy, X., Burridge, S. M., Cowley, E. A., and Lindell, P. (2002) J. Physiol. 540, 39–47
8. Anderson, M. P., Gregory, R. J., Thompson, S., Souza, D. W., Paul, S., Mulligan, R. C., Smith, A. E., and Welsh, M. J. (1991) Science 253, 202–205
9. Chen, J. M., Cutler, C., Jacques, C., Beauf, G., Denamur, E., Lecointre, G., Mercier, B., Cramb, G., and Feree, C. (2001) Mol. Biol. Ecol. 18, 1771–1788
10. Botreau, K., Papageorgiou, E., Georgiou, G., Angastiniotis, M., Middleton, L. T., and Constantinou-Deltas, C. D. (1996) Hum. Genet. 99, 529–532
11. Teafter, M., and Hartl, F. (1999) EMBO J. 18, 6290–6298
12. Smith, S. S., Liu, X., Zhang, Z. R., Sun, F., Kriewall, T. E., McCarty, N. A., and Dawson, D. C. (2001) J. Gen. Physiol. 118, 407–431
13. von Heijne, G. (1991) J. Mol. Biol. 218, 499–503
14. Woolfson, D. N., and Williams, D. H. (1990) FEBS Lett. 277, 185–188
15. Hurley, H. J., Mason, D. A., and Matthews, B. W. (1992) Biopolymers 32, 1443–1446
16. Williams, K. A., and Deber, C. M. (1991) Biochemistry 30, 8919–8923
17. Yohannan, S., Yang, D., Faham, S., Boulting, G., Whitelegge, J., and Bowie, J. U. (2004) J. Mol. Biol. 341, 1–6
18. Deber, C. M., and Therien, A. G. (2002) Nat. Struct. Biol. 9, 318–319
19. Liu, L.-P., and Deber, C. M. (1998) Biopolymers (Peptide Sci.) 47, 41–62
20. Mackey, R. A., Partridge, A. W., and Deber, C. M. (2001) Biochemistry 40, 11106–11113
21. Benharouga, M., Sharma, M., So, J., Haarrit, M., Dryzmala, L., Popov, M., Schwapach, B., Grinstein, S., Du, K., and Lukacs, G. L. (2003) J. Biol. Chem. 278, 22079–22089
22. Sharma, M., Benharouga, M., Lechardre, D., Kartner, N., and Lukacs, G. L. (1999) J. Biol. Chem. 274, 21873–21877
23. Sharma, M., Benharouga, M., Hu, W., and Lukacs, G. L. (2001) J. Biol. Chem. 276, 8942–8950
24. Lukacs, G. L., Mohamed, A., Kartner, N., Chang, X. B., Riordan, J. R., and Grinstein, S. (1994) EMBO J. 13, 6076–6080
25. Mohamed, A., Ferguson, D., Seibert, B., Cai, H. M., Kartner, N., Grinstein, S., Riordan, J. R., and Lukacs, G. L. (1997) Biochem. J. 322, 259–265
26. Deber, C. M., Wang, C., Liu, L. P., Prior, A. S., Agrawal, S., Muskat, B. L., and Cutichio, A. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 212–219
27. Liu, L.-P., and Deber, C. M. (1998) J. Biol. Chem. 273, 23645–23648
28. Liu, L.-P., and Deber, C. M. (1999) Bioorg. Med. Chem. 7, 1–7
29. Therien, A. G., Grant, F. E., and Deber, C. M. (2001) Nat. Struct. Biol. 8, 597–602
30. Adair, B. D., and Engelman, D. M. (1994) Biochemistry 33, 5539–5544
31. Jain, S., Fritsch, J., Lehmann-Che, J., Bali, M., Aroua, N., Goossens, M., Edelman, A., and Ferec, C. (2001) J. Biol. Chem. 276, 9045–9049
32. Sharma, M., Pampinella, F., Nemes, C., Benharouga, M., So, J., Du, K., Bache, K. G., Papsin, B., Zerangue, N., Stenmark, H., and Lukacs, G. L. (2004) J. Cell Biol. 164, 923–933
33. Carveth, K., Buck, T., Anthony, V., and Skach, W. R. (2002) J. Biol. Chem. 277, 39507–39514
34. Sanders, R. C., and Myers, J. K. (2004) Annu. Rev. Biophys. Biomol. Struct. 33, 25–51
35. Chang, G. (2003) J. Biol. Chem. 330, 419–430
36. Locher, K. P., Lee, A. T., and Rees, D. C. (2002) Science 296, 1091–1098
37. Ward, C. L., and Kopito, R. R. (1994) J. Biol. Chem. 269, 25710–25718
38. Ellgaard, L., and Helenius, A. (2003) Nat. Rev. Mol. Cell Biol. 4, 181–189
39. White, S. H., and Wimley, W. C. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 319–365
40. Popot, J. L., and Engelman, D. M. (2000) Annu. Rev. Biochem. 69, 881–922
41. Partridge, A. W., Therien, A. G., and Deber, C. M. (2002) Biopolymers 66, 350–358
42. Partridge, A. W., Therien, A. G., and Deber, C. M. (2004) Proteins 54, 648–656
43. Cheung, M., and Akasah, M. H. (1997) J. Gen. Physiol. 109, 289–299
44. Linsdell, P., Evagelidis, A., and Hanrnan, J. W. (2000) Biophys. J. 78, 2973–2982
45. Linsdell, P. (2001) J. Physiol. 531, 51–66
46. Oblatt-Montal, M., Reddy, G. L., Iwamoto, T., Tomich, J. M., and Montal, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1495–1499
Destabilization of the Transmembrane Domain Induces Misfolding in a Phenotypic Mutant of Cystic Fibrosis Transmembrane Conductance Regulator
Mei Y. Choi, Anthony W. Partridge, Craig Daniels, Kai Du, Gergely L. Lukacs and Charles M. Deber

J. Biol. Chem. 2005, 280:4968-4974.
doi: 10.1074/jbc.M410069200 originally published online November 10, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410069200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 17 of which can be accessed free at http://www.jbc.org/content/280/6/4968.full.html#ref-list-1