CFTR transmembrane segments are impaired in their conformational adaptability by a pathogenic loop mutation and dynamically stabilized by Lumacaftor

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Georg Kainer1,2, ¶ Mathias Schenkel1‡, Andreas Hartmann‡, Dorna Ravamehr-Lake‡4,5, Charles M. Deber5,4,6, and ¶ Michael Schlierf1,11

From the 1B CUBE–Center for Molecular Bioengineering, Technische Universität Dresden, Tatzberg 41, 01307 Dresden, Germany, the 2Division of Molecular Medicine, Research Institute, Hospital for Sick Children, Toronto, Ontario MSG 0A4, Canada, the 3Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada, and the 4Cluster of Excellence Physics of Life, TU Dresden, 01062 Dresden, Germany

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The cystic fibrosis transmembrane conductance regulator (CFTR) is an ion channel protein that is defective in individuals with cystic fibrosis (CF). To advance the rational design of CF therapies, it is important to elucidate how mutational defects in CFTR lead to its impairment and how pharmacological compounds interact with and alter CFTR. Here, using a helical-hairpin construct derived from CFTR’s transmembrane (TM) helices 3 and 4 (TM3/4) and their intervening loop, we investigated the structural effects of a patient-derived CF-phenotypic mutation, E217G, located in the loop region of CFTR’s membrane-spanning domain. Employing a single-molecule FRET assay to probe the folding status of reconstituted hairpins in lipid bilayers, we found that the E217G hairpin exhibits an altered adaptive packing behavior stemming from an additional GXXG helix–helix interaction motif created in the mutant hairpin. This observation suggested that the misfolding and functional defects caused by the E217G mutation arise from an impaired conformational adaptability of TM helical segments in CFTR. The addition of the small-molecule corrector Lumacaftor exerts a helix stabilization effect not only on the E217G mutant hairpin, but also on WT TM3/4 and other mutations in the hairpin. This finding suggests a general mode of action for Lumacaftor through which this corrector efficiently improves maturation of various CFTR mutants.

This article contains support for Experimental procedures and Figs. S1–S3.

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed: Dept. of Chemistry, University of Cambridge, Lensfield Rd., Cambridge CB2 1EW, United Kingdom. E-mail: gk422@ch.cam.ac.uk.
3 Recipient of a RESTRACOMP research studentship from the Hospital for Sick Children.
4 To whom correspondence may be addressed: Division of Molecular Medicine, Research Institute, Hospital for Sick Children, 686 Bay St., Toronto, Ontario MSG 0A4, Canada. E-mail: debe@sickkids.ca.
5 To whom correspondence may be addressed: B CUBE–Center for Molecular Bioengineering, Technische Universität Dresden, Tatzberg 41, 01307 Dresden, Germany. Tel.: 49-351-463-43050; E-mail: michael.schlierf@tu-dresden.de.

The cystic fibrosis transmembrane conductance regulator (CFTR) is an ion channel protein that is defective in patients suffering from cystic fibrosis (CF). This inherited disease affects ~1 of every 3,000 newborns among people of European ancestry and therefore represents the most common life-limiting genetic disorder in the Western world (1). >2,000 CFTR mutations have been reported to date (Cystic Fibrosis Mutation Database; http://www.genet.sickkids.on.ca/cftr/app; accessed June 20, 2018), a majority of which impair maturation of CFTR (e.g. at the endoplasmic reticulum (ER)) or alter its channel activity at the cell surface. In addition to the most common phenotypic mutation, a deletion of Phe-508 in the first nucleotide-binding domain, the membrane-associated portions of CFTR, comprising two six-strand transmembrane (TM) domains with adjacent intervening intra- and extracellular loop regions, represent particularly vulnerable hot spots and frequent targets of CF mutations. Strikingly, ~33% of CFTR’s disease-causing mutations are found in these segments, which themselves cover only <20% of CFTR’s total residues (3).

Despite considerable progress in understanding CFTR pathology on a cellular level (4–7), the mechanisms by which mutations trigger misfolding and cause channel dysfunction remain largely obscure. In particular, there is very limited information on the underlying structures and conformational states that lead to an altered topology or dysfunctional state. Moreover, enormous efforts are currently being made in developing small-molecule compounds that correct the underlying misfolding or functional defect to increase the amount of matured protein at the cell surface or modulate CFTR activity (8–11). Recently, a binding site for two CFTR potentiators, ivacaftor and GLPG1837, has been found by cryo-EM (12). However, the mechanisms of action of many CFTR modulators still remain largely elusive. This lack of knowledge is mainly rooted in the...
ACCELERATED COMMUNICATION: Effects of a CFTR loop mutation

**Figure 1.** a, structure of human CFTR (44) (Protein Data Bank code SUAK) highlighting the position of the E217G mutation in the interconnecting loop ECL2 (blue) of TM3/4 (yellow/red). Visualization was generated using Visual Molecular Dynamics (VMD) (47). b, schematic representation of the E217G (left) and WT (right) TM3/4 helical-hairpin motifs comprising CFTR’s TM helices TM3 (yellow) and TM4 (red) and the intervening extracellular loop ECL2 (blue). The residues at position 217 are represented as van der Waals surfaces. The lengths of TM3 and TM4 are indicated for WT TM3/4, estimated from the cryo-EM structure (44) (Protein Data Bank code SUAK). Visualization was generated using VMD (47). c, schematic of the single-molecule FRET approach for investigating hairpin conformations. Shown are single fluorescently labeled TM3/4 hairpin molecules reconstituted into phospholipid vesicles (not to scale) freely diffusing through the observation volume of the confocal microscope.

Challenges of studying folding of full-length CFTR. On the one hand, the WT protein is already notoriously difficult to obtain in sufficient quantities and purities for in vitro scrutiny, and proteins carrying destabilizing mutations are even less available. On the other hand, CFTR with its 1,480 amino acid residues is too large and too complex to pinpoint the local structural effects of a single point mutation, particularly for classical ensemble biochemical and biophysical techniques, which are often limited in their ability to resolve the structural heterogeneities of misfolded states.

To overcome these difficulties, we recently introduced a single-molecule approach that exploits helical-hairpin constructs derived from full-length CFTR to gain insights into the structural effects of misfolding and drug rescue (13). Helical hairpins, comprising two TM helices and their intervening loop region, are readily prepared in sufficient amounts for biophysical analysis. They constitute the smallest units that can be inserted autonomously by the translocon, since CFTR topogenesis in the ER is based on the pairwise integration of helical segments (6), and therefore represent minimal in vitro folding units of tertiary contacts between two helices in a membrane (14, 15). In tandem with single-molecule FRET (16), which serves as a spectroscopic ruler (17) to probe the end-to-end distances of hairpins reconstituted in lipid bilayers, these minimalistic folding units thus constitute versatile platforms to characterize the molecular events that link CF disease to structural effects of mutations and drug rescue, mimicking in vivo processes of CFTR misfolding and fold recovery. We have recently applied this approach to study misfolding of the CF-phenotypic TM mutation V232D in TM helix 4 (TM4) and the impact of the pharmacological corrector VX-809 (also known as Lumacaftor) (18) on hairpin misfolding by exploiting the TM3/4 hairpin construct, a helix-loop-helix hairpin comprising CFTR’s third and fourth TM helices (human CFTR residues 194–241) and their intervening extracellular loop region 2 (ECL2) (13).

Herein, we exploit the TM3/4 hairpin construct to delineate structural effects of a pathogenic loop mutation and the impact of Lumacaftor on helical packing. Extramembranous loop regions represent key folding determinants (3, 19) and are crucial for the normal functioning of membrane proteins. Mutations in these regions can decrease the stability of a protein and alter topogenesis and are even capable of inducing a change in the secondary structure of TM segments (20–23). They have also been shown to impair the functionality of membrane proteins (24, 25) and membrane channels in particular (26), yet how the removal of a single amino acid in CFTR’s loop region compromises the structure/function so extensively that a disease state ensues is still largely unclear. Among the two CFTR mutations found in the extracellular loop region connecting TM3 and TM4, we focus here on the disease-causing loop mutation E217G (Fig. 1a), which causes a mild form of the disease and drastically alters net charge and hydrophobicity of the loop region. Previous cell-based experiments have shown that E217G drastically impairs the maturation to the fully glycosylated, full-length protein (27, 28) and alters CFTR’s activity at the cell surface (22). More recently, biophysical studies have yielded insights into secondary structure changes upon mutation and found that the helical content increases upon mutation (27). Yet the influence of the E217G mutation on tertiary structure changes (i.e. helical packing) has received limited study. Moreover, we and others have previously shown that Lumacaftor efficiently targets the first membrane-spanning domain of CFTR, including the TM3/4 hairpin, to rescue misfolding of mutations located in TM helices (13, 29–33), yet the potential effects of Lumacaftor on misfolding mutations located in loop regions, such as E217G, are unexplored.

Results and discussion

To probe the effects of the E217G mutation and Lumacaftor action on TM3/4 hairpin folding, we made use of our single-molecule FRET approach and engineered a hairpin construct carrying the E217G mutation and labeled the hairpin with FRET donor and acceptor dyes at its N- and C-terminal ends (Fig. 1b). For reference, we prepared a WT hairpin construct as described previously (13). We reconstituted both variants into
phosphatidylcholine (PC) lipid vesicles and employed confocal fluorescence spectroscopy to probe end-to-end distance changes of hairpins by monitoring FRET efficiencies from individual hairpin molecules (Fig. 1c). We first probed hairpin conformations in bilayers composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (i.e., POPC, 16:0–18:1 PC) (Fig. 2a), whose thickness ($d_h = 29.2$ Å (34)) mimics that of the ER membrane (35) and matches well the length of TM3/4’s helical segments (i.e., $\sim 30$ Å for TM3 and $\sim 28$ Å for TM4; see Fig. 1b). The FRET efficiency histograms of both hairpins exhibited a bimodal distribution with a major high-FRET and a minor low-FRET population, indicating that both hairpins exist in an equilibrium between a compact, folded structure and an open-state conformation with the equilibrium lying on the side of the compact conformation determined by tight helix–helix interactions. Introduction of the E217G mutation did not cause a change in the occupancy of the two states compared with the WT hairpin. Quantification of open-state and closed-state fractions using probability-distribution analysis (PDA) (Fig. 2a, red cityscapes) showed that the closed state in the E217G hairpin is, within error, indeed equally populated as in the WT hairpin ($f_{C,WT,POPC} = 0.76 \pm 0.01$; $f_{C,E217G,POPC} = 0.76 \pm 0.004$). Accordingly, closed hairpin stability in POPC, as reflected in the Gibbs free-energy change of hairpin closing, is favorable in both WT and E217G TM3/4 ($\Delta G_{WT,POPC} = -2.85$ kJ/mol; $\Delta G_{E217G,POPC} = -2.85$ kJ/mol). This suggests that the Glu-to-Gly exchange at position 217 in the loop region of CFTR does not affect the open/closed state equilibrium of the TM3/4 hairpin in POPC membranes.

Whereas the E217G loop mutation might not alter packing stability per se, it might change the conformational adaptability of the helical segments, which is an important factor in the topogenesis and functioning of membrane proteins (36–38). Segmental malleability, for example, is required during co-translational folding and insertion when newly synthesized TM segments seek contacts with neighboring helices, but also for conformational cycling between the different functional states of a membrane protein. The adaptations required in these processes typically also depend on the given bilayer environment. For example, CFTR is embedded in bilayers of increasing thickness on its way from the ER membrane to the plasma membrane. Because loop regions represent hinges connecting TM segments, mutations in these regions may therefore alter the responsiveness of helical segments to changes in bilayer properties.

To test this hypothesis, we probed hairpin conformations in lipid bilayers composed of PCs of different acyl chain lengths and thus of varying hydrophobic thickness. We reconstituted hairpins into vesicles composed of thin 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC; 12:0 PC) and thick 1,2-dieicosenoyl-sn-glycero-3-phosphocholine (DEiPC; 20:1 PC) lipid bilayers to create a positive and negative hydrophobic mismatch situation between the hydrophobic stretches of the TM3/4 hairpins and the thickness of the lipid bilayers, respectively. In particular, the hydrophobic thickness of DLPC is $\sim 6–8$ Å smaller ($d_h = 21.9$ Å (34)), whereas the hydrophobic thickness of DEiPC is $\sim 3–5$ Å larger ($d_h = 32.6$ Å (39)) than the length of TM3/4’s $\alpha$-helices. FRET efficiency histograms in DLPC yielded almost identical equilibrium distributions for the open and closed states of both hairpins (Fig. 2b), indicating that both hairpins respond in a similar fashion to a decrease in membrane thickness and, thus, positive hydrophobic mismatch. By contrast, a marked difference in the adaptability was discernible between the two hairpins in DEiPC (Fig. 2c). Upon increasing bilayer thickness, the WT hairpin responded with a more pronounced opening behavior ($f_{C,WT,DEiPC} = 0.30 \pm 0.01$) than the E217G hairpin, in which the open and closed state were almost equally populated ($f_{C,E217G,DEiPC} = 0.48 \pm 0.01$). Accordingly, hairpin stability is more favorable in E217G TM3/4 ($\Delta G_{E217G,DEiPC} = 0.20$ kJ/mol) than in WT TM3/4 ($\Delta G_{WT,DEiPC} = 2.09$ kJ/mol). The same stabilization effect is also observed at physiological temperatures (Fig. 2d). At $37^\circ$C, the E217G TM3/4 hairpin remains relatively more stabilized ($\Delta G_{E217G,DEiPC,37^\circ} = 1.21$ kJ/mol) than WT TM3/4.

**Figure 2.** a–c, FRET efficiency histograms of E217G TM3/4 (pink) and WT TM3/4 (cyan) in POPC (16:0–18:1 PC) (a), DLPC (12:0 PC) (b), and DEiPC (20:1 PC) (c) lipid vesicles at room temperature. d, further experiments were performed in DEiPC lipid vesicles at $37^\circ$C. PDA fits to the histograms are shown as red cityscapes. Right panels, fraction of closed hairpin ($f_C$) as function of hydrophobic thickness ($d_h$) for E217G TM3/4 (pink) and WT TM3/4 (cyan) as determined by PDA fits. $d_h$ represents the thicknesses of the entire hydrocarbon region, as determined at $20^\circ$C (POPC and DLPC) (34) or $30^\circ$C (DEiPC) (39). Errors are S.D. of the PDA $\chi^2$ minimization algorithm calculated from 10 iterations.
interhelical interactions with other TM segments. This may inhibit maturation of CFTR because the protein is trapped in a partially folded, intermediate state at the ER, where it may be targeted for degradation by the ER’s quality control machinery.

Changes in the conformational plasticity may also help in rationalizing the impaired functioning of the E217G CFTR channel at the cell membrane. Previous electrophysiological experiments have shown that the E217G CFTR mutant exhibits a decreased Cl⁻ efflux compared with WT CFTR upon stimulation, which has been attributed to transient rather than stable openings of E217G CFTR compared with WT CFTR (22). Because the extracellular loop connecting TM3 and TM4 seems to be involved in the open-state stabilization of the CFTR pore (22), our observation of reduced conformational/dynamic adaptability of the E217G containing TM3/4 segment may provide a mechanistic basis for the open-state destabilization of E217G CFTR causing channel dysfunction. Moreover, channel opening of CFTR is linked to a conformational change upon phosphorylation and ATP binding (43), and recent cryo-EM structures of human CFTR have provided further structural details of this opening mechanism (44, 45). Upon pore opening, the TM domains of CFTR rearrange such that the TM3/4 hairpin is in a more compact conformation (45), which appears functionally important. This conformational switch to the closed state may be disturbed in the mutant protein, trapping the CFTR pore in another closed intermediate state or leading to the disfavoring of the conformational change from closed to open state in general.

After having explored the influence of the E217G mutation on TM3/4 hairpin folding, we tested the effect of Lumacaftor on helical packing. Lumacaftor (Fig. 4a) is a corrector drug that has been developed to rescue misfolding of the most common CFTR mutation F508del (18), which is located in the nucleotide-binding domain of CFTR. Yet the promiscuity of Lumacaftor for correcting various CF-causing mutants at different loca-
The stabilization effect of Lumacaftor on the TM3/4 hairpin likely stems from its ability to reside in a phospholipid-bilayer membrane to modulate helical packing of TM domains. It has been proposed that Lumacaftor either interacts with the membrane-spanning stretches of CFTR and promotes tertiary interactions for tighter helix–helix packing or, through its membrane-stabilizing properties (46), aids the adaptation of a more compact fold by decreasing the hydrophobic mismatch between TM segments and the membrane. This could originate from a change in lateral pressure profiles and/or a more disordered hydrocarbon core layer, promoting more dynamic interhelical interactions and allowing polar solvent molecules (e.g. water) to penetrate deeper into the membrane, thereby locally decreasing bilayer hydrophobic thickness.

Indeed, we observed that Lumacaftor imposes changes on the stability of the phospholipid bilayer. Using a terbium(III)/dipicolinic acid fluorescence assay that sensitively reports on ion leakage from terbium-loaded liposomes, we found that Lumacaftor efficiently permeabilizes the membrane yet keeps the vesicles intact and causes a release of ion content in a dose-dependent manner up to 20 μM (Fig. 5a), at which point the disruptive properties of Lumacaftor reach a plateau at ~40%. This suggests that the corrector perturbs the bilayer order and alters membrane permeability but does not disrupt the vesicles. Furthermore, we observed an increased dynamic behavior of TM segments for all hairpin variants after the addition of Lumacaftor, as judged from correlative analysis of relative donor fluorescence lifetime versus FRET efficiency (Fig. 5b). Accordingly, the hairpin molecules appear less constrained and more dynamic in the presence of Lumacaftor, while still being in a closed conformation. Overall, our results create a picture in which Lumacaftor disorders the membrane in such a way that, on the one hand, it supports native interactions between the two helices of TM3/4 and, on the other hand, intensifies dynamics between closely related compact conformations.

Figure 5. a, terbium(III)/dipicolinic acid (DPA) fluorescence assay with increasing concentrations of VX-809 (Lumacaftor) in POPC (16:0–18:1 PC) liposomes. Measurements were performed at room temperature, and data were normalized to treatment with 0.1% Triton-lysed control. Significance at all doses is compared with the central point of 20 μM (***, p < 0.0001; n.s., not significant). Error bars, S.D. b, contour plots of relative donor fluorescence lifetime versus FRET efficiency of E217G TM3/4 (magenta), Q220R TM3/4 (orange), and WT TM3/4 (cyan) in POPC vesicles without (top row) and with the addition (bottom row) of 1 μM VX-809. All measurements were performed at room temperature. Models for “static FRET” (48, 49) (black line) and reconformation dynamics of a Gaussian chain (2) (red line) were fitted. In the presence of VX-809, all TM3/4 variants follow the reconformation dynamics of a Gaussian chain more prominently, which indicates that the corrector disorders the lipid bilayer and intensifies dynamics within the compact state. The position of the folded state is indicated by a black arrow.
This mode of action may be the dominant factor in the observed stabilization of WT TM3/4, E217G TM3/4, and Q220R TM3/4.

**Conclusion**

Taken together, our findings suggest that both the folding defect and the channel dysfunction caused by the E217G mutation stem from an altered adaptive packing behavior of the mutant TM3/4 hairpin. The additional GXXXG helix-interaction motif created by the loop mutation at the C-terminal end of TM3 may restrict the conformational freedom of the TM3/4 helical segments during their folding and insertion and alter the conformational plasticity during ion channeling. The stabilization effect exerted by Lumacaftor on the WT TM3/4, V232D TM mutant, as well as E217G and Q220R loop mutant hairpins, support the idea that this corrector has a rather broad mode of action likely connected to its membrane-stabilizing properties, where it efficiently improves CFTR folding stability to recover misfolding from various CF mutations.

**Experimental procedures**

Details on experimental procedures including hairpin design (Fig. S2) and protein purification (Fig. S3) are given in the supporting information.

**Author contributions**—G. K., C. M. D., and M. Schlierf conceptualization; G. K., M. Schenkel, A. H., and D. R.-L. formal analysis; G. K. and M. Schlierf supervision; G. K., M. Schenkel, and A. H. validation; G. K., M. Schenkel, A. H., and M. Schlierf investigation; G. K., M. Schenkel, A. H., and M. Schlierf methodology; G. K., M. Schenkel, and M. Schlierf writing—original draft; G. K., M. Schenkel, D. R.-L., C. M. D., and M. Schlierf writing—review and editing; M. Schenkel and D. R.-L. data curation; M. Schenkel visualization; A. H. software; C. M. D. resources; M. Schlierf funding acquisition; M. Schlierf project administration.

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