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CFTR structure, stability, function and regulation

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Abstract: Cystic fibrosis transmembrane conductance regulator (CFTR) is a unique member of the ATP-binding cassette family of proteins because it has evolved into a channel. Mutations in CFTR cause cystic fibrosis, the most common genetic disease in people of European origin. The F508del mutation is found in about 90% of patients and here we present data that suggest its main effect is on CFTR stability rather than on the three-dimensional (3D) folded state. A survey of recent cryo-electron microscopy studies was carried out and this highlighted differences in terms of CFTR conformation despite similarities in experimental conditions. We further studied CFTR structure under various phosphorylation states and with the CFTR-interacting protein NHERF1. The coexistence of outward-facing and inward-facing conformations under a range of experimental conditions was suggested from these data. These results are discussed in terms of structural models for channel gating, and favour the model where the mostly disordered regulatory-region of the protein acts as a channel plug.

Keywords: ABC transporter; CFTR; electron microscopy; ion channel; membrane protein structure.

Introduction: the cystic fibrosis transmembrane conductance regulator (CFTR)

CFTR is an ATP-gated chloride channel present in the apical membranes of cells lining the externally-facing surfaces of several organs (Riordan, 2008). Phosphorylation

of a highly charged regulatory region (R-region) of CFTR by protein kinase A or C activates the channel, which can then open, allowing chloride ion flux, provided ATP is available (Chappe et al., 2003). Chloride ions pass (passively) through the protein along their concentration gradient. Typically, this means that the CFTR channel opening is associated with the loss of chloride ions from the cell and their accumulation in the extracellular milieu. The charge imbalance caused by the movement of chloride is corrected by the concomitant flux of sodium ions in the same direction, and the resultant osmotic imbalance is accommodated by the flux of water into the extracellular space. Excess CFTR activity and hence water flux can lead to diarrhoea (in the intestinal tract). Sub-normal CFTR activity can lead to constipation in the gut and also stasis of the mucociliary clearance mechanism, vital for maintaining healthy lungs (Donaldson et al., 2006). This latter outcome is the most serious symptom for cystic fibrosis (CF) patients who have significantly reduced CFTR activity due to inheritance of two mutated and defective copies of the *cftr* gene (Riordan et al., 1989). In CFTR, channel opening is associated with the outward-facing state, where the ATP-binding domains are dimerised (Vergani et al., 2005; Cai et al., 2011; Yeh et al., 2017). CFTR channel function is also activated and controlled by phosphorylation, and here new structural data have provided different models of how phosphorylation could affect CFTR activity (Zhang et al., 2017; Fay et al., 2018).

Mutations in the *cftr* gene are varied, although the predominantly-observed mutation in patients of European descent is a deletion of three bases that results in the loss of a phenylalanine residue at position 508 in the protein's amino acid sequence (Riordan et al., 1989; Veit et al., 2016). Interestingly, deletion of this single residue (F508del) leads to a protein that is only a little worse in terms of its intrinsic channel activity (Meng et al., 2017a,b). However, it is noticeably less stable at physiological temperatures (Meng et al., 2017a,b). The unstable product is recognised and degraded by endoplasmic reticulum (ER) quality control mechanisms, and this occurs so efficiently that little or no F508del CFTR protein reaches the apical membrane (Brodsky, 2001). The high rates of occurrence of the F508del mutation (90% of patients have at least one copy) and disease (~1 in 2000 sufferers, carriage rate ~1 in 25) point to a prior selective advantage of being a carrier in humans with European origins. The exact nature of that advantage is debated (Gabriel et al., 1994; Wiuf, 2001), but

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there is broad agreement that it is likely to be related to a strong selective pressure imposed by intestinal diseases associated with high rates of infant mortality in the past.

Biochemical and biophysical measurements of CFTR have been crucial in our understanding of the effects of the various CF-causing mutations such as F508del (Meng et al., 2017a,b; Vernon et al., 2017; Yang et al., 2018a). Moreover, the development of fluorescence-based assays for CFTR channel activity has allowed the discovery of small molecules that can potentiate the channel activity and also rescue the instability defect (Galiotta et al., 2001). There are now molecules available for the treatment of most patients (Yu et al., 2012; Ren et al., 2013; Davies et al., 2018; Keating et al., 2018) (subject to cost). CFTR is somewhat unusual in that it is clearly a channel that has evolved from a group of proteins that are otherwise active transporters (Dean et al., 2001). Our understanding of the CFTR protein is, to a large extent, based on prior studies with these other transporters that couple ATP hydrolysis to substrate transport across a membrane (Higgins, 1992). When compared using sequence homology and phylogenetics, CFTR has been classified into the C sub-family of the eukaryotic ATP-binding cassette transporters (Dean et al., 2001). There are 38 transporter-like proteins belonging to this overall family in humans of which 13 are in the C sub-family. Two other C sub-family members are also

non-transporters, and this pair have evolved as switches that control another channel (Mikhailov et al., 2005). The remaining 10 members of the C sub-family are transporters and many of them are involved in xenobiotic clearance and hence are associated with multi-drug resistance (Higgins, 2007; Rosenberg et al., 2010).

Comparison of cryo-EM structures for CFTR

There are now several structures that have been published for CFTR. A summary of the various structures, their overall conformation and the experimental conditions employed to obtain them is proposed in Table 1.

An immediate observation based on Table 1 is that different conformations of the protein can be observed under very similar experimental conditions: For example, the fully activated (phosphorylated) state of the protein in the presence of ATP has been reported as being in the outward-occluded conformation in two studies (Zhang et al., 2017, 2018), but in another structural study, activated CFTR was found to be in the inward-facing state (Fay et al., 2018). Similarly, the absence of nucleotide was correlated with the inward-facing state in most studies

Table 1: Summary of sub-nanometre resolution cryo-EM structures of full-length CFTR that have been deposited in the EMDB and PDB.

Ortholog	EMDB code	PDB code	Experimental conditions	Mutations	Overall State(s)	Ref.
Human CFTR	1966	4a82	Phosphorylation state unknown. No nucleotide. Detergent micelle Epitaxial 2D crystals. N-linked glycosylation present	WT with C-term His tag	OF	1
Zebrafish CFTR	8461	5uar	Dephosphorylated. No nucleotide. Detergent micelle. SPA. N-linked glycosylation	WT	IF	2
Human CFTR	8516	5uak	Dephosphorylated. No nucleotide. Detergent micelle. SPA. N-linked glycosylation	WT	IF	3
Zebrafish CFTR	8782	5w81	Phosphorylated. ATP present. Detergent micelle. N-linked glycosylation	E1371Q	OO	4
Chicken CFTR	7793	6d3r	Dephosphorylated. No nucleotide. Detergent micelle. SPA. N-linked glycosylation present	Stabilising: Δ RI/H1404S/1441X C-term His tag	IF ^a	5
Chicken CFTR	7794	6d3s	Phosphorylated. ATP present. Detergent micelle. N-linked glycosylation	Stabilising: H1404S/1441X/ Δ RI/ C-term His tag	IF	5
Human CFTR	9230	6msm	Phosphorylated. ATP present. Detergent micelle. N-linked glycosylation	E1371Q	OO	6

CFTR, Cystic fibrosis transmembrane conductance regulator; EMDB, Electron Microscopy Databank (density map data); PDB, Protein Databank (atomic model); SPA, single particle averaging; WT, wild type sequence; OF, outward-facing state; IF, inward-facing state; OO, outward/occluded state; IF^a, evidence for particles in the inward facing state co-existing with the major state; ECL, extracellular loop; C-term, C-terminal; Δ RI, deletion of residues 405–436; 1441X, truncation at this residue. References: (1) (Rosenberg et al., 2012); (2) (Zhang and Chen, 2016); (3) (Liu et al., 2017); (4) (Zhang et al., 2017); (5) (Fay et al., 2017); (6) (Zhang et al., 2018).

(Zhang and Chen, 2016; Liu et al., 2017; Fay et al., 2018), but in one study the outward-facing state was found instead (Rosenberg et al., 2011). We would argue that consideration of the plasticity of ABC transporters must be taken into account. There are now several studies where a range of conformations within a population of an ABC transporter under the same conditions has been observed (Moeller et al., 2015). These studies show that the separation between the nuclear binding domains (NBDs) can vary considerably for inward-facing conformations, and that the NBD dimerised state (outward and outward-occluded states) are sampled very rarely, even in the presence of nucleotide and non-hydrolysable nucleotide analogues. Mutagenesis may also throw some light on these discrepancies (Table 1, column 5). Catalytic silencing of ATPase activity alone does not seem to be capable of stabilising the outward-facing or outward-occluded states, as the H1404S mutation when combined with ATP and phosphorylation was nevertheless associated with the inward-facing state in chicken CFTR (Fay et al., 2018). It seems possible, therefore, that the E1371Q mutation may favour the outward-facing state by changing the charge repulsion at the NBD-NBD interface.

CFTR channel function can be monitored at the single channel level. After activation and the addition of ATP, single CFTR channels can open and ‘bursts’ of current can be detected (Meng et al., 2017a,b). The bursts are punctuated by inter-burst intervals of quiescence. In activated human WT CFTR at physiological temperatures and with ATP present, the channels are closed for about 50% of the time (Meng et al., 2017a,b). Even during the bursts of current, there are rapid ‘flickery’ closures of extremely short duration. Rationalising these single channel measurements using the structural data summarised in Table 1 is not easy and clearly all the cryo-EM structures are not determined under physiological conditions. One could propose that the outward/occluded state could represent the state of CFTR in the inter-burst duration. Alternatively, although flickery closures are transient at body temperature, low temperature may favour this state, hence the outward-occluded state could be representative of CFTR during a flickery closure.

Epitaxial two-dimensional (2D) crystals (Rosenberg et al., 2011) are unlikely to be formed by CFTR molecules with mixed conformational states, hence the crystallisation process itself may select for a given conformation, perhaps explaining the observation of the outward-facing state in the absence of nucleotides. Although the phosphorylation state in the 2D crystal study could not be readily controlled, in all the other studies listed in Table 1, the phosphorylation state was imposed before structural data were

collected. In all the structural studies, full phosphorylation has been associated with the disappearance of a weak sinuous density that was observed for the fully dephosphorylated state between the NBDs and intracytoplasmic loops (ICLs). This weak density was proposed to be part of the 200-residue long disordered regulatory (R) region that links the first NBD to the second transmembrane domain (TMD) (Zhang and Chen, 2016; Fay et al., 2018). Hence, models for how phosphorylation of the R-region regulates the channel activity could be proposed: In one model a simple steric blocking of NBD dimerisation in the dephosphorylated state would prevent formation of the outward-facing state and hence a channel opening (Zhang and Chen, 2016). In the second model the dephosphorylated R-region would not only prevent dimerisation but would also insert itself into the cytoplasmic entrance of the channel, blocking it like a plug (Fay et al., 2018). In both these models, phosphorylation of the R-region would cause increased disorder and its dissociation from its position in the dephosphorylated state. Thus, from Table 1, it would appear that a refined model for ABC transporter or CFTR conformation/function relationships is needed. We have proposed that inward-facing, outward-occluded and outward-facing states are always in equilibrium with each other and that the presence of ATP can push the balance in one direction or another (Meng et al., 2018). Similarly, in CFTR, phosphorylation can influence the equilibrium, especially between the inward-facing and outward-occluded states. Whether a similar phospho-regulation mechanism exists in other ABC transporters remains to be explored.

Further regulation of the activity of CFTR

Apart from regulation via the linking R-region, CFTR activity may also be influenced via the disordered C-terminal region of the protein. In the structures listed in Table 1, no density was observed for the last 40 amino acids, and indeed for the chicken CFTR studies, the C-terminal region was deliberately truncated at residue 1441 (Fay et al., 2018). It is known that the CFTR C-terminus displays a PDZ-binding motif, and this allows its interaction with either of the two PDZ domains of NHERF1, a cytoplasmic soluble protein (Moyer et al., 2000). NHERF1 can mediate CFTR-protein interactions with other PDZ binding motif-containing proteins via its two PDZ domains. It can also mediate interaction with the cell cytoskeleton via the ezrin-binding domain of NHERF1 (Moyer et al., 1999). This has been associated with the formation of a non-mobile

pool of CFTR in epithelial cells (Haggie et al., 2004; Valentine et al., 2012). Links have also been proposed between CFTR-NHERF1-cytoskeleton interactions and the recycling of CFTR in the cell (Cushing et al., 2008). From a structural perspective, the details of the interaction between the NHERF1-PDZ1 domain and the last five residues of the CFTR C-terminal region have been well characterised (Karthikeyan et al., 2001), but the overall effects of NHERF1 binding on CFTR conformation have been little studied. There is some evidence that NHERF1 binding may favour the inward-facing state of the channel (Al-Zahrani et al., 2015), and it is suspected that the CFTR C-terminus may associate with the R-region when it is phosphorylated (Bozoky et al., 2013). These data would point to a regulatory system where NHERF1 would bind to the dephosphorylated CFTR protein in its quiescent state.

F508 deletion

Noticeable by its absence in Table 1 is a structure for the F508del mutated version of CFTR. Although the effects of this mutation have been examined in the isolated NBD1 (Atwell et al., 2010), only minor local changes in the conformation were noted. Furthermore, these studies relied on a stabilised, more soluble NBD1 construct that may represent a ‘rescued’ state. Examination of the available atomic models and the maps from which they are derived suggests that the F508 residue sits at an important interface between NBD1 and TMD2. This location was predicted well before the higher resolution structures of CFTR became available (Dawson and Locher, 2007). Surprisingly, the overall configuration of this interface remains unchanged throughout the large conformational transitions associated with channel activation (Meng et al., 2018). From an engineering perspective, this implies that F508 is part of a locking-pin component rather than being part of a ball and socket joint. Hence, F508 deletion may destabilise the protein by weakening the link between TMD and NBD.

In the studies described below we have tested the effects of the F508del mutation on CFTR using a biophysical and a biochemical assay. The former probes for the native folded state as well as the thermostability of the protein. The latter probes for the native folded state and can also be applied to non-purified CFTR in the membrane it was expressed in. We have also examined the effects of phosphorylation on CFTR conformations using low resolution EM and further explored the link between structure/activity and regulation with the CFTR-interacting protein NHERF1.

Results and discussion

Protein purification and characterisation

The expression and purification of full-length CFTR construct with an N-terminal His-SUMOstar tag and a C-terminal GFP-His tag has been described previously (Pollock et al., 2014; Meng et al., 2017a,b). WT, F508del and G551D versions of this construct were employed for the CFTR stability studies. The generation of a CFTR construct with no GFP-His tag and an authentic C-terminus was carried out for the structural studies and a purification gel is shown in Supplementary Figure 1A. The purified protein was shown to be partly phosphorylated and could be readily dephosphorylated or fully phosphorylated by treatment with the relevant enzymes (Supplementary Figure 1C, D). Full-length NHERF1, containing two PDZ binding domains and an ezrin-binding domain, was expressed in *Escherichia coli*. A gel for the final purification step (size exclusion chromatography) is shown in Supplementary Figure 1B.

CFTR Stability: the CPM assay

We have employed a fluorescence-based assay for the stability of purified proteins that is based on the labeling of exposed Cys residues by a 4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) dye (Alexandrov et al., 2008; Meng et al., 2017a,b). Figure 1 summarises the data for many experiments with purified CFTR under various conditions. CFTR can be denatured thermally, whereupon more CPM will bind (to previously buried Cys residues) giving rise to a fluorescence increase from which a mid-point unfolding temperature can be extracted (T_m). Initial labelling of exposed Cys residues at the surface of the folded protein at the start of the experiment is also a useful indicator of the native folded state, reporting on batch-to-batch quality and can detect the effects of chemical denaturation (e.g. by detergent or chaotropes). For example, increasing concentrations of guanidium HCl reduce the thermal unfolding transition temperature as well as having a major effect on the native folded state as indicated by the high initial labelling by CPM (Figure 1). Using this assay one can clearly distinguish the lower thermal stability of F508del CFTR (Figure 1A, red symbols), although there is no clear difference in terms of its initial folded state (within batch-to-batch variability). This supports earlier studies implying that F508del CFTR NBD1 was unaltered in terms of its overall three-dimensional (3D) structure (Meng et al., 2017a,b). Acute addition

Figure 1: Thermal unfolding of CFTR.

(A) Midpoint of thermal unfolding transition (T_m) is plotted against the % of initial labelling at 10°C for several batches of CFTR and under different experimental conditions. Red, black and blue data points represent F508del, WT and G551D CFTR, respectively, purified in DDM detergent. Green points are for G551D CFTR in the presence of LPG14 detergent. Round, square and triangle symbols show the effects of no addition, VX809 (2 μ M) addition or VX770 (2 μ M) addition, respectively. Crosses and numbers show the effects of GuHCl addition, with the number indicating the final GuHCl concentration in mol/L. Where no clear thermal unfolding transition was detected (bottom right), the T_m was arbitrarily assigned to the initial labeling temperature (10°C). (B) Exemplar thermal unfolding profiles for CFTR. The data series shown are for CFTR purified in LPG and at different concentrations of GuHCl (numbers indicate molarity).

of the F508del corrector drug (VX809) appears to have an effect on the initial labelling of the protein, with less exposure of Cys residues implied (square symbols), and there is a small thermostabilisation as reported before (Meng et al., 2017a,b). In contrast, the potentiator compound VX770 appears to have a general destabilising effect without significantly affecting the initial labelling (triangles, Figure 1A). Interestingly, the G551D mutation, which induces the closed channel state, has a slight thermostabilising effect on the protein (blue symbols). Although the negatively charged lysophosphatidylglycerol (LPG) detergent has been shown to be destabilising for isolated CFTR NBD1 (Yang et al., 2018b) [relative to the non-ionic n-dodecyl β -D-maltoside (DDM) detergent], we find that the thermostability of the G551D full-length CFTR protein is somewhat enhanced in this detergent (Figure 1A, green symbols). However, the CPM labelling shows an additional transition prior to the main higher temperature transition (Figure 1B). This minor transition was not included in the analysis presented in Figure 1A, but it may indicate some localised low-temperature unfolding by LPG.

CFTR stability: limited proteolysis assay

The CPM assay requires purified protein, hence, it was useful to compare purified CFTR in LPG with CFTR expressed in membranes. For this we employed limited

proteolysis at low temperature followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figures 2 and 3). Proteolytic fragments were visualised using a C-terminal GFP tag as well as an N-terminal SUMO tag (probed with a commercial antibody). Figure 2 shows the effects of limited trypsin concentrations on C-terminal fragments generated from WT, F508del and G551D versions of the protein as described already, with the data for CFTR in microsomes on the left and for purified protein on the right. Figure 3 shows the effects of limited (25 μ g/ml) and high (250 μ g/ml) concentrations of a different protease (thermolysin) and compares WT and F508del versions of the protein in microsomes and after purification. Both the N-terminal fragments (upper panels) and the C-terminal fragments (lower panels) are compared.

The largely unstructured R-region represents a major target for proteolytic enzymes, and hence large fragments around 100 kDa, representing TMD2-NBD2-GFP and SUMO-TMD1-NBD1 can be observed at low protease levels for all CFTR variants. Lower mass C-terminal fragments can also be detected at 25 kDa (GFP) and 50–55 kDa (NBD2 + GFP) with both proteases, implying common protease-sensitive sites at the TMD2-NBD2 linker (55 kDa fragment) and at the CFTR C-terminus (25 kDa GFP fragment). Despite the presence of F508 in the N-terminal end of CFTR, no obvious differences can be detected in the N-terminal proteolytic cleavage patterns of WT and

Figure 2: Limited proteolysis of CFTR variants.

Protease was at a ratio of 1:75 (0.3 $\mu\text{g}/\text{ml}$ trypsin) with microsomes (left) and purified proteins (right) and detection of C-terminal fragments using the GFP fluorescence. The 25 kDa lowest mass fragment is GFP alone (based on excess trypsin experiments and GFP-only controls). GFP is not unfolded by the SDS-PAGE conditions. Full-length protein runs just above the 250 kDa marker and is much more protected in microsomes (arrow). The dashed rectangle outlines bands that correspond to GFP with part of NBD2, discussed in the main text.

F508del CFTR (Figure 3), but the N-terminal patterns are much more complex than the equivalent C-terminal patterns. This applies to both purified CFTR as well as membrane-bound CFTR and implies that the C-terminal end of CFTR may be susceptible to cleavage at a few, particularly sensitive, sites. Some differences in the C-terminal proteolytic cleavage patterns of F508del and WT CFTR were detected when thermolysin was employed, but not with trypsin. The formation of SDS-resistant thermolysin-GFP or thermolysin-SUMO complexes, especially at high thermolysin levels may explain these differences (Supplementary Figure 1). Overall, the conclusions from these limited proteolysis data are that F508del, G551D and WT CFTR display broadly similar sensitivity to proteases at low temperatures and that membrane-bound and detergent-purified CFTR are also similar in terms of their ability to be cleaved by soluble proteases. The discrete nature of the N-terminal and C-terminal bands with masses indicative of cleavage at major domain interfaces strongly supports the hypothesis that CFTR expressed in yeast microsomes is folded correctly and that after purification it retains a very similar overall conformation, at least at low temperatures.

Figure 3: Limited proteolysis.

Digestion with thermolysin at a concentration of 25 $\mu\text{g}/\text{ml}$ and 250 $\mu\text{g}/\text{ml}$ with microsomes (left) or LPG-purified proteins (right). The mass ratio was 1:1 or 10:1 with microsomes and protein. The gel was probed by GFP fluorescence scan (lower panels) or subsequently Western blotted and probed with a SUMO antibody to detect the N-terminal fragments. Note that for microsomes many native yeast SUMO-ylated microsomal proteins are detected in addition to CFTR (left upper panel, no thermolysin). The overall fragmentation patterns with thermolysin are similar although some differences can be observed for the lowest mass C-terminal fragments (lower panels).

These data also support the hypothesis that F508 deletion mainly affects the thermostability of the protein, rather than affecting the overall fold or topology of the protein (Meng et al., 2017a,b).

CFTR structure: conformational plasticity and interaction with NHERF1

Both inward-facing and outward/occluded-facing conformations of CFTR have been reported under similar conditions (+ATP and after phosphorylation, Table 1). In order to understand this dichotomy, we have proposed a model (Meng et al., 2018) where CFTR can sample

both conformations continuously in the presence or absence of ATP. In this model the equilibrium between inward- and outward-facing states is influenced by the presence of nucleotide and phosphorylated residues in the R-region. In order to explore this further, we studied LPG-purified CFTR single particles by negative-stain EM after phosphorylation and dephosphorylation. We also investigated whether the C-terminal interacting protein, NHERF1 had any effects on the CFTR conformational states. Figure 4A shows an example of data recorded for dephosphorylated CFTR. Single particles can be observed (circled) and these could be selected semi-automatically (Figure 4C), classified and averaged (Figure 4D). For dephosphorylated CFTR, but for none of the other

Figure 4: Electron microscopy of phosphorylated and dephosphorylated CFTR.

(A) Negatively stained micrograph of LPG-purified dephosphorylated CFTR showing 14 nm diameter single particles (circled) and some linear aggregates (rectangle). (B) Examples of linear aggregates. (C) Examples of single particles. (D) 2D class averages for phosphorylated CFTR showing a mixture of inward-facing and outward-facing projection classes. Box size for panels (B–C) is 29 nm. (E) Structures of inward-facing (top) and outward-facing (bottom) 3D classes derived from (l to r): dephosphorylated CFTR; dephosphorylated CFTR + NHERF1; phosphorylated CFTR + NHERF1; phosphorylated CFTR. (F) Fitting of the dephosphorylated human CFTR structure (purple ribbon trace) to the dephosphorylated CFTR + NHERF1 map (mesh). Two orthogonal views are displayed. Additional density can be identified close to the NBD2 location after fitting (arrows).

conditions, short linear aggregates were also detected in these samples (Figure 4A, rectangle, Figure 4B). Similar linear aggregates were previously reported for LPG-purified CFTR where the phosphorylation status was not controlled (Al-Zahrani et al., 2015), and the aggregates may be mediated by intermolecular NBD to R-region interactions which are promoted after dephosphorylation of the R-region (Bozoky et al., 2013). Examination of the projection class averages for all samples implied a mixture of inward- and outward-facing states, as expected, hence a multi-reference refinement was carried out, employing references corresponding to inward-facing and outward/occluded states that had been filtered to low resolution (40 Å). Figure 4E shows the resulting 3D representations. No condition resulted in a single conformational state, indeed even dephosphorylated CFTR in the absence of ATP contained particles that gave an outward-facing structure (magenta volume) whilst phosphorylated CFTR in the presence of ATP also had a recognisably inward-facing sub-population of particles with separated NBDs (sky blue volume). Particles corresponding to the inward-facing state of dephosphorylated CFTR with NHERF1 (grey volume) had additional density between the NBDs (ellipse outline, Figure 4E and arrows, Figure 4F). It seems possible that this could represent the location of

NHERF1. No other maps showed a plausible additional density close to the expected position of the C-terminal region. If correct, this would imply that NHERF1 may preferentially bind to the dephosphorylated state and may take up a discrete location in the inward-facing conformation. This concurs with earlier data showing an association of the C-terminal 40 residues with the R-region of CFTR in the phosphorylated but not dephosphorylated state (Bozoky et al., 2013).

Figure 5 shows the fitting of the inward-facing human CFTR structure (PDBID 5uak) within the equivalent low-resolution map obtained from dephosphorylated CFTR. Despite the low resolution of the negative-stain structure, the overall fit is very good, with similar separation distances between the NBDs. The LPG micelle is partly delineated and at lower density thresholds takes on the characteristic toroidal shape (shaded area and dashed red outline). Similarly, the outward/occluded CFTR structural model (PDBID 6msm) fits reasonably well within the equivalent map obtained from phosphorylated CFTR in the presence of ATP. Here the detergent micelle appears somewhat smaller and less symmetric (red dashed line).

Global 3D structures representative of all particles across the various experiments were also generated

Figure 5: CFTR maps.

(A) Fitting of the inward-facing (purple, 5uak) and outward/occluded (blue, 6msm) molecular CFTR models to the equivalent low resolution maps obtained from dephosphorylated CFTR (blue mesh) and phosphorylated CFTR + ATP (brown mesh). The lower panel shows the same models/maps after rotation of each by 90° around the vertical axis. At lower density threshold (shaded regions) the boundaries of the upper LPG micelle can be partially delineated (dashed red outline). (B) As in panel a, but the maps shown are global averages of all particles classified as inward-facing (left) or outward-facing (right).

(Figure 5B). These are for significantly larger datasets and show better-defined molecular outlines. The quality of the maps after global averaging suggests that CFTR is not sampling a spectrum of conformations under the varying experimental conditions employed (at least to the low resolution limits obtained here). However, the inward-facing global 3D map does show some smearing of one of the NBDs, whilst the other NBD density is well defined. This could reflect some conformational flexing in the inward-facing state, but also could arise from NHERF1 binding to the CFTR C-terminal PDZ binding motif in the dephosphorylated state (Figure 4F). For the global 3D map of the outward-facing conformation, one would expect very similar conformations as the NBDs should be tightly dimerised.

Conclusions

The studies suggest that the most common CFTR mutation (F508 deletion) mainly affects the stability of the protein rather than its overall 3D conformation. The structural studies suggest that the protein is able to sample inward-facing and outward-facing conformations in both the activated and inactivated states, and that the CFTR-interacting protein, NHERF1 may preferentially associate with the inward-facing, dephosphorylated state. The observation of the outward-facing configuration, even for the inactivated/dephosphorylated state implies that the R-region may have a loose association between the NBDs and its steric hindrance of the NBD dimer formation observed in some structural studies is not permanent. Similarly, the presence of ATP in phosphorylated CFTR does not appear to result in a uniformly outward-facing population of molecules. Hence, phosphorylation of the R-region may influence the balance between inward-facing and outward-facing conformations under physiological conditions, but does not appear to impose the outward-facing state. When comparing the various models for CFTR regulation and channel gating, the idea of the R-region acting as a phospho-regulated channel plug appears to be more consistent with the data presented here (Fay et al., 2018). The plug could have a ‘back-stop’ function to limit unwanted leakage of chloride ions while CFTR is in the closed state rather than acting as the main channel gate. Once the plug is removed (perhaps by processive kinase activity on the multiple R-region phosphorylation sites), the unblocked protein is also free to sample conformations consistent with the open channel state.

Materials and methods

NHERF1 expression and purification

The cDNA for human NHERF1 was synthesised by Geneart Inc. (Burlingame, CA, USA) and cloned into a pSY5 vector (Chumnamsilpa et al., 2009) using standard ligation independent cloning methods (Aslanidis and de Jong, 1990). Plasmids were transformed into BL21 (DE3) competent *E. coli* (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Starter cultures were grown overnight at 37°C with shaking at 250 rpm in Luria broth (5 g/l yeast extract, 10 g/l peptone, 10 g/l NaCl) supplemented with 100 µg/ml ampicillin. Starter cultures were inoculated at 20 ml/l into auto-induction media [12 g/l tryptone, 24 g/l yeast extract, 9.4 g/l K_2HPO_4 , 2.2 g/l KH_2PO_4 , 8 ml/l glycerol 50 mM NH_4Cl , 20 mM $MgSO_4$, 5 mM Na_2SO_4 , 0.3% (w/v) α -lactose, 0.015% (w/v) D-glucose] supplemented with 100 µg/ml ampicillin. Cultures were grown overnight at 15°C. Cultures were harvested by centrifugation at 4200 g, 1 h, 4°C. Pellets were resuspended in 50 mM Tris.HCl pH 8.0, 500 mM NaCl, 20 mM imidazole and lysed using sonication then centrifuged at 19 000 g, 1 h, 4°C. Filtered (0.45 µm) lysates were loaded onto a 5 ml HisTrap FF Ni-NTA column (GE Healthcare, Gloucester, UK) washed with the same buffer and elution was with 50 mM Tris.HCl pH 8.0, 500 mM NaCl, 250 mM imidazole. The eluted material was desalted with 50 mM Tris.HCl pH 8.0 on a HiPrep 26/10 desalting column (GE Healthcare, Gloucester, UK) and loaded onto 1 mL HiTrap Q HP columns (GE Healthcare, Gloucester, UK). Following washing, bound NHERF1 was eluted using a gradient of 0–500 mM NaCl. Fractions were collected and further purified by size-exclusion chromatography (SEC) in 50 mM Tris.HCl pH 8.0, 150 mM NaCl using a HiLoad 16/600 Superdex 200 column (GE Healthcare, Gloucester, UK). Fractions were combined and concentrated using 30 kDa molecular weight cut-off concentrators and the final NHERF1 concentration was determined using the Pierce Bicinchoninic Acid (BCA) Protein Assay kit (Thermo Scientific, Runcom, UK) according to the manufacturer's instructions.

CFTR expression and purification

C-terminal eGFP* and StrepII tags were removed from opti-hCFTR within the pTR vector (Rimington, 2014) using the Site Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. The sequence was confirmed by DNA sequencing and transformed into competent FGY217 *Saccharomyces cerevisiae* for expression and purification as previously described (O’Ryan et al., 2012; Pollock et al., 2014).

Phosphorylation and dephosphorylation

Purified CFTR in 50 mM Tris, pH8, 10% glycerol (v/v), 50 mM NaCl, 1 mM DTT, 0.05% LPG-14 was treated with protein kinase A (PKA) catalytic subunit (New England Biolabs, P6000, Ipswich, MA, USA) as previously described (Zhang and Chen, 2016; Liu et al., 2017). PKA was diluted to 1.6 µM and incubated with CFTR at 30°C for 1 h with 0.2 mM MgATP. The dephosphorylation reaction was done in the presence of Lambda Phosphatase diluted 1:40 (w/w) at 22°C for 1 h (Lin et al., 2017). Protein phosphorylation was detected with Pro-Q Diamond stain, as described in the manufacturer's protocol (Steinberg et al., 2003) and scanned using Trans-UV illumination.

CFTR stability assay

The coumarin maleimide (CPM) assay was adapted from (Alexandrov et al., 2008). DTT, which interferes with the assay, was removed from the protein sample at the SEC purification step. The assay was done either in a conventional fluorimeter connected to a temperature-controlled water bath; or in an Unchained Labs UNCLE instrument using the UV laser (Meng et al., 2017a,b). For the latter, emission spectra were scanned every minute at 10°C for 30 min followed by a temperature ramp to 90°C and spectra were collected at 2°C increments with a heating rate of approximately 1.5°C min⁻¹. After subtraction of buffer controls, data were analysed by integrating the tryptophan (323 nm to 350 nm) and CPM (445 nm to 463 nm) emission peaks. The ratio of the integrals CPM/Trp was employed to compensate for temperature-dependent fluorescence quenching effects and any spikes in the laser output. One microgram of purified CFTR and 0.1 µg CPM were used in each experiment.

Limited proteolysis assay

Limited proteolysis reactions were carried out at 4°C in a 20 µl reaction volume. Thermolysin (*Geobacillus stearothermophilus*) was from Sigma CAS # – 9073-78-3. For thermolysin, protein and microsomes were incubated with the enzyme for 15 min at 4°C and then the reaction was halted with 12 mM EDTA. Purified protein was at 25 µg/ml and microsomes 50 µg/ml while thermolysin was at 12.5 µg/ml or 50 µg/ml for pure protein reactions, and 25 µg/ml or 250 µg/ml for microsome reactions. The enzyme was diluted in a calcium-containing thermolysin buffer (50 mM Tris, 0.05 mM CaCl₂). SDS buffer (50 mM Tris, pH7.6, 5% glycerol, 5 mM EDTA, 0.02% bromophenol blue, 4% SDS, 50 mM DTT) was then added to the reaction 1:1 v:v and left at room temperature for 30 min before being run on a 12% polyacrylamide gel for 90 min at 130V. Limited proteolysis experiments with trypsin were conducted at 4°C for 15 min and stopped with a protease inhibitor cocktail (5 µg/ml E64, 48 µg/ml AEBSF, 174 µg/ml PMSF, 8.25 µg/ml lupeptin, 8.25 µg/ml pepstatin, 1.75 µg/ml chymostatin, 2 µg/ml bestatin). Protease was at a ratio of 1:75 (0.3 µg/ml trypsin) with both microsomes and purified proteins.

For Sumo identification, the gel proteins were transferred using the semi-dry method using anode (Anode I – 30 mM Tris, 20% methanol, Anode II – 300 mM Tris, 20% methanol) and cathode (40 mM 6-amino hexanoic acid, 25 mM Tris, 0.01% SDS, 20% methanol) transfer buffers. Following transfer, the membrane was incubated in 5% w/v BSA in TBST buffer (Tris buffered saline – 20 mM Tris (pH7.5), 150 mM NaCl, 1% Tween-20) for 1 h at room temperature. The membrane was then incubated with an anti-Sumo primary antibody (Abcam ab176485) overnight at 4°C. Following incubation, the membrane was washed with TBST buffer 5 times for 5 min each. The membrane was incubated with a secondary antibody (Dnk pAb to Ms IgG (IRD® 800 cw Abcam ab216774) for an hour at room temperature followed by three washes with TBST buffer for 5 min. The membrane was then viewed on a LiCor Odyssey machine.

Electron microscopy and structural analysis

Samples were diluted to ~25 µg/ml in 50 mM NaCl, 1 mM DTT, 0.05% LPG-14 (Mg-ATP, 2 mM, was added for the phosphorylated sample) and

then applied to glow-discharged EM grids and negatively-stained as described earlier (Al-Zahrani et al., 2015). Image processing was carried out with the EMAN2 software package (Ludtke et al., 2004). Single particles were selected using the EMAN2 interactive particle-picking tool. Classification of the projection classes allowed the removal of non-CFTR particles (discriminated by size) and aggregates (discriminated by size and shape). For most specimens this resulted in elimination of about 50% of the automatically-selected particles; about 75% were excluded for the dephosphorylated CFTR sample where linear CFTR aggregates were present. Further reclassification of the included particles identified projection classes consistent with both outward- and inward-facing CFTR particles surrounded by an annular detergent micelle. The particles were therefore further classified using the EMAN2 multirefine tool using two 3D reference structures (EMDB_8516 and EMDB_9230 – corresponding to inward-facing and outward-facing conformations respectively filtered to ~40Å resolution). Finally, particles classified with inward-facing or outward-facing references were separately re-refined using the single map refinement tool: Here, each dataset was split into two separately-refined subsets to allow the assessment of the resolution of the final map by Fourier shell correlation (FSC). A combination of all the particles classified with the inward-facing reference across all datasets was also carried out and this large dataset (~14 000 particles) was used for a global 3D structure refinement. A similar procedure was performed for all particles associated with the outward-facing state (~13 000 particles).

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