NMR evidence for differential phosphorylation-dependent interactions in WT and Δ F508 CFTR

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The most common cystic fibrosis (CF)-causing mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) is deletion of Phe508 (Δ F508) in the first of two nucleotide-binding domains (NBDs). Nucleotide binding and hydrolysis at the NBDs and phosphorylation of the regulatory (R) region are required for gating of CFTR chloride channel activity. We report NMR studies of wild-type and Δ F508 murine CFTR NBD1 with the C-terminal regulatory extension (RE), which contains residues of the R region. Interactions of the wild-type NBD1 core with the phosphoregulatory regions, the regulatory insertion (RI) and RE, are disrupted upon phosphorylation, exposing a potential binding site for the first coupling helix of the N-terminal intracellular domain (ICD). Phosphorylation of Δ F508 NBD1 does not as effectively disrupt interactions with the phosphoregulatory regions, which, along with other structural differences, leads to decreased binding of the first coupling helix. These results provide a structural basis by which phosphorylation of CFTR may affect the channel gating of full-length CFTR and expand our understanding of the molecular basis of the Δ F508 defect.

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Introduction

Cystic fibrosis (CF) is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator

(CFTR) (Rommens *et al*, 1989), a 1480-residue multi-domain, integral membrane protein that functions as a chloride channel. CFTR belongs to the ABC transporter superfamily of proteins and consists of two repeats, each composed of a membrane-spanning domain (MSD) followed by a cytosolic nucleotide-binding domain (NBD) (Supplementary Figure 1). A large intrinsically disordered regulatory (R) region, unique to CFTR, is located between the first NBD and the second MSD. Intracellular domains (ICDs) extend beyond the transmembrane helices to link the MSDs with the NBDs. The ICDs are composed of residues between the transmembrane segments and residues N-terminal to the NBDs that extend the transmembrane helices into the cytoplasm. Connecting these long α -helical segments of the ICDs are short helical elements or coupling helices (Supplementary Figure 1a). Coupling helices 1 and 3 are located between the second and third transmembrane helices in MSD1 and MSD2, respectively. Coupling helices 2 and 4 are between the fourth and fifth transmembrane segments in MSD1 and MSD2, respectively.

CFTR channel gating is regulated by ATP binding and hydrolysis at the NBDs, and phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) at multiple Ser residues within the R region (Anderson *et al*, 1991; Cheng *et al*, 1991; Kartner *et al*, 1991; Tabcharani *et al*, 1991; Picciotto *et al*, 1992; Ma *et al*, 1996). During the gating cycle, two ATP molecules bind at the interface of a proposed NBD1/NBD2 heterodimer, as shown by crystal structures of homologous bacterial NBD homodimers (Smith *et al*, 2002) and crosslinking studies in CFTR (Mense *et al*, 2006). Conformational changes associated with the formation and disruption of the NBD dimer are suggested to be transmitted to the MSDs through the ICDs, allowing the channel to open and close (Wang *et al*, 2004; Ward *et al*, 2007). Currently, over 1500 mutations in the CFTR gene (<http://www.genet.sickkids.on.ca/cftr>) have been associated with CF with different symptoms and varying severities. The most common, and a severe CF-causing mutation, is deletion of Phe508 (Δ F508) located in the first nucleotide-binding domain (NBD1), which affects the folding (Du *et al*, 2005; Thibodeau *et al*, 2005), maturation (Cheng *et al*, 1990; Denning *et al*, 1992), gating (Hwang *et al*, 1994; Schultz *et al*, 1999; Cui *et al*, 2006), and cell-surface stability (Lukacs *et al*, 1993) of CFTR.

Crystal structures of wild-type (WT) and mutant CFTR NBD1, including variants of Δ F508, have been solved (Lewis *et al*, 2004, 2005; Thibodeau *et al*, 2005). The structure of the NBD core, which consists of the α/β subdomain (that contains the ATP-binding site) and the ABC-specific α -helical and β -sheet subdomains, is similar to that of NBDs from other ABC transporters. NBD1 from CFTR also contains a unique regulatory insertion (RI) that has one site for PKA phosphorylation (Dahan *et al*, 2001). The RI links the first two strands of the β -sheet subdomain and is composed of two short α -helices separated by a disordered linker, most of which is

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not observed in the various crystal structures of CFTR NBD1 (Supplementary Figure 1b). The constructs that have been used for most of the crystallization studies (residues 389–673) include a C-terminal regulatory extension (RE) (Lewis *et al*, 2004) that extends beyond the C-terminus of NBD1, which is defined as residue 650 by sequence alignment with NBDs from bacterial and other eukaryotic ABC transporters (Gibson *et al*, 1991). By this definition, the RE comprises the first ~25 residues of the R region (Supplementary Figure 1a). A recent crystal structure of the NBD1 that lacks the RI and extends only to Gly646 contains the entire canonical NBD fold (PDB code 2PZE). Recent NMR data from our laboratory indicate significant conformational flexibility of the C-terminus of this construct (Chong and Forman-Kay, unpublished results). Therefore, the R region in CFTR likely begins before Gly646, possibly as far back as Leu633, which was defined as the functional C-terminus of NBD1 (Chan *et al*, 2000). Thus, our construct of NBD1, which extends to 653, likely still contains some of the R region, although it lacks all phosphorylation sites of the R region of CFTR.

Many of the crystallization studies to date have used murine CFTR NBD1 because of its increased solubility relative to the human protein (Lewis *et al*, 2004; Thibodeau *et al*, 2005). Solubilizing mutants of human NBD1 used for crystallization lead to partial correction of $\Delta F508$ (Pissarra *et al*, 2008), making it difficult to elucidate the structural differences between true human WT and $\Delta F508$ NBD1. Murine and human NBD1 share nearly 80% sequence identity. Further, there is strong conservation of PKA phosphorylation sites among CFTR proteins from mammalian species (Dahan *et al*, 2001) and conservation of domain–domain interactions in related ABC proteins even from bacteria (Dawson and Locher, 2006, 2007; Hollenstein *et al*, 2007; Ward *et al*, 2007; Aller *et al*, 2009). Although human NBD1 constructs can now be obtained with reasonable solubility and yield, they require deletions of the phosphoregulatory regions, notably the RI, thus precluding analysis of the structural basis of phosphorylation-dependent interactions. We have chosen to perform NMR studies on the natural murine WT and $\Delta F508$ NBD1 sequences that do not contain any other substitutions or deletions of the phosphoregulatory regions. Notably, a recent study indicates that a reduction in open probability in human, mouse, and pig $\Delta F508$ CFTR occurs by the same mechanism (Ostedgaard *et al*, 2007). Thus, the natural murine WT and $\Delta F508$ NBD1 sequences can be used to elucidate the molecular basis of some regulatory features of CFTR.

Our previous NMR work on the isolated human R region (Baker *et al*, 2007) showed a dynamic interaction between murine NBD1 and multiple R region segments with significant helical propensity. Phosphorylation by PKA at multiple Ser residues alters the conformational ensemble of the R region to reduce the helical propensity and disrupt interactions with NBD1. In this study, we describe NMR studies of regions of murine WT and $\Delta F508$ CFTR that contain NBD1 (residues 389–653) and the RE (NBD1–RE; residues 389–673). We have made resonance assignments for WT NBD1–RE and probed changes in the conformation of the protein and its interactions with coupling helix 1 upon phosphorylation and deletion of Phe508, shedding light on the mechanisms underlying regulation of CFTR and its dysfunction in CF.

Results

NMR spectra of WT NBD1–RE

The NMR spectrum of WT NBD1–RE that is bound to ATP is presented in Figure 1A and B, showing dispersion characteristics of a folded protein. Identical spectra of WT NBD1–RE recorded at different concentrations (0.10, 0.25, and 0.42 mM) or different times (over 7 days) indicate that there is no concentration-dependent aggregation, change in oligomeric state, or increase in disorder occurring under these conditions (data not shown). NMR resonances of WT NBD1–RE have different intensities, varying from sharp to broad, weak signals (Figure 1C). The sharp resonances, which are centred at ~8.2 p.p.m. (Figure 1B), are due to the disordered segments of NBD1–RE, and the number of these peaks (~20) suggests that significant segments of the protein are disordered in solution, including the RI and RE, with only transient sampling of ordered conformations. The significant broadening observed for some resonances is indicative of motion on the μ s–ms timescale, as shown by elevated R_2 relaxation rates (Supplementary Table 1) and different peak intensities at different magnetic field strengths, as broadening due to μ s–ms dynamic processes is field dependent (Supplementary Figure 2). Spectra of WT NBD1–RE in the ATP-bound state recorded with varying concentrations of glycerol and at different temperatures and buffer conditions were very similar to the spectrum shown in Figure 1A (data not shown). Therefore, the variable peak intensity that is observed likely reflects the general motional properties of NBD1–RE rather than specific solution conditions.

Resonance assignment of G550E/R553M/R555K NBD1–RE

The weak intensity of many of the resonances and the limited stability of the WT NBD1–RE NMR samples precluded resonance assignment. Therefore, we used a variant of NBD1–RE containing the revertant mutations, G550E (DeCarvalho *et al*, 2002), R553M (Teem *et al*, 1993), and R555K (Teem *et al*, 1996). These mutations increase the amount of soluble recombinantly expressed NBD1–RE and the solubility of the purified protein. The G550E/R553M/R555K mutant NBD1–RE could be concentrated to ~600 μ M and was stable for >20 days, allowing NMR data for backbone resonance assignment to be recorded. More resonances are present in the spectra of the G550E/R553M/R555K mutant compared with WT NBD1–RE (Supplementary Figure 3), pointing to less severe broadening than in the spectra of WT protein because of differences in motion on the μ s–ms timescale. Although not as extensive as observed for the WT NBD1–RE, spectra of the G550E/R553M/R555K mutant also show broadening, with some of the weak resonances having elevated R_2 rates from μ s–ms timescale motion (Supplementary Table 1). Relaxation data recorded on 360 and 550 μ M samples of the G550E/R553M/R555K mutant were very similar for most residues, indicating that the elevated R_2 rates are not caused by sample aggregation at high concentrations (Supplementary Table 1). Many resonances are weak, especially in the spectra of the lower concentrated sample of the G550E/R553M/R555K mutant (i.e., Val562, Asp572, and Ser573), precluding reliable R_2 values from being obtained for these residues. Importantly, for resonances observed for both the WT and G550E/R553M/R555K mutant forms of the protein,

Figure 1 Conformational and dynamic changes in NBD1-RE with the $\Delta F508$ mutation. (A) Comparison of ^{15}N - ^1H correlation spectra for WT (400 μM) and $\Delta F508$ (175 μM) NBD1-RE with 5 mM Mg^{2+} and 5 mM ATP in 20 mM Na^+ phosphate, pH 7, 150 mM NaCl, 2% glycerol, 5 mM DTT, 10% (v/v) D_2O at 20 $^\circ\text{C}$ at 600 MHz, with selected residues indicated. The spectrum of WT NBD1-RE is shown in the foreground with resonances of backbone nuclei, as well as those from side chain nuclei from Trp, Asn, and Gln residues, in black. The blue resonances are of opposite sign, caused by spectral aliasing, and are from side chain Arg $\text{N}_\text{C}\text{H}_2$ correlations and one backbone NH correlation. The spectrum of $\Delta F508$ NBD1-RE is shown in the background. Resonances coloured red and green in the $\Delta F508$ NBD1-RE spectrum correspond to those coloured black and blue in the WT NBD1-RE spectrum, respectively. (B) Selected region of the spectrum in (A) with assigned residues in the RI and RE highlighted. The asterisks indicate sharp resonances that are overlapped from residues in the RI or RE and residues in the NBD core. (C) Selected regions of the spectra with peaks of different intensities. A trace through the approximate centre of the peak is shown at the bottom of each spectrum, illustrating the lineshape. (D) The combined chemical shift difference, $\Delta\delta(\text{tot})$, from deletion of F508 is plotted as a function of residue number. The combined chemical shift difference is calculated with the equation $((\Delta\text{H.p.p.m.})^2 + (\Delta\text{N.p.p.m.}/5.3)^2)^{1/2}$. The $\Delta\text{N.p.p.m.}$ value was divided by 5.3 to account for the difference in digital resolution between proton and nitrogen dimensions in our spectra. Significant chemical shift changes defined as values higher than the average of all $\Delta\delta(\text{tot})$ values plus 1 s.d. are shown by the red dashed line. $\Delta\delta(\text{tot})$ values greater than the maximum value shown on the y axis reflect very large chemical shift changes that limit our ability to assign that residue in $\Delta F508$ NBD1-RE. For situations in which there are multiple possibilities for the $\Delta F508$ assignment, the closest peak was used to calculate the $\Delta\delta(\text{tot})$ value. (E) A schematic ribbon diagram of the crystal structure of the WT NBD1-RE from murine CFTR (PDB code 1R0X) is shown. The ribbon is coloured blue for residues for which we have resonance assignments, light grey for those not assigned, and dark grey for those assigned in the G550E/R553M/R555K mutant but not transferable to WT NBD1-RE. The C α atoms for residues that show chemical shift changes in $\Delta F508$ NBD1-RE are shown as spheres coloured with a linear gradient from light pink ($\Delta\delta(\text{tot}) = 0.04$) to magenta ($\Delta\delta(\text{tot}) \geq 0.1$), as indicated by the bar in the top right. The C α atom of Phe508 is coloured yellow. All structure figures were created using MOLMOL (Koradi *et al*, 1996).

backbone chemical shifts are very similar (Supplementary Figure 3), allowing the straightforward transfer of assignments for most resonances. Using triple resonance experiments and specific labelling on Leu, the combination of Gly, Ser, Asp, and Asn residues, or aromatic residues, we

have assigned 70% of the $^1\text{H}^\text{N}$ and ^{15}N resonances in the G550E/R553M/R555K mutant and 60% of the $^1\text{H}^\text{N}$ and ^{15}N resonances in WT NBD1-RE (Supplementary Figure 4a). Fewer assignments for the WT are due to chemical shift changes primarily for residues Glu543-Ala559 near the sites

of mutations. Similarly, transfer of many assignments was possible to the Δ F508 NBD1-RE, to the WT NBD1 (lacking the RE), and to the phosphorylated states of WT and Δ F508 proteins. The more the changes in spectral positions, however, the fewer the assignments that could be transferred. The secondary structures of the G550E/R553M/R555K mutant, WT, and Δ F508 NBD1-RE were determined using ^1H and ^{15}N chemical shifts, as well as $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, and ^{13}CO chemical shifts where available (Supplementary Figure 5). As expected from the similarity of the NMR spectra, secondary structures of the G550E/R553M/R555K mutant, WT, and Δ F508 NBD1-RE proteins are very similar and largely agree with that of the crystal structures.

The resonance assignments and different peak intensities give insights into the motional properties of the CFTR NBD1-RE. Consistent with the crystal structures of CFTR NBD1-RE (Lewis *et al*, 2004, 2005; Thibodeau *et al*, 2005), many of the sharp resonances located near 8.2 p.p.m. in the ^1H dimension are from residues in the RE and the disordered linker of the RI (Figure 1A–C and Supplementary Table 1). Additional peaks in this region, which are not as intense as those from the disordered linker but are more intense than the average, are from residues in the H1c helix of the RI, implying increased mobility of this fragment (Supplementary Table 1). This observation is in contrast with the predominantly ordered structure of the H1b and H1c helices of the RI when bound to the NBD core that is observed in crystal structures of CFTR (Lewis *et al*, 2004, 2005; Thibodeau *et al*, 2005).

Interestingly, the unassigned residues in the G550E/R553M/R555K mutant map to distinct regions on NBD1-RE (Supplementary Figure 4b). One set of residues involved includes those in the N-terminus of helix H1, the N-terminus of helix H6, the N-terminus of helix H7, the C-terminus of helix H8, and the N-terminus of helix H9. The N-termini of helices H1, H6, and H7 form part of the NBD1/NBD2 dimerization interface (Smith *et al*, 2002; Mense *et al*, 2006). The H1b helix of the RI and the H9b helix of the RE are also not assigned and, when bound to the NBD core, these elements form a nearly contiguous surface with helices H1 and H6. This surface changes when the RE adopts an alternate conformation and contacts only helices H6 and H7, as observed in the crystal structure of human NBD1-RE F508A (Lewis *et al*, 2004, 2005). Helix H4, which is located in the α -helical subdomain and is adjacent to helix H6, is also not assigned. The few broadened resonances that could be assigned and for which R_2 rates could be extracted (Ser478, Ser490, Cys524, Val591, and Ser641) map to sites around the unassigned residues, implicating dynamic interactions of the RI and RE with multiple surfaces of the NBD core on the μs –ms time-scale. In addition, the broadening of residues near the Walker A sequence in the triple resonance assignment experiments (data not shown) implicates motion at the ATP-binding site, possibly caused by exchange of ATP on and off of the binding site and/or the effects of dynamic interactions of the RI and RE at this site.

Effect of the Δ F508 mutation on NBD1-RE

The spectra of NBD1-RE for WT and Δ F508, containing no additional mutations, are very similar (Figure 1A and D), showing that WT and Δ F508 NBD1-RE adopt similar structures, as previously observed (Lewis *et al*, 2004, 2005). Exceptions include the loss of resonance for Phe508 in the

mutant, as well as chemical shift changes for the backbone amides of Ile506, Ile507, and Val562–Ala566, all located in the α -helical subdomain and within 6 Å of Phe508 (Figure 1D and E). Resonances of Asn505 and Gly509–Ser511 are overlapped, masking any chemical shift changes. The sharp resonances observed in the spectrum of Δ F508 indicate that the RI and RE are also significantly disordered in Δ F508 NBD1-RE.

Chemical shift changes are also observed for some residues that are not close to Phe508 (Figure 1D and E). These include residues at the interface between the α/β and α -helical subdomains (Val488, Cys491, and Gln493), residues located on the other side of the α -helical subdomain (Cys524, Asp529, Thr531, Lys532, Ala534, Glu535, Asp537, Asn538, Val540, and Val591), as well as the RI residue Gly437 and α/β subdomain residues Ile616, Gly622, and Arg647, which are 30–40 Å away from Phe508 in the various crystal structures (Lewis *et al*, 2004, 2005; Thibodeau *et al*, 2005). These chemical shift differences may result from conformational changes in Δ F508 compared with WT, including, but not limited to, changes in the relative orientation of the α -helical and α/β subdomains and/or different interactions of the RI and/or RE with the NBD core. Significant conformational changes, apart from differences in the local surface properties at the mutation site, were not observed in the crystal structures of Δ F508 NBD1-RE (also containing F429S, F494N, and Q637A mutations required for protein solubility and crystallization) (Lewis *et al*, 2004, 2005) and of Δ F508 NBD1 lacking the RI and the RE (PDB code 2PZF).

Differential effect of phosphorylation of WT and Δ F508 NBD1-RE

Our previous work on the isolated R region indicates that PKA phosphorylation decreases the helical propensity of many residues in the R region and disrupts binding of NBD1 to most segments of the full-length phospho-R region (Baker *et al*, 2007). To probe the structural basis of these differences from the perspective of NBD1, we have compared the spectra of phosphorylated (phospho)-WT NBD1-RE, phospho-WT NBD1 lacking the RE, and phospho- Δ F508 NBD1-RE with their non-phospho counterparts (Figures 2 and 3). Constructs of CFTR NBD1 lacking the RE (residues 389–653) are referred to as NBD1 to distinguish them from NBD1-RE. The murine WT and Δ F508 NBD1-RE contain four PKA phosphorylation sites, with three located in the RE at Ser659, Ser660, and Ser670 and one in the RI at Ser422, while NBD1 lacking the RE has only the single RI phosphorylation site. Human NBD1-RE does not have a phosphorylation site at position 659. Analysis of tryptic fragments using mass spectrometry indicated complete phosphorylation of Ser422 in the RI and Ser659 and Ser670 in the RE, with almost complete phosphorylation of Ser660 in the RE (Supplementary Figure 6). The NMR spectra of two different samples of phospho-WT NBD1-RE are very similar (data not shown), illustrating the reproducibility of our protocol.

Large differences between the spectra of phospho- and non-phospho-WT NBD1-RE (Figure 2A and D) result from the introduction of four phosphate groups that significantly change the chemical environment around Ser422, Ser659, Ser660, and Ser670, and neighbouring residues. Chemical shift changes are observed for Ser423 and Asp424 in the RI, and Thr604 and Tyr625 in the NBD core (Figure 2G, top),

Figure 2 Phosphorylation of the RI and RE disrupts interactions with the NBD core. ^{15}N - ^1H correlation spectra of (A) non-phospho-WT NBD1-RE (400 μM) and phospho-WT NBD1-RE (150 μM), (B) non-phospho-WT NBD1-RE (400 μM) and non-phospho-WT NBD1 (85 μM), and (C) non-phospho-WT NBD1 (85 μM) and phospho-WT NBD1 (25 μM) are overlaid. The solution conditions for each sample are identical to those described in the legend to Figure 1. The spectrum of non-phospho-WT NBD1-RE is in the foreground in (A, B), with resonances coloured in black or blue as in Figure 1. The spectra of phospho-WT NBD1-RE and non-phospho-WT NBD1 are in the background in (A, B), respectively, with resonances coloured red and green as in Figure 1. Blue circles in (A, B) highlight chemical shift changes in non-phospho-WT NBD1-RE upon both phosphorylation and removal of the RE, respectively. The spectrum of non-phospho-WT NBD1 is shown in the foreground in black and blue in (C) whereas that of phospho-WT NBD1 is shown in the background in red and green. Light blue circles in (C) highlight the subset of resonances in non-phospho-WT NBD1 with chemical shift changes upon phosphorylation common to both phosphorylation of WT NBD1-RE and removal of the RE. The green squares highlight resonances in non-phospho-WT NBD1 that show chemical shift changes that are common to phosphorylation of WT NBD1-RE but which are not observed with removal of the RE, indicating that these chemical shift changes are specific to phosphorylation of the RI. Selected regions of the spectra in (A-C) are shown in (D-F), respectively. Phosphorylation of NBD1-RE (D) and NBD1 (F) results in an increased number of resonances at approximately 8.2 p.p.m. in the ^1H dimension, as shown by the asterisks (*). (G) The combined chemical shift difference, $\Delta\delta(\text{tot})$, is plotted as a function of residue. $\Delta\delta(\text{tot})$ is plotted for non-phospho- and phospho-WT NBD1-RE (top), non-phospho-WT NBD1-RE, and non-phospho-WT NBD1 (middle), and non-phospho-WT NBD1 and phospho-WT NBD1 (bottom). The sparseness of the data in the bottom panel is because of the lack of definitive assignments for non-phospho-WT NBD1 that can be obtained by transferring resonance assignments from non-phospho-WT NBD1-RE.

Figure 3 Differential effect of phosphorylation on WT NBD1-RE and $\Delta F508$ NBD1-RE. (A) ^{15}N - ^1H correlation spectra of non-phospho- $\Delta F508$ NBD1-RE (175 μM) and phospho- $\Delta F508$ NBD1-RE (125 μM) are overlaid. The spectrum of non-phospho- $\Delta F508$ NBD1-RE is shown in the foreground in black and blue, whereas that of phospho- $\Delta F508$ NBD1-RE is shown in the background in red and green. (B) The combined chemical shift difference, $\Delta\delta(\text{tot})$, for non-phospho- $\Delta F508$ NBD1-RE and phospho- $\Delta F508$ NBD1-RE (top) and for non-phospho-WT NBD1-RE and phospho-WT NBD1-RE (bottom; repeated from Figure 2G for comparison) is plotted as a function of residue number. (C) ^{15}N - ^1H correlation spectra of phospho-WT NBD1-RE (150 μM) and phospho- $\Delta F508$ NBD1-RE (125 μM) are overlaid. The spectrum of phospho-WT NBD1-RE is shown in the foreground in black and blue, whereas that of phospho- $\Delta F508$ NBD1-RE is in the background in red and green. Resonances that appear upon phosphorylation of WT NBD1-RE but not $\Delta F508$ NBD1-RE are indicated by purple boxes. (D) Schematic ribbon diagram with C α atoms for residues that show chemical shift differences between phospho-WT NBD1-RE and phospho- $\Delta F508$ NBD1-RE shown as spheres coloured as in Figure 1E. The backbone is coloured by resonance assignment as in Figure 1E, with additional residues coloured in dark grey that have been assigned in WT NBD1-RE but cannot be transferred to $\Delta F508$ NBD1-RE. The C α atom of Phe508 is coloured yellow.

which are close in space to the phosphorylation sites in the crystal structures of murine NBD1-RE (Lewis *et al*, 2004). Other residues close to these Ser positions have not been assigned.

Phosphorylation of NBD1-RE also results in an increase in the number of sharp resonances (~ 15) centred around 8.2 p.p.m. in ^1H chemical shift (Figure 2D), indicating a greater number of disordered residues or population of molecules with regions that sample disordered conformations. Furthermore, phosphorylation of WT NBD1-RE induces specific chemical shift changes that are also observed upon deletion of the RE (Figure 2A, B, D and E, blue circles), indicating that phosphorylation disrupts interactions of the RE with the NBD core. Many of the residues showing phosphorylation-dependent chemical shift changes

(Supplementary Table 1) are in the proposed dimer interface (Figure 4C). Our current observations are consistent with our previous data on the isolated full-length R region (Baker *et al*, 2007). In addition, peak intensities are more uniform for phospho-WT NBD1-RE than the non-phosphorylated protein (Figure 2D and Supplementary Table 1), reflecting decreased broadening because of decreased fluctuating interactions of the RI and RE with the NBD core on the μs -ms timescale. Interestingly, phosphorylation of the RI and RE, or removal of the RE, causes chemical shift changes in the NBD core on the opposite side of the molecule from the ATP-binding site (Supplementary Figure 7). Formed in part by Lys593, Arg611, Lys612, Tyr627, Phe630, and Glu632, this surface may define an RE (and thus an R region) interacting site not previously observed in any of the crystal structures of CFTR

Figure 4 Differences in phosphorylation-dependent changes in WT and $\Delta F508$ NBD1-RE. Schematic ribbon diagrams of WT NBD1-RE from murine CFTR (PDB codes 1R0X for **A**, **C**, **D**, **F**, and PDB code 1XMI for **B**, **E**) with α atoms that show chemical shift differences between non-phospho-WT NBD1-RE and phospho-WT NBD1-RE (**A–C**) and non-phospho- $\Delta F508$ NBD1-RE and phospho- $\Delta F508$ NBD1-RE (**D–F**). As in Figures 1E and 3D, residues are coloured based on the resonance assignment data and chemical shift changes with phosphorylation. Residues with phosphorylation-dependent chemical shift changes that are specific to WT NBD1-RE are labelled in (**A**, **B**), whereas residues that show chemical shift changes only with phosphorylation of $\Delta F508$ NBD1-RE are labelled in (**D**, **E**). Phosphorylation of WT NBD1-RE results in chemical shift changes for more residues than in $\Delta F508$ NBD1-RE, many of which are located in the central β -sheet of the ATP-binding subunit and the β -sheet subdomain. A total of 61 residues show significant chemical shift changes with phosphorylation in WT NBD1-RE compared with only 51 residues in $\Delta F508$ NBD1-RE. Further, phosphorylation of WT NBD1-RE results in large chemical shift changes ($\Delta\delta$ (tot) ≥ 0.1) for 37 residues but only for 26 residues upon phosphorylation of $\Delta F508$ NBD1-RE, based on our assignments of the phosphorylated proteins. Panels (**C**, **F**) show schematic ribbon diagrams shown in (**A**, **D**), respectively, but lacking the RI and RE to reveal the putative NBD1/NBD2 dimerization interface (denoted by the dashed box).

NBD1-RE. Alternatively, these shifts might reflect conformational changes transmitted through the domain by RE binding or release. Thus, phosphorylation of the RI and RE seems to alter their dynamic properties, including fluctuations in their conformations and their interactions with the hydrophobic NBD core.

Phosphorylation of NBD1 lacking the RE (Figure 2C) and of NBD1-RE (Figure 2A) results in the appearance of a cross-peak corresponding to phospho-Ser422 (pSer422). Additional spectral changes observed for NBD1 upon phosphorylation include some chemical shift changes for resonances located throughout the spectrum, but most notably for a few resonances near ~ 8.2 p.p.m. from Ser423, Asp424, Glu425, Leu 435, and Asn438. Chemical shift changes in Ser423, Asp424, and Glu425 are likely due to their proximity with the phosphorylation site. However, chemical shift changes for residues Leu435 and Asn438, which are located in the H1c helix of the RI and are at least 10 residues C-terminal to Ser422, may be due to structural changes in

the RI. Phosphorylation-dependent chemical shift changes in NBD1-RE are also observed for Leu435 and Asn438 (Figure 2G). Note that changes in the spectra of NBD1 upon phosphorylation are not as dramatic as those for NBD1-RE because the RE has been deleted. In addition, phosphorylation of NBD1 results in the appearance of ~ 7 resolved sharp resonances at ~ 8.2 p.p.m., some of which are indicated by the asterisks in Figure 2F. Although our ability to quantify the increase in disordered resonances for phospho-WT NBD1 and NBD1-RE is somewhat limited because of overlap in this region of the 2D ^1H - ^{15}N correlation spectra, these spectral changes clearly show that phosphorylation disrupts the interaction of the RI with the NBD core, changing the equilibrium so that the RI samples more disordered conformations.

Phosphorylation of $\Delta F508$ NBD1-RE (Figure 3A and B) also results in spectral changes that include changes in peak positions, peak intensity, and the appearance of sharp resonances at ~ 8.2 p.p.m. in the ^1H dimension, although the number of resonances reflecting disorder that are observable

from phosphorylation of Δ F508 NBD1-RE is ~ 5 , compared with ~ 15 for WT NBD1-RE. In addition, other phosphorylation-dependent resonances that are observed in the spectra of phospho-WT NBD1-RE are not observed or are very weak in the spectra of phospho- Δ F508 NBD1-RE (Figure 3C, purple boxes). The spectra of phospho- Δ F508 also show markedly different intensities, similar to the spectra of non-phospho-WT and Δ F508 (Supplementary Table 1). Interestingly, most of the greatest chemical shift differences between the phospho-WT and phospho- Δ F508 NBD1-RE are in the α/β subdomain and β -sheet subdomain, whereas there are only small chemical shift differences for residues near F508 (Figure 3D). The large chemical shift differences that are observed between phospho-WT and phospho- Δ F508 NBD1-RE (Figure 3C) are due to changes that occur upon phosphorylation of the WT NBD1-RE, but that do not occur for Δ F508 NBD1-RE (Figures 3B and 4). The distinct spectral properties and phosphorylation-dependent chemical shift changes of phospho-WT and phospho- Δ F508 NBD1-RE suggest different conformations for these proteins, including less disorder of the RI and RE induced by phosphorylation and greater residual interactions of the phosphorylated RI and RE with the NBD core in phospho- Δ F508 NBD1-RE (Figure 4D and E) versus WT (Figure 4A and B). We have been unable to obtain NMR spectra of phospho- Δ F508 NBD1 lacking the RE because of limited protein solubility.

Interactions of the first coupling helix and WT NBD1 require phosphorylation

The crystal structures of seven full-length bacterial ABC transporters (Locher *et al*, 2002; Dawson and Locher, 2006, 2007; Hollenstein *et al*, 2007; Oldham *et al*, 2007; Pinkett *et al*, 2007; Ward *et al*, 2007; Gerber *et al*, 2008; Kadaba *et al*, 2008) and murine P-glycoprotein (Aller *et al*, 2009) in various nucleotide-bound states show ICDs linking the MSDs and NBDs. These interactions are via short helical segments, or coupling helices, located at the ends of the ICDs. Electron microscopy (EM) studies of human CFTR are consistent with similar interactions of the NBDs and the coupling helices in CFTR (Rosenberg *et al*, 2004; Awayn *et al*, 2005; Zhang *et al*, 2009). However, at 21–35 Å the EM data do not provide information regarding the specific residues involved in these interactions. To guide our experimental studies of NBD1 interactions with the coupling helices of the ICDs, homology models of WT CFTR (Supplementary Figure 8) were generated based on the crystal structure of Sav1866, in which NBD1 and NBD2 form a productive dimer (Dawson and Locher, 2006, 2007). A structure-based alignment using multiple sequences from the C-family of ABC proteins was used to optimize the results (see Materials and methods). The crystal structure of the human F508A NBD1-RE (Lewis *et al*, 2005), in which the RI is not bound to the NBD core, was also used as a template. Residues Gln634–Asp673 were excluded in the template to avoid steric clashes in the modelled dimer interface.

Coupling helix 4 interacts with Phe508 in our homology model and as shown by other modelling and crosslinking data (Mendoza and Thomas, 2007; Serohijos *et al*, 2008). Interactions analogous to those between NBD1 and coupling helix 4 of CFTR were also observed in the crystal structure of the bacterial lipid exporter MsbA (Ward *et al*, 2007), shown by specific crosslinks in P-glycoprotein (Zolnericiks

et al, 2007) and observed in the recent crystal structure of P-glycoprotein (Aller *et al*, 2009). In addition, NBD1 interacts with coupling helix 1 in our homology model and in crystal structures of the bacterial exporters Sav1866 (Dawson and Locher, 2006, 2007), MsbA (Ward *et al*, 2007), and P-glycoprotein (Aller *et al*, 2009). Coupling helices 1 and 4 are located within 10 Å of their respective binding sites on NBD1 in our homology models and crystal structures of full-length ABC transporters. As few studies have probed the interactions between NBD1 and coupling helix 1 (He *et al*, 2008; Serohijos *et al*, 2008), in this study we focused on the binding of coupling helix 1 to WT and Δ F508 NBD1. A synthetic peptide (with an acetylated N-terminus and amidated C-terminus) comprising the NBD1-interacting region of coupling helix 1, determined from our homology model, was generated (Lys166–Val181, see Materials and methods), and binding to WT and Δ F508 NBD1 was tested using NMR spectroscopy (Table I, Figures 5 and 6).

Surprisingly, coupling helix 1 binds to phospho-WT NBD1-RE and phospho-WT NBD1 (Figure 5A and C, respectively) but not to their non-phosphorylated counterparts (Figure 5B), or at least the interactions are much weaker (Table I). Together, these results indicate that binding of coupling helix 1 with NBD1 is promoted by phosphorylation of the RI or a combination of the RI and RE. Multiple residues across the domain show chemical shift changes upon binding coupling helix 1 (Figure 5D), which are due to the direct interaction of NBD1 residues with coupling helix 1 and/or conformational changes transmitted to secondary sites. Many of the residues with large chemical shift changes upon addition of coupling helix 1 are close in space in the β -sheet subdomain (Ala477, Glu479, Gly480, and Lys483) and the α/β subdomain (Leu468 and Gly622), and are consistent with the interaction surface between NBD1 and coupling helix 1 observed in crystal structures of Sav1866, MsbA, P-glycoprotein, and our homology model (Figure 5D and Supplementary Figure 8). This interface is occluded in crystal structures of the murine WT and human Δ F508 NBD1-RE in which the RI is bound to the NBD core, but is exposed in the human F508A NBD1-RE structure (Lewis *et al*, 2004, 2005). Therefore, phosphorylation of the RI seems to promote exposure of this site for binding to coupling helix 1.

Additional residues in NBD1-RE that show chemical shift changes upon binding coupling helix 1 include Thr501, Ala534, Thr539, Val562, and Tyr563 in the α -helical subdomain, Met453, Lys598, and Arg611 in the α/β subdomain, as well as Gly437 in the RI. These sites are distributed across the domain and are likely due to changes in conformation of the phospho-NBD1 and NBD1-RE upon binding coupling helix 1, such as further displacement of the RI and RE, as some of these residues also show chemical shift changes upon phos-

Table I Summary of WT and Δ F508 NBD1 interactions with coupling helix 1 observed from NMR data

Construct	Wild type	Δ F508 mutant
NBD1-RE	No binding	—
NBD1	No binding	—
Phosphorylated NBD1-RE	Binding	No binding
Phosphorylated NBD1	Binding	—

A dash (—) indicates that binding was not tested.

Figure 5 Differential binding of coupling helix 1 to phospho-WT NBD1 and NBD1-RE, and non-phospho-WT NBD1. (A) Comparison of ^{15}N - ^1H correlation spectra at 500 MHz for phospho-WT NBD1-RE in the unbound state (90 μM) and bound to coupling helix 1 (295 μM). The solution conditions are identical to those described in the legend to Figure 1. The significant conformational flexibility of phospho-WT NBD1-RE results in the broadening of many resonances that are observed at higher field (Figure 2A). The spectrum of phospho-WT NBD1-RE in the unbound state is in the background with resonances coloured in black or blue as in Figure 1. The spectrum of phospho-WT NBD1-RE bound to coupling helix 1 is in the foreground with peaks coloured red and green corresponding to the black and blue peaks in the spectrum of the unbound state, respectively. (B) ^{15}N - ^1H correlation spectra at 600 MHz of non-phospho-WT NBD1 (85 μM) in the presence and absence of coupling helix 1 (1 mM) are overlaid. The spectrum of WT NBD1 without coupling helix 1 is shown in the background with resonances coloured in black or blue as in Figure 1. The spectrum of WT NBD1 in the presence of coupling helix 1 is shown in the foreground with peaks coloured red and green as in Figure 1. Note that the spectra of WT NBD1 in the presence and absence of coupling helix 1 are identical, with no resonances changing in chemical shift and no new peaks appearing, indicating that non-phospho-WT NBD1 does not bind coupling helix 1. (C) Selected region of the ^{15}N - ^1H correlation spectra at 600 MHz of non-phospho-WT NBD1 (85 μM), phospho-WT NBD1 (25 μM), and phospho-WT NBD1 bound to coupling helix 1 (250 μM). The spectrum of non-phospho-WT NBD1 is in the background with resonances coloured in black. The spectra of phospho-WT NBD1 is in the middle, whereas that of phospho-WT NBD1 bound to coupling helix 1 is in the foreground. Resonances coloured red and cyan in spectra of phospho-WT NBD1 without and with coupling helix 1, respectively, correspond to the black resonances in the spectrum of non-phospho-WT NBD1. Arrows highlight specific linear chemical shift changes upon phosphorylation of NBD1 and binding of coupling helix 1 to phospho-WT NBD1. Asterisks indicate new peaks that appear in spectra of phospho-WT NBD1 with coupling helix 1 binding. The T_1 noise at ~ 8.2 p.p.m. in the ^1H dimension (very small peaks) in spectra of phospho-WT NBD1 and phospho-WT NBD1 + coupling helix 1 is because of the presence of large amounts of ATP (2 mM) versus protein (25 μM). These noise peaks are of much lower intensity than peaks from disordered regions of the protein. (D) The interaction of NBD1-RE and coupling helix 1 from the homology model based on Sav1866 is shown as a schematic ribbon diagram, with the WT NBD1-RE structure coloured as in Figure 1 and the interacting peptide in red. The C α atoms for residues that show chemical shift changes upon binding of coupling helix 1 in phospho-WT NBD1-RE and/or phospho-WT NBD1 are shown as magenta spheres. The C α atom of Phe508, which does not show chemical shift changes upon addition of coupling helix 1 (C, D), is highlighted in yellow. Although G437 disappears upon addition of coupling helix 1 in phospho-WT NBD1-RE, other residues of the RI, such as N427 (C), do not show chemical shift changes. Chemical shifts observed with addition of coupling helix 1 reflect both binding of coupling helix 1 to the NBD1 surface and conformational changes in the protein, which may include displacement of the RI and RE from the NBD1 core.

phorylation. Interestingly, a few resonances show linear chemical shift changes (Pufall *et al*, 2005) when comparing non-phospho-WT NBD1, phospho-WT NBD1, and phospho-

WT NBD1 bound to coupling helix 1 (Figure 5C). Such linear chemical shift changes reflect fast exchange on the NMR timescale between two distinct states, here being (1) the RI

Figure 6 Comparison of ^{15}N - ^1H correlation spectra at 600 MHz for phosphorylated ΔF508 NBD1-RE in the absence (125 μM) and presence of coupling helix 1 (750 μM). The solution conditions are identical to those described in the legend to Figure 1 with the exception that 4% (v/v) glycerol was used rather than 2% (see Results). The spectrum of phospho- ΔF508 NBD1-RE in the absence of coupling helix 1 is in the background with resonances coloured in black or blue as in Figure 1. The spectrum of phospho- ΔF508 NBD1-RE in the presence of coupling helix 1 is in the foreground with peaks coloured red and green as in Figure 1. Note the very strong T_2 noise in this spectrum at 6.8, 8.2, and 8.5 p.p.m., derived from ATP resonances (see legend to Figure 5).

and RE tightly interacting with the NBD core and (2) the RI and RE fully displaced from the NBD core (Figure 7). This observation, therefore, is most simply explained by a direct linkage between binding of NBD1 and coupling helix 1 and disruption of RI and RE interactions with the NBD core. Binding coupling helix 1 to phospho-WT NBD1 also results in the appearance of ~ 8 resonances centered at approximately 8.2 p.p.m. in the ^1H dimension (Figure 5C), indicating that binding of coupling helix 1 increases the number of disordered residues in phospho-WT NBD1 (Figure 7).

In contrast with the WT protein, coupling helix 1 does not bind phospho- ΔF508 NBD1-RE or binds with much lower affinity, with no significant chemical shift changes upon addition of coupling helix 1 (Table I, Figure 6). The spectral features of phospho- ΔF508 indicate greater RI and RE interactions with the NBD core compared with phospho-WT NBD1-RE (Figures 3A, 4D, and E). Thus, the lack of exposure of the binding site for coupling helix 1 may explain the reduced binding of coupling helix 1 to phospho- ΔF508 NBD1-RE. Further support for this hypothesis is provided by our observation that residues showing chemical shift differences between phospho-WT and phospho- ΔF508 NBD1-RE overlap with our proposed interaction surface coupling helix 1 (Figures 4 and 5D). Binding studies between coupling helix 1 and phospho- ΔF508 NBD1-RE were conducted in the presence of 4%, not 2%, glycerol in the sample buffer, because of the decreased stability of NBD1-RE

imparted by deletion of Phe508 and phosphorylation. The significant destabilization of all forms of phosphorylated NBD1-RE (WT, ΔF508 , and the G550E/R553M/R555K) precludes NMR resonance assignment required to further test this hypothesis.

Discussion

Regulation of CFTR requires phosphorylation of the channel and ATP binding and hydrolysis at the NBDs, with interactions of the NBDs and ICDs likely involved in transferring these cytoplasmic events to the MSDs. Altered intramolecular interactions involving the NBDs, ICDs, and MSDs would affect channel gating in ΔF508 CFTR. Our results for WT and ΔF508 NBD1-RE show the interdependence of NBD1 interactions with the RI and RE phospho-regulatory elements and the first coupling helix in the N-terminal ICD.

NMR data for WT NBD1-RE, WT NBD1, and their phosphorylated counterparts (Figure 2A, B, D, and E) indicate that the RE and RI do not form stable structures in solution, but are rather mobile elements that bind transiently to the NBD core in the β -sheet and α/β subdomains (Figure 4A–C). However, in contrast with the X-ray data, in which phosphorylation of murine NBD1-RE leads to ordering of residues Lys420–Val428 in the RI and only a modest loss of helix in the RE, with no differences in interactions of the phosphoregulatory elements and the NBD1 core (Lewis *et al*, 2004), our work indicates that interactions of the RI (Figure 2C, F, and G) and RE (Figure 2A, B, D, E, and G) are disrupted upon phosphorylation. These results are consistent with our previous data on the R region (Baker *et al*, 2007) showing that both the helical structure of these phosphoregulatory elements and their interactions with the NBD1 core are disrupted upon phosphorylation. The result is an increased number of disordered residues or population of molecules with segments that sample disordered conformations (Figures 2A–F, 4A, and B). Notably, a 180° rotation in position of the RE and RI between the murine WT and human F508A crystal structures (Lewis *et al*, 2004, 2005) (Supplementary Figure 1b) also indicates significant conformational flexibility of the phosphoregulatory elements of CFTR. The spectra of non-phospho- and phospho- ΔF508 NBD1-RE (Figure 3A) also show that the RI and RE phosphoregulatory elements are mobile and have fluctuating interactions with the NBD1 core, although the disruption of interactions of the RI and RE in ΔF508 with phosphorylation is not as extensive as in the WT protein (Figures 3C and 4).

Consistent with functional studies of full-length CFTR (Tabcharani *et al*, 1991; Ma *et al*, 1997; Winter and Welsh, 1997; Mathews *et al*, 1998), our current and previous results indicate that phosphorylation of the R region regulates channel gating in part by disrupting its interaction with NBD1 to enhance ATP binding and hydrolysis (Baker *et al*, 2007). Because many of the interacting residues for the non-phosphorylated RI and RE in the NBD1 are in the NBD1/NBD2 dimerization interface (Figure 4C and F), our NMR data may explain the enhancement of ATP binding and hydrolysis in phosphorylated CFTR compared with non-phosphorylated CFTR (Li *et al*, 1996; Csanady *et al*, 2000). Although conformational changes upon phosphorylation cannot be ruled out, our data are also suggestive of a potential binding site for the RE on the side of the α/β subdomain, opposite from the

Figure 7 Model of dynamic interactions of the RI and RE with the NBD1 core. A schematic diagram of CFTR NBD1-RE is shown with the NBD1 core in dark blue and the RI and RE as red curves. Interaction surfaces for the RI and RE on the NBD1 core are represented by light blue ovals. The two states of NBD1-RE, in which the RI and RE are bound or unbound to the NBD core, represent the two extremes of the conformational ensemble of NBD1-RE. Different states of NBD1-RE are shown at their relative positions in the conformational ensemble. The average positions of the dynamic equilibrium from G550E/R553M/R555K and WT NBD1 were determined from the percentage of elevated R_2 rates measured for each protein (Supplementary Table 1), whereas that of $\Delta F508$ NBD1 was determined from the number of broadened residues compared with WT. The average position of the dynamic equilibrium for phospho-WT NBD1 and phospho-WT + coupling helix 1 were determined from four resonances that show linear chemical shifts for WT NBD1, phospho-WT NBD1, and phospho-WT NBD1 + coupling helix 1, assuming that we have achieved 80% saturation in the coupling helix 1 binding experiment. The width of the bars is determined from the s.d. of the chemical shift changes. The average position of phospho- $\Delta F508$ NBD1-RE (and phospho- $\Delta F508$ NBD1-RE + coupling helix 1, for which no chemical shift changes are observed compared with phospho- $\Delta F508$ NBD1-RE alone) is determined from the number of sharp resonances that reflect increased disorder compared with phospho-WT NBD1. Note that positions on the equilibrium are affected by the type of data used in each case. Because we do not have full assignments of these proteins, some of the parameters are underestimated, such as the percentage of elevated R_2 rates. In addition, the number of sharp resonances from disordered residues, used to determine the position of phospho- $\Delta F508$ NBD1-RE, may also be underestimated because of spectral overlap in this region. Although the exact positions of the species may change depending on the type of data used, these data reflect the relative positions of the G550E/R553M/R555K mutant, WT, and $\Delta F508$ NBD1-RE proteins.

ATP-binding site and dimerization interface (Supplementary Figure 7), which would still allow NBD1/NBD2 dimer formation with binding of the R region. As no one PKA phosphorylation site is necessary for CFTR activity, the presence of an additional binding site for the RE, and perhaps for other segments of the R region, may provide a structural explanation for CFTR activity of partially phosphorylated channels and for differential effects of the R region (Winter and Welsh, 1997; Xie *et al*, 2002; Chappe *et al*, 2005).

Chemical shift differences are observed between the WT and $\Delta F508$ NBD1-RE in both non-phosphorylated and phosphorylated states, reflecting conformational and dynamic differences, especially differences in the structural properties of the RI and RE and their association with the core NBD. For example, although far from Phe508, a number of residues in the β -sheet subdomain and the α/β (ATP-binding) subdomain only show chemical shift changes upon phosphorylation in WT NBD1-RE and not in $\Delta F508$ NBD1-RE, and fewer residues in the β -sheet subdomain show chemical shift changes in $\Delta F508$ NBD1-RE upon phosphorylation compared with WT (Figures 3D and 4). These observations suggest a difference in conformation of the RI and RE in the two proteins that may include greater interactions of the RI and RE with the NBD1 core in $\Delta F508$ NBD1-RE compared with WT. In contrast, structural differences, including in the interactions of the RI and RE with the NBD1 core, were not observed in the crystal structures of $\Delta F508$ NBD1-RE (Lewis *et al*, 2005) and

WT NBD1-RE (Lewis *et al*, 2004). The additional mutations (F494N, Q637A or F429S, F494N, and Q637R) in the $\Delta F508$ NBD1-RE construct required for protein solubility and crystallization (Lewis *et al*, 2005) also partially rescue the trafficking and gating defects of full-length $\Delta F508$ CFTR, suggesting that the crystal structure of $\Delta F508$ NBD1-RE may correspond to a partially corrected conformation (Pissarra *et al*, 2008). It is noteworthy that the F429S mutation is in the RI and further promotes the revertant effect produced by the F494N/Q637 mutant (Pissarra *et al*, 2008). These data hint that deletion of the RI (and RE) may also be partially correcting in that WT can more easily displace the RI and RE from the NBD core by phosphorylation than $\Delta F508$, facilitating a shift in the equilibrium towards heterodimer formation that could stabilize the full-length protein. In addition, the increased interactions of the RI and RE in $\Delta F508$ may explain the slower rate of PKA activation in $\Delta F508$ CFTR compared with WT (Drumm *et al*, 1991; Wang *et al*, 2000) and the increased ATP dependence of channel gating (Schultz *et al*, 1999).

Our NMR data and homology modelling also indicate that binding of coupling helix 1 to WT NBD1 and NBD1-RE is promoted by phosphorylation of the RI or the combination of the RI and RE (Table I), which may be required to expose the interaction surface for coupling helix 1 on NBD1. Phosphorylated $\Delta F508$ NBD1-RE does not bind or binds with weaker affinity (Table I), likely because of the increased

RI or RI and RE association with the NBD core. Because the phosphoregulatory regions interact with NBD1 in a dynamic manner, fluctuating on and off of the surface of the NBD core (Figure 7), some binding of coupling helix 1 possibly also occurs with non-phospho WT NBD1, particularly in full-length CFTR. Our negative binding results for non-phospho NBD1 and coupling helix 1 are likely because of the weakness of the interaction for isolated polypeptides. Although such an interaction is expected to occur between the coupling helix 1 and the surface of the NBD core based on analogous interactions in other ABC transporters (Locher *et al*, 2002; Dawson and Locher, 2006, 2007; Hollenstein *et al*, 2007; Oldham *et al*, 2007; Pinkett *et al*, 2007; Ward *et al*, 2007; Gerber *et al*, 2008; Kadaba *et al*, 2008), crosslinking studies of full-length CFTR show interactions of coupling helix 1 with two residues in helix H1b of the RI in a phosphorylation-independent manner (He *et al*, 2008). Although our NMR data show that the phosphorylated RI samples more disordered conformations, the RI has significant dynamic fluctuations even in the non-phosphorylated state (Figure 7) that could bring the RI into proximity with any NBD1-interacting region of CFTR, potentially explaining this unexpected observation. Note that we do see chemical shift changes in Gly437 with binding of coupling helix 1 to phospho-WT NBD1-RE (Figure 5A). However, no other RI residues show chemical shift changes upon interaction with coupling helix 1 (i.e., Asn427; Figure 5C), indicating that the RI does not form a stable interaction with coupling helix 1.

The multiple phosphorylation sites in the R region provide a level of redundancy in regulation of CFTR activity (Cheng *et al*, 1991; Chang *et al*, 1993). Phosphorylation of many residues in the R region results in active channels, with no requirement for a specific site. This redundant mode of regulation likely also encompasses the RI and therefore its removal would have little effect on phosphorylation-dependent activation of CFTR (Csanady *et al*, 2005), provided that phosphorylation sites in the R region are still intact. However, CFTR with complete removal of the R region is still slightly activated by PKA (Csanady *et al*, 2000), implicating the RI in regulation of CFTR activity. As summarized by our model (Figure 7), phosphorylation seems to regulate CFTR in part by altering the conformational ensemble and fluctuating interactions of the phospho-regulatory elements with NBD1. Our interaction experiments using isolated NBD1 or NBD1-RE and coupling helix 1 show a dependence on phosphorylation of the RI or the RI and RE together, thus highlighting one aspect of this redundant and dynamic mode of regulation.

The dynamic aspect of the interaction between NBD1 and coupling helix 1 likely extends to the entire NBD1/ICD interface and through structural symmetry across CFTR to the NBD2/ICD interface. Recent crosslinking studies indicate that interactions of NBD1 and coupling helix 4 are also dynamic and that flexibility across the interface between NBD1 and coupling helix 4 is necessary for channel gating (Serohijos *et al*, 2008). As interactions of coupling helix 4 and NBD1 are mediated by Phe508, binding of coupling helix 4 is likely disrupted or altered in $\Delta F508$ CFTR. Although they are far in the primary sequence and interact with different residues in NBD1, coupling helices 1 and 4 are close in space (with the closest residues within 6 Å) in our homology model of CFTR. Therefore, changes in the dynamic association between NBD1 and any one of these loops may result in conforma-

tional changes throughout CFTR. Notably, recent EM structures of CFTR show variability in the NBD/ICD interface (Zhang *et al*, 2009), which is possibly caused by dynamic interactions of the NBDs and coupling helices.

CF-causing mutations are not limited to $\Delta F508$ but are found throughout CFTR, including residues that comprise the coupling helices (Seibert *et al*, 1996, 1997) (<http://www.genet.sickkids.on.ca/cftr>), showing the importance of intramolecular interactions involving the ICDs. Mutation of Lys166, Arg170, Ile175, Ile177, Gly178, or Gln179 of the coupling helix 1 peptide region leads to CF (<http://www.genet.sickkids.on.ca/cftr>), likely because of disrupted maturation and/or altered activity of CFTR (Seibert *et al*, 1997), thereby supporting the role of intra-domain interactions involving coupling helix 1 in the folding and function of CFTR. Among other roles, the dynamic interaction between coupling helix 1 and NBD1, which is regulated by phosphorylation of the RI or RI and RE, is expected to be important during the gating cycle of CFTR. Removal of the RI results in CFTR channels with shorter open bursts and faster closing from locked-open states in the presence of PKA (Xie *et al*, 2002; Csanady *et al*, 2005). Although its effect on the formation of the NBD1/NBD2 heterodimer is likely critical, the RI may also modulate the open-burst state of the channel by altering interactions of NBD1 and coupling helix 1 in a phosphorylation-dependent manner. Altered binding of coupling helix 1 to $\Delta F508$ NBD1 would decouple these events, and possibly other NBD1/ICD interactions, and therefore may help explain the functional defect of the mutant channel, which includes altered whole cell Cl^- conductance because of a decreased open probability (Schultz *et al*, 1999; Wang *et al*, 2000; Ostedgaard *et al*, 2007). The availability of NMR assignments of NBD1 enables the exploration of additional interactions to provide further insights into both the function and misfunction of CFTR.

Materials and methods

Sample preparation

NBD1 from murine CFTR (residues 389–673 or 389–653) with the WT sequence, lacking Phe508 ($\Delta F508$), or containing the revertant mutations G550E, R553M, and R555K (G550E/R553M/R555K) (Teem *et al*, 1993, 1996; Roxo-Rosa *et al*, 2006), was expressed as a 6x-His-Smt (SUMO) (Mossessova and Lima, 2000) fusion at 16 °C in BL21(DE3) Codon Plus cells grown in minimal media with ^{15}N - NH_4Cl , ^{13}C -glucose, and/or 70% $^2\text{H}_2\text{O}$ as required for NMR studies, and purified using standard chromatographic techniques, as previously described (Lewis *et al*, 2004, 2005). Similar to previous work, proteins are kept in solutions containing 12.5% glycerol for sample stability until the final NMR sample is made (see below). Constructs ending at residue 673 contain the regulatory extension (RE) and are referred to as NBD1-RE, whereas those lacking the RE are referred to as NBD1. Note that binding and phosphorylation experiments used WT and $\Delta F508$ only. The G550E/R553M/R555K NBD1-RE mutant was used only for backbone resonance assignment (see Results). Purified WT, $\Delta F508$, and G550E/R553M/R555K mutant proteins were exchanged into NMR buffer containing 20 mM Na phos, pH 7.0, 150 mM NaCl, 5 mM MgCl_2 , ATP or AMP-PNP, with 2 or 4% (v/v) glycerol. Saturating concentrations of nucleotides were used, considering a K_d value of 150 μM , in each sample (Lewis *et al*, 2004). The protein concentration in each NMR sample reported in the text is the highest concentration that could be achieved because of protein expression levels or aggregation.

PKA phosphorylation of WT and $\Delta F508$ NBD1

Purified WT and $\Delta F508$ NBD1 proteins were exchanged into 50 mM Tris, pH 7.6, 50 mM ATP, 50 mM MgCl_2 , 5 mM DTT, and 5%

glycerol. Phosphorylation reactions were performed using 400 U PKA per mg NBD1 at 4 °C with NBD1 concentrations of 15–20 µM, and were allowed to proceed overnight. Aliquots containing 20 µg of protein were incubated with trypsin at a final concentration of 0.02 µg/ml at 37 °C for 2 h. The trypsin digestion was stopped with 0.5% (final concentration) formic acid. NBD1-RE digested peptides were subjected to LC-MS to identify sites of phosphorylation in the NMR samples. Mass spectrometric analysis of multiple phosphorylated samples indicated complete phosphorylation of Ser422 in the RI and Ser659 and Ser670 in the RE, with almost complete phosphorylation of Ser660 in the RE (Supplementary Figure 6). We saw no evidence of phosphorylation of any other Ser or Thr residues in the protein. Phosphorylated proteins were also detected using Pro-Q Diamond fluorescent phosphoprotein gel stain (Molecular Probes). Gels were imaged on a 300 nm UV transilluminator and subsequently counterstained with SYPRO Ruby Protein gel stain (Molecular Probes) to image total protein. The phosphorylated proteins (NBD1-RE, NBD1, and ΔF508) were exchanged into the NMR buffer described above.

Coupling helix 1 peptide

Homology models of CFTR were generated using the programme Modeller (Sali and Blundell, 1993). A structure-based sequence alignment (Supplementary Figure 5) of C-family ABC transporters based on the available structural data was generated using Clustal W (Thompson *et al*, 1994). The crystal structures of Sav1866 (PDB code 2HYD) and the human CFTR F508A NBD1-RE (PDB code 1XMI), excluding residues Gln634–Asp673, were used as templates. A total of 100 models were generated, from which the 15 lowest energy models were selected for analysis.

A peptide comprising the residues in the N-terminal ICD that include coupling helix 1 (Lys166–Val181) acetylated at the N-terminus and amidated at the C-terminus was synthesized by the Hospital for Sick Children/Advanced Protein Technology Centre (Toronto, Canada). Crude peptide was dissolved in 6 M guanidium hydrochloride with 0.1% TFA and incubated at room temperature for 1 h before purification. The peptide was purified by reverse-phase high performance liquid chromatography (RP-HPLC) using a C18 column with an acetonitrile gradient. Mass and purity were confirmed using electrospray mass spectrometry. The concentration of the peptide used for binding experiments was determined using amino acid analysis.

NMR spectroscopy

NMR experiments were carried out at 20 °C on a Varian 500, 600, or 800 MHz spectrometer equipped with pulsed field gradients and a triple resonance cryo-probe (600 MHz) or a triple resonance room temperature probe (500, 800 MHz) with actively shielded z-gradients. Data were processed and analyzed using nmrPipe/nmrDraw (Delaglio *et al*, 1995) and nmrView (Johnson and Blevins, 1994).

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Backbone H, N, C, and Cα, and side chain Cβ assignments for G550E/R553M/R555K NBD1-RE were obtained from standard triple resonance TROSY-based experiments (Sattler *et al*, 1999; Kanelis *et al*, 2001) and a ¹⁵N-edited NOESY-HSQC spectrum (200 ms) recorded on samples of 0.5–0.6 mM G550E/R553M/R555K NBD1-RE that were uniformly ¹⁵N and ¹³C labelled and fractionally ²H labelled to ~50%. These data were supplemented with ¹⁵N-¹H TROSY-HSQC spectra recorded on samples specifically ¹⁵N labelled on Leu residues, ¹⁵N labelled on aromatic residues (Phe, Tyr, and Trp), or ¹⁵N labelled on Gly, Ser, Asp, and Asn residues. The ¹⁵N-Leu-labelled sample was obtained by growing *E. coli* BL21(DE3) Codon Plus cells on M9 minimal media supplemented with ¹⁵N-labelled Leu (200 mg/l) and a mixture of 19 unlabeled amino acids. A similar approach was used to obtain the ¹⁵N-Phe/Tyr/Trp sample. The ¹⁵N-Gly/Ser/Asp/Asn labelled sample was expressed in the *E. coli* strain DL39 deficient in the aromatic (TyrB), branched chain (IlvE), and aspartate (AspC) transaminases. The cells were grown in M9 minimal media supplemented with ¹⁵N-labelled Gly (500 mg/ml) and ¹⁵N-labelled Asp (250 mg/ml) and a mixture of 16 unlabeled amino acids lacking Ser and Asn. The DL39 strain was kindly supplied by L McIntosh (University of British Columbia).

NMR assignments

NMR resonance assignments for G550E/R553M/R555K NBD1-RE, WT NBD1-RE, and ΔF508 NBD1-RE have been deposited in the BioMag Res Bank under the accession codes 16367, 16393, and 16394, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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