NMR Evidence for differential phosphorylation-dependent interactions in WT and ΔF508 CFTR

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1st Editorial Decision 23 February 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has now been evaluated by three referees whose comments are enclosed below. As you will see from their reports the referees express interest in your study and find that it provides further conceptual insight that will be of interest to the readers of EMBO J. From their reports, referee #1 would like to see more experimental evidence for the specific binding of ICL1 (coupling helix) to NBD1 upon release of RI and would like to see more peptides including the effects of disease-related mutants tested. Referee #3 requires that the resonance assignments be included in the manuscript and an accession code provided. Also importantly, both referee #2 and #3 feel that the manuscript and the general readership would benefit from clearer representation of the data and direct comparison between different experimental conditions and provide a number of ways this can be achieved. Should you be able to address all the raised concerns we would be willing to consider a revised version of your manuscript.

I would like to remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your revisions included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal
REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript describes the results of a large body of meticulous investigations and insightful if sometimes exaggerated interpretation. It is emphasized that the experiments use constructs with natural unmodified murine NBD1 sequences, but in fact one with the original 3 suppressor mutations is also used to improve expression and solubility so that more complete resonance spectral assignments could be made. This was helpful, but since fewer residues could be assigned without the suppressors, it cannot strictly be concluded that the structures are identical. This point is raised only because a major outcome of the overall study is that the ΔF508 mutation does indeed change structural features of the domain in contrast to crystallography results that have essentially indicated that it does not. This is not to argue against or question the apparent differences caused by the mutation reported here, but only to point out that perhaps even more information could have been obtained from comparison of wild-type and ΔF508 with suppressors as well as without. In addition to the assignments of a large proportion of the residues in the wild-type structure, the first significant observation is that residues in the Hθ helix of the RI may be more mobile and less ordered than when bound to the rest of the domain as in the crystal structure. Furthermore, several unassigned residues are localized within RI and RE as apparently are some of the broadened assigned resonances. In fact the multiple different crystal structures now available already indicated that much of the RI may be unstructured or able to assume different structures. However these new data do set the stage for focus on the RI and RE regions, which with their phosphorylation sites are candidates for involvement in CFTR regulation.

However, before focusing on phosphorylation, the influence of the ΔF508 mutation was explored. Although the NMR spectra of the mutant and wild-type NBD1 were generally similar, chemical shift changes were observed not only locally near Phe 508, but also more distantly and regions at the interface between the and α/β domains as well residues in the RI and RE are emphasized. This is highly significant because it constitutes the first biophysical evidence of NBD1 structural changes due to ΔF508 although differences in protease sensitivity have been observed.

In the phosphorylated wild-type NBD1 containing RE, large spectral differences in residues at and near the 4 PKA phosphorylation sites in RI and RE were observed, suggesting greater mobility or disorder in these regions. Such changes were also observed at distant locations including a region containing residues between K593 and E632 indicated to interact with the RE. A reference in support of the latter suggestion (Ma et al?) seems inappropriate or the meaning is obscure and should be clarified.

Importantly when RE is not present, these phosphorylation induced changes seem to have already occurred, possibly indicating that either the absence of RE or its phosphorylation when present may influence order in the interacting region. In ΔF508 NBD2 RE many of these phosphorylation dependent differences were not observed. Unfortunately, it was not possible to directly test whether changes due to just RI phosphorylation at ser 422 were also abrogated by ΔF508. However, a peptide corresponding to a loop between membrane spanning helices in CFTR did cause spectral changes in regions indicating its binding when the domain had been phosphorylated. This is interpreted as reflecting the freeing of this binding surface by phosphorylation of the RI which may normally block it. This is the least convincing of the claims made from the data presented. Evidence for an ICL1 binding site or surface is very indirect with this surface defined by sets of residues exhibiting spectral resonance changes on binding of a synthetic peptide. Most of these residues are localized together in NBD1 but one of them, Thr547 does not appear to be and as groups they don't appear to immediately contact ICL1. There are no control experiments employing any other peptides not corresponding to ICL1 or ICL1 peptides with disease-associated mutations such as those cited. Some such experiments are required to indicate the relevance of the interpretation to events in the intact CFTR protein.

The overall interpretation of the investigation, consistent with the previous NMR study of the R
domain by this group, is that phosphorylation control is elicited by further disrupting relatively unordered regions which alters their interaction with and influence on other segments of the protein. Thus, several important messages are conveyed that bear on understanding of both the control of this ion channel by phosphorylation and how this may be compromised by the major cystic fibrosis causing mutation. However it remains entirely unclear to what extent the particular limited set of phosphorylation sites studied control chain gating, how much the NBD1 interaction with ICL1 influences gating and how much of the ΔF508 defect can be explained on this basis. In this regard there is a general tendency to extrapolate interpretations beyond what is firmly supported by the NMR data from one isolated domain and a short peptide from a large five domain protein. Nevertheless, the concepts presented are valuable and can aid further elucidation of the complex molecular underpinning of these essential biological functions and how they are compromised by disease-causing mutations.

Referee #2 (Remarks to the Author):

General
The authors' previous NMR measurements using labeled CFTR R domain peptide showed that phosphorylation diminished the helical propensity of R-domain segments near phosphoserines, which, in co-incubations with CFTR NBD1 peptide, resulted in weakened binding interactions between the R domain and NBD1. The present work instead uses labeled NBD1 (from mouse CFTR) that includes a short extension (called RE) containing the N-terminal three (two in human) consensus phosphorylation serines of the R domain. The NBD1 construct also includes the regulatory insertion (RI) that is not found in any other ABC protein nucleotide binding domain; it is called regulatory because it too contains a consensus phosphorylation serine. Between one half and two thirds of the NMR resonances could be assigned to specific NBD1-RE residues, allowing the authors to interpret spectral characteristics, and their changes on phosphorylation or in response to mutation, to extract information about structural changes with single residue resolution. Consistent with prior observations from X-ray crystal structures suggesting mobility and disorder of the RI and RE, the present work confirms disorder of some RI and RE residues, and shows that phosphorylation increases the mobility of the RI and RE as a result of weakened interaction between the RE and the rest of NBD1. This meshes with the authors' previous work using the entire R domain. The second major conclusion is that the coupling helix from the first intracellular loop (ICL1) binds to NBD1 in a phosphorylation-dependent manner, conceptually linking an effect of R-domain phosphorylation on NBD1 interaction with the transmembrane domains that form the channel pore. The third major conclusion is that the structural dynamics of the phosphorylated F508 mutant NBD1-RE (of clinical importance in cystic fibrosis) more closely resemble those of non-phosphorylated NBD1-RE than those of phosphorylated WT-NBD1-RE, interpreted to explain known functional deficits of this common cystic fibrosis mutant. Unfortunately, however, problems with the presentation of the experimental data preclude evaluation of how well they support the conclusions drawn, as indicated below.

#1. In general, the resolution and size of the many images showing spectra, and the paucity of labels, make it almost impossible to discern, let alone evaluate, the chemical shift changes described in the text. In addition, there are no direct comparisons of spectra under some of the critical conditions from which major conclusions are drawn. For instance, just how different is the effect of phosphorylation on WT NBD1-RE and WT NBD1 lacking RE (of crucial importance for deciphering the relative contributions of RI vs. RE)? And exactly how different are the chemical shift changes upon addition of ICL1 peptide to ΔF508 NBD1-RE than to WT NBD1-RE? Even for some conditions that are directly compared the full difference between the spectra is unclear, e.g. for phosphorylation of ΔF508 NBD1-RE vs. WT NBD1-RE (despite the few differences boxed in Fig. 1c). To overcome these shortcomings, more convincing ways to present and compare the spectral information are required, including presentation of all chemical shift data (as supplementary material or via a link to a database deposit), additional direct comparisons of spectra, additional illustration of amplified regions from some of the 2D spectra (hopefully more convincing than the examples in Fig. 4c,d), and replacement of the binary classification (‘no change’ or ‘change’) in Figs. 1b,d, 3a-d, and 4g with more informative color-coded illustrations that present the magnitude of chemical shift changes. A further problem with Fig. 1d is its inconsistency with Suppl Table I, since there are several residues with appropriate characteristics in the table that are not labeled in Fig. 1d, and vice versa.

#2. Many of the structural differences between protein species presented are characterized by sharp
peaks at chemical shifts indicative of a disordered or unfolded region. Providing the chemical shift data (as requested in point #1) would allow readers to evaluate which (and how many) of the assigned residues are considered disordered based on where in the spectrum they resonate vs. which (and how many) are considered disordered based on relaxation properties. Also, given the stated low stability of these proteins, reassurance should be provided that their spectra are not changing over time, to distinguish a protein with an increasingly disordered region from one that is slowly denaturing. This seems especially important since the more soluble revertant triple mutant appears to have fewer of these peaks than the wild-type (judging from Suppl Fig. 3) and phosphorylated samples are necessarily older and subjected to more manipulation than non-phosphorylated samples.

#3. Throughout the text, imprecise and qualitative observations ("many residues", "some residues", "a few residues", "very similar") make the significance of the results hard to judge.

#4. Binding of the ICL1 peptide to NBD1 was said (p15) to be "tested using NMR titration experiments", which implies collection of multiple spectra each at a different protein:ligand ratio. Spectra are presented, however, only of NBD1 protein in the presence of a ten-fold excess of ICL1 peptide. If full titrations were performed, the data should be included since they would allow evaluation of the strength of the binding as well as its specificity.

#5. "ICL1" is an unfortunate name for the peptide representing the short coupling helix in the middle of the first intracellular loop. The name 'coupling helix' was introduced some years ago by crystallographer Kaspar Locher and is now well established in the field. In fact, the authors themselves cite (on p22) Seibert et al. papers from over a decade ago that use the term ICL to mean everything linking one membrane-spanning helix to the next. That long-accepted definition came from membrane topology diagrams years before the first X-ray crystal structure of any ABC transporter, and is more like what the present authors call "intracellular domains" (ICDs).

#6. Comparing Fig. 4a with Fig. 4b,c by eye, the changes on ICL1 binding appear to be substantially larger on binding to phospho-NBD1-RE than to phospho-NBD1 without the RE, implying that it is the RE, not the RI, whose displacement by phosphorylation allows ICL1 to bind. This is in contrast to the authors' emphasis on displacement of RI, on p15-16 in the Results and p20-22 in the Discussion.

#7. Fig. 5 presents the dynamic equilibrium model proposed to account for the results, but the six different protein species are positioned in the equilibrium used four different parameters. The authors should clarify how use of these distinct metrics influences the accuracy of the position estimates.

#8. Suppl Fig. 2 describes the important result that broadening of some signals may reflect ATP binding (and dissociation), which is pertinent to interpretation of the all the other results and so should be given more prominence.

#9. For readers unfamiliar with NMR, the authors should clarify that the spectra show varying amounts of T1 noise (and explain its source), including a signal band close to the important "sharp resonances, centered at ~8.2 ppm" which could otherwise be misleading.

#10. Relaxation data on the triple-mutant at different concentrations are described (p7) as "very similar", whereas in Suppl Table 1 elevated R2 rates for many positions seem consistently higher above uncertainty for the more concentrated sample. The authors should briefly mention the expected result if aggregation rather than, e.g., sample viscosity affected the relaxation rates.

Specific
Add references: (on p3) the sentence "Conformational changes associated...open and close" needs a citation; (on p15) "As few studies have probed...." should cite the He et al., 2008 and Serohijos et al., 2008 references listed in the bibliography.

p6: "suggests that portions of the RI and RE are likely disordered"; which aspect of the data implicates the RI and RE segments?

Inconsistent labeling: "phospho-WT" in Fig 1c denotes the WT NBD1-RE construct, but in Fig 4b "non-phospho-WT" presumably means WT NBD1 lacking the RE.

p14, top half: the two sentences on EM data add nothing (because of low resolution, as the authors state) and should be deleted; the sentence about "lack of sequence similarity" should also be deleted since the authors negate its message by using homology to model the structure of CFTR.

p18, line 8: should be Figure 3c,d instead of "Figure 3b,c"?

p18, 2nd para: "to enhance ATP binding and hydrolysis", and "requirement for phosphorylation prior to ATP binding and hydrolysis"; what is the evidence that phosphorylation is required for ATP binding and hydrolysis?
p22: "Removal of the RI results in CFTR channels with shorter open bursts" is true only in the presence of PKA, not for phosphorylated channels after PKA removal.
p22: "altered Cl- conductance with a decreased open probability" is misleading, and should be replaced with 'altered whole-cell Cl- conductance due to a decreased open probability'.
p24: It is not clear how the authors determined that phosphorylation of Ser422, Ser660, and Ser670 was "complete", nor what is meant by "almost complete" phosphorylation; if by MS, then the LC-MS data should be included in the supplementary material, since tryptic cleavage sites are sensitive to phosphorylation.
p30, Fig. 3 legend: "Analogous to Figure 1(c)" should probably refer to "Figure 1(b)" or "Figure 1(d)" but, even then, "analogous" doesn't sound right.
Suppl Table 1 legend: "divided by 5.3 to account for the difference in resolution", should be 'difference in amplification'.

Referee #3 (Remarks to the Author):

This paper reports an analysis of NMR chemical shifts in wild type and ΔF508 murine CFTR NBD1 with the C-terminal regulatory extension (RE), interactions of the wild-type NBD1 core with the regulatory insert (RI), and with the RE are disrupted upon phosphorylation. This represents a significant amount of effort on a medically very important problem with results that will be of interest for the general readership of EMBO Journal.

However, the paper in its present form does not meet the requirements for publication. The major accomplishment of the work is the NMR resonance assignments. However, the authors do not appear to have deposited the resonance assignments in the public data base (BioMagResBank) or provided the assignments as supporting information. The authors have not even labeled any of the spectra with assignments. In my view, the paper cannot not be published with provision of shift assignments.

Minor comments:

1. The abstract should mention that the murine protein is used.

2. In addition to mapping shift perturbations onto structures, the authors could provide graphs of perturbations vs. sequence, which aid in quantitative assessment of the effects.

3. The authors could provide (in supporting information) graphs of 13C secondary shifts. This would help support correctness of assignments (at least relative to known secondary structure) and help highlight regions lacking structure or that are highly dynamic.

1st Revision - authors' response 08 June 2009

Below please find a description of these changes made in response to the reviewers' suggestions. For clarity, the reviewer's comment is included before our response.

Reviewer 1:
#1. The manuscript describes the results of a large body of meticulous investigations and insightful if sometimes exaggerated interpretation. It is emphasized that the experiments use constructs with natural unmodified murine NBD1 sequences, but in fact one with the original 3 suppressor mutations is also used to improve expression and solubility so that more complete resonance spectral assignments could be made. This was helpful, but since fewer residues could be assigned without the suppressors, it cannot strictly be concluded that the structures are identical. This point is raised only because a major outcome of the overall study is that the ΔF508 mutation does indeed change structural features of the domain in contrast to crystallography results that have essentially indicated that is does not. This is not to argue against or question the apparent differences caused by the mutation reported here,
but only to point out that perhaps even more information could have been obtained from comparison of wild-type and ΔF508 with suppressors as well as without.

We acknowledge the potential interest in comparison of the ΔF508 NBD1 with and without suppressors. Thus, we have included a comparison of the secondary structural elements in the G550E/R553M/R555K mutant, WT and ΔF508 NBD1-RE based on chemical shift index (CSI) analysis. In light of the large amount of data currently in this manuscript, we did not further extend our analyses. The results from CSI secondary structural description are included in Supplementary Figure 5. We note that, for residues which could be assigned, the CSI analysis shows that the secondary structure is similar to that expected from the crystal structures. The similarity of spectra of the G550E/R553M/R555K mutant, WT and ΔF508 NBD1-RE also indicate the overall structural similarities of the proteins. In order to avoid confusion, we have replaced the phrase "structural differences" with "conformational changes" throughout the manuscript.

#2. In addition to the assignments of a large proportion of the residues in the wild-type structure, the first significant observation is that residues in the Hlc helix of the RI may be more mobile and less ordered than when bound to the rest of the domain as in the crystal structure. Furthermore, several unassigned residues are localized within RI and RE as apparently are some of the broadened assigned resonances. In fact the multiple different crystal structures now available already indicated that much of the RI may be unstructured or able to assume different structures. However these new data do set the stage for focus on the RI and RE regions, which with their phosphorylation sites are candidates for involvement in CFTR regulation.

However, before focusing on phosphorylation, the influence of the ΔF508 mutation was explored. Although the NMR spectra of the mutant and wild-type NBD1 were generally similar, chemical shift changes were observed not only locally near Phe 508, but also more distantly and regions at the interface between the β and αβ- domains as well residues in the RI and RE are emphasized. This is highly significant because it constitutes the first biophysical evidence of NBD1 structural changes due to ΔF508 although differences in protease sensitivity have been observed.

We appreciate the reviewer's sense of the significance of our results.

#3. In the phosphorylated wild-type NBD1 containing RE, large spectral differences in residues at and near the 4 PKA phosphorylation sites in RI and RE were observed, suggesting greater mobility or disorder in these regions. Such changes were also observed at distant locations including a region containing residues between K593 and E632 indicated to interact with the RE. A reference in support of the latter suggestion (Ma et al?) seems inappropriate or the meaning is obscure and should be clarified.

We thank the reviewer for observing the mis-referencing of the Ma et al, 1996 paper which has now been removed from this section.

#4. Importantly when RE is not present, these phosphorylation induced changes seem to have already occurred, possibly indicating that either the absence of RE or its phosphorylation when present may influence order in the interacting region. In ΔF508 NBD1-RE many of these phosphorylation dependent differences were not observed. Unfortunately, it was not possible to directly test whether changes due to just RI phosphorylation at ser 422 were also abrogated by ΔF508. However, a peptide corresponding to a loop between membrane spanning helices in CFTR did cause spectral changes in regions indicating its binding when the domain had been phosphorylated. This is interpreted as reflecting the freeing of this binding surface by phosphorylation of the RI which may normally block it. This is the least convincing of the claims made from the data presented. Evidence for an ICL1 binding site or surface is very indirect with this surface defined by sets of residues exhibiting spectral resonance changes on binding of a synthetic peptide. Most of these residues are localized together in NBD1 but one of them, Thr547 does not appear to be and as groups they don't appear to immediately contact ICL1.
The intramolecular interaction of the CFTR NBDs with the ICLs has previously been described in the literature. In particular, a cross-link between ICL1 and the NBD1 has been observed (He et al.) and all of the published models of CFTR demonstrate such an interaction. Thus, our manuscript is not arguing for the interaction of NBD1 and ICL1 but rather for its dependence on phosphorylation. We agree that our interpretation of the role of phosphorylation of the RI and its concomitant release from the surface of the NBD1 core was difficult to see in our original figures. (Note that the phosphorylated proteins are very insoluble, particularly the phospho-WT NBD1. Therefore, these experiments were conducted with very low concentrations of protein, 90 µM for phospho-WT NBD1-RE and only 25 µM phospho-WT NBD1.) In order to provide more convincing evidence to support our interpretation of the ICL1 interaction with the NBD1 core following phosphorylation of the phosphoregulatory elements, we have remade figures to more effectively show the results of these experiments (Figure 5). The figure showing binding of ICL1 to phospho-WT NBD1-RE is larger and we have blown up a region of our figure in which we have overlayed spectra of non-phospho-WT NBD1, phospho-WT NBD1, and phospho-WT NBD1 + ICL1. We have highlighted additional disordered resonances that appear in spectra of phospho-WT NBD1 upon ICL1 addition by asterisks. In addition, we have altered the figure in which we map changes with ICL1 binding onto the NBD1 structure to include the magnitude of chemical shift changes, as was suggested by Reviewer 1 for this and other figures. Furthermore, we state that residues in NBD1 that appear to contact ICL1 based on chemical shift changes are ~10Å away from the peptide in our homology model and crystal structures of ABC transporters.

#5. There are no control experiments employing any other peptides not corresponding to ICL1 or ICL1 peptides with disease-associated mutations such as those cited. Some such experiments are required to indicate the relevance of the interpretation to events in the intact CFTR protein.

While we agree with the reviewer that binding studies of ICL1 peptides with disease-associated mutations would be very interesting, we feel that the internal controls of our experiments provide evidence relating our results to the intact CFTR protein. We have performed experiments with both non-phosphorylated and phosphorylated protein and with both WT and ∆F508, an obvious disease-associated mutation, showing a dependence of binding on both of these perturbations, albeit with the perturbations in the NBD1 and not ICL1.

[Paragraph removed at authors’ request]

In addition, as there are many ICL1 mutations, some of which may not affect NBD1 binding, and it is extremely difficult to work with phosphorylated NBD1 samples, we feel that further peptide binding work is beyond the scope of the current manuscript.

#6. The overall interpretation of the investigation, consistent with the previous NMR study of the R domain by this group, is that phosphorylation control is elicited by further disrupting relatively unordered regions which alters their interaction with and influence on other segments of the protein. Thus, several important messages are conveyed that bear on understanding of both the control of this ion channel by phosphorylation and how this may be compromised by the major cystic fibrosis causing mutation. However it remains entirely unclear to what extent the particular limited set of phosphorylation sites studied control chain gating, how much the NBD1 interaction with ICL1 influences gating and how much of the ∆F508 defect can be explained on this basis. In this regard there is a general tendency to extrapolate interpretations beyond what is firmly supported by the NMR data from one isolated domain and a short peptide from a large five domain protein.

Again, we are pleased by the supportive comments regarding the importance of our work. In addition, we do agree with the reviewer that our experiments on isolated NBD1 and ICL1 cannot directly probe the effect of phosphorylation on channel gating. Therefore, all
interpretations of our results with respect to function of full length CFTR have been removed from the results and appear only in the discussion, in the context of suggestive comments. None-the-less, we do feel that our experiments on isolated NBD1 and ICL1 can yield insights of relevance to the full-length protein. CFTR is a modular protein and studies of interactions of peptides with protein domains from modular proteins have proven very powerful for modeling protein-protein interactions in general and yielding insights of relevance to function. The data presented support our interpretations and will prove useful for an understanding of CFTR function and the effect of ΔF508.

Reviewer 2:

General comments: .... Consistent with prior observations from X-ray crystal structures suggesting mobility and disorder of the RI and RE, the present work confirms disorder of some RI and RE residues, and shows that phosphorylation increases the mobility of the RI and RE as a result of weakened interaction between the RE and the rest of NBD1. This meshes with the authors' previous work using the entire R domain. The second major conclusion is that the coupling helix from the first intracellular loop (ICL1) binds to NBD1 in a phosphorylation-dependent manner, conceptually linking an effect of R-domain phosphorylation on NBD1 interaction with the transmembrane domains that form the channel pore. The third major conclusion is that the structural dynamics of the phosphorylated ΔF508 mutant NBD1-RE (of clinical importance in cystic fibrosis) more closely resemble those of non-phosphorylated NBD1-RE than those of phosphorylated WT NBD1-RE, interpreted to explain known functional deficits of this common cystic fibrosis mutant. Unfortunately, however, problems with the presentation of the experimental data preclude evaluation of how well they support the conclusions drawn, as indicated below.

We agree with the reviewer that we poorly presented much of the data in our first version of the manuscript and appreciate the specific suggestions offered. Our largest changes to this manuscript have been in the way we present our data. We have remade most of the figures in this new version so that our conclusions are more obvious and have clarified the links between the data presented and our interpretations throughout the manuscript. Detailed changes are described in response to specific comments of the reviewer, below.

#1a. In general, the resolution and size of the many images showing spectra, and the paucity of labels, make it almost impossible to discern, let alone evaluate, the chemical shift changes described in the text. In addition, there are no direct comparisons of spectra under some of the critical conditions from which major conclusions are drawn. For instance, just how different is the effect of phosphorylation on WT NBD1-RE and WT NBD1 lacking RE (of crucial importance for deciphering the relative contributions of RI vs. RE)? And exactly how different are the chemical shift changes upon addition of ICL1 peptide to ΔF508 NBDRE than to WT NBD1-RE? Even for some conditions that are directly compared the full difference between the spectra is unclear, e.g. for phosphorylation of ΔF508 NBD1-RE vs. WT NBD1-RE (despite the few differences boxed in Fig. 1c). To overcome these shortcomings, more convincing ways to present and compare the spectral information are required, including presentation of all chemical shift data (as supplementary material or via a link to a database deposit), additional direct comparisons of spectra, additional illustration of amplified regions from some of the 2D spectra (hopefully more convincing than the examples in Fig. 4c,f), and replacement of the binary classification ('no change' or 'change') in Figs. 1b,d, 3a-d, and 4g with more informative color-coded illustrations that present the magnitude of chemical shift changes. A further problem with Fig. 1d is its inconsistency with Suppl Table I, since there are several residues with appropriate characteristics in the table that are not labeled in Fig. 1d, and vice versa.

We have increased the size of many of the spectra. For example, Figure 1a shows an overlay of WT and ΔF508 NBD1-RE. A number of resonances that change with deletion of F508 are labeled in Figure 1a. In Figure 1b we present a selected region of Figure 1a intended to highlight the number of disordered resonances in spectra of WT and ΔF508 NBD1-RE, identified with asterisks. Figure 1c shows three different resonances displaying the differences
in resonance intensity in spectra of WT NBD1-RE. In Figure 1d, we have plotted chemical shift changes as a function of residue number and in Figure 1e, we have mapped these changes onto the structure. As also suggested by Reviewer 2 (see below), we have used a gradient to illustrate the magnitude of chemical shift changes with deletion of F508, phosphorylation, and ICL1 binding. Similar changes were made to our figure showing differential interactions between ICL1 and phosphorylated and non-phosphorylated NBD1, as described above.

We have also made changes to figures that show the effects of phosphorylation (Figure 2af). In addition to full spectra, we have shown selected regions of spectra comparing nonphospho-WT NBD1-RE and phospho-WT NBD1-RE, and non-phospho-WT NBD1-RE and non-phospho-WT NBD1. A comparison between spectra of non-phospho-WT NBD1 and phospho-WT NBD1 is also included in order to illustrate the differences between phosphorylation of WT NBD1-RE and WT NBD1. The selected regions illustrate that phosphorylated WT NBD1-RE and WT NBD1 proteins have an increased number of disordered residues compared with their non-phosphorylated counterparts. Disordered resonances that appear with phosphorylation are indicated with an asterisk. Importantly, we have now added a series of graphs displaying the magnitude of chemical shift changes as a function of residue throughout the manuscript, including specifically those observed with phosphorylation and removal of the RE presented in Figure 2g.

Chemical shift differences of WT NBD1-RE upon binding ICL1 are now clearly demonstrated in Figure 5a and directly compared to spectra of ∆F508 NBD1-RE upon ICL1 addition where we do not see significant chemical shift changes in Figure 5b. The lack of significant chemical shift changes for ∆F508 NBD1-RE upon ICL1 addition is stated in the text more clearly. In addition, to increase clarity we have now included a table summarizing the ICL1 binding data (Table 1).

We have also addressed the extent of differences in the phosphorylated WT and ∆F508 NBD1-RE. Along with a figure comparing spectra of non-phospho and phospho-∆F508 NBD1-RE (Figure 3a), we have included a plot showing chemical shift changes in ∆F508 NBD1-RE with phosphorylation as a function of residue number and compared it to a plot of chemical shift changes in WT NBD1-RE with phosphorylation (Figure 3b). We have made our figure of the overlayed spectra of phospho-WT and phospho-∆F508 larger (Figure 3c), keeping the boxes to highlight the number of resonances in phospho-WT NBD1-RE with no counterpart in spectra of phospho-∆F508 NBD1-RE in order to guide the eye of the reader to these changes. The use of visual guides is particularly important for readers who are not accustomed to looking at NMR data and we have employed them in our other figures (i.e. Figure 2a-f). Mapping of chemical shift differences between phospho-WT and phospho-∆F508 NBD1-RE onto the structure of WT NBD1-RE has been changed to account for the magnitude of chemical shift differences between these proteins (Figure 3d).

We have remade our figure in which chemical shift changes in WT and ∆F508 NBD1-RE (Figure 4) upon phosphorylation are mapped onto the structure. The surface representation has been replaced by ribbon diagrams and chemical shift changes for both proteins are mapped onto two different crystal structures. We feel that these new figures more accurately represent the magnitude of phosphorylation-dependent chemical shift changes in WT and ∆F508 NBD1-RE, as most residues are visible. Again, the magnitude of chemical shift changes are illustrated on the structure and the number of residues with chemical shift changes in each protein is reported in the text.

As requested by both this reviewer and Reviewer 3, we will submit our chemical shifts for the G550E/R553K/R555M mutant, WT NBD1-RE, and ∆F508 NBD1-RE to the BMRB. Until release of these files, we have also included the chemical shifts as Supplementary Material for the reviewers.

We have thoroughly compared Figure 1d and Supplementary Figure 1 and ensured that everything is consistent. This has also been done for other columns in the table and Figures in the text.

#2. Many of the structural differences between protein species presented are characterized by sharp peaks at chemical shifts indicative of a disordered or unfolded region. Providing the chemical shift data (as requested in point #1) would allow readers to evaluate which (and how many) of the assigned residues are considered disordered based on where in the spectrum they resonate vs. which (and how many) are considered disordered based on relaxation properties. Also, given the stated low stability of these proteins, reassurance
should be provided that their spectra are not changing over time, to distinguish a protein with an increasingly disordered region from one that is slowly denaturing. This seems especially important since the more soluble revertant triple mutant appears to have fewer of these peaks than the wild-type (judging from Suppl Fig. 3) and phosphorylated samples are necessarily older and subjected to more manipulation than non-phosphorylated samples.

As stated above, we have provided the chemical shifts for the G550E/R553M/R55K mutant, WT, and ∆F508 NBD1-RE proteins. We have also stated in the text (page 6) that spectra do not change over time, as assessed by recording \[^{15}N\]H TROSY HSQC spectra. For the G550E/R553M/R55K mutant and WT NBD1-RE proteins, \[^{15}N\]H TROSY HSQC spectra were recorded after each triple resonance experiment (i.e. every 2-3 days for at least one week).

As for the phosphorylated samples, the phosphorylation reaction adds an additional day (out of ~6 days for preparation of non-phosphorylated samples. During the purification and phosphorylation, the samples are kept in higher concentrations of glycerol (12.5% for purification and 5% for phosphorylation reaction) for stability. This is more clearly explained in the methods.

#3. Throughout the text, imprecise and qualitative observations ("many residues", "some residues", "a few residues", "very similar") make the significance of the results hard to judge.

We have replaced the words "many residues", "some residues", "a few residues", "very similar" with concrete numbers or numerical comparisons or with explicit listing of residue numbers. For example, in the legend for Figure 4, we state the following: "A total of 61 residues display significant chemical shift changes with phosphorylation in WT NBD1-RE compared with only 51 residues in ∆F508 NBD1-RE." In addition, residues with chemical shift changes in WT upon phosphorylation are labeled in Figure 4a and/or Figure 4b. Similarly, residues with chemical shift changes upon phosphorylation of ∆F508 NBD1-RE are labeled in Figure 4d and/or Figure 4e. In places were we do not give a numerical count in the text, we list every residue with chemical shift changes that we have assigned, such as our description of chemical shift changes with deletion of F508 on page 9.

#4. Binding of the ICL1 peptide to NBD1 was said (p15) to be "tested using NMR titration experiments", which implies collection of multiple spectra each at a different protein:ligand ratio. Spectra are presented, however, only of NBD1 protein in the presence of a ten-fold excess of ICL1 peptide. If full titrations were performed, the data should be included since they would allow evaluation of the strength of the binding as well as its specificity.

NMR titration experiments (with multiple additions of peptide) were not possible for the ICL1 binding experiments due to the low solubility of the samples. Therefore, we simply added saturating amounts of ICL1 peptide. We have therefore taken the word "titration" out of our description and state that "...binding to WT and ∆F508 NBD1 was tested using NMR spectroscopy."

#5. "ICL1" is an unfortunate name for the peptide representing the short coupling helix in the middle of the first intracellular loop. The name 'coupling helix' was introduced some years ago by crystallographer Kaspar Locher and is now well established in the field. In fact, the authors themselves cite (on p22) Seibert et al. papers from over a decade ago that use the term ICL to mean everything linking one membrane-spanning helix to the next. That long-accepted definition came from membrane topology diagrams years before the first Xray crystal structure of any ABC transporter, and is more like what the present authors call "intracellular domains" (ICDs).

Because both terms, "ICLs" and "coupling helices," are used in the literature, we have included both terms in the manuscript to describe the NBD-interacting regions of the intracellular domains. We agree with the reviewer that "ICL" with the "L" for "loop" is a rather unfortunate name for a helical element but a number of researchers doing active work
on interactions of these peptides use either "ICL" or "CL" and thus we have chosen to be consistent with this nomenclature. We now also note the problem with the term when introducing it: "Connecting these long a-helical segments of the ICDs are short helical elements or coupling helices that are frequently referred to in the literature (somewhat confusingly) as intracellular loops (ICLs) ...."

#6. Comparing Fig. 4a with Fig. 4b,c by eye, the changes on ICL1 binding appear to be substantially larger on binding to phospho-NBD1-RE than to phospho-NBD1 without the RE, implying that it is the RE, not the RI, whose displacement by phosphorylation allows ICL1 to bind. This is in contrast to the authors' emphasis on displacement of RI, on p15-16 in the Results and p20-22 in the Discussion.

We agree with the reviewer that we cannot rule out a combination of RI and RE effects since the NBD1 without the RE is partially mimicking a phosphorylated RE state. Thus, we have altered our description to state that ICL1 binding is "promoted by phosphorylation of the RI or the combination of the RI and RE" and have modified our discussion throughout to refer to phosphorylation of both the phosphoregulatory elements.

#7. Fig. 5 presents the dynamic equilibrium model proposed to account for the results, but the six different protein species are positioned in the equilibrium used four different parameters. The authors should clarify how use of these distinct metrics influences the accuracy of the position estimates.

We have included additional descriptions in the legend for this figure (now Figure 7) to explain the source of data for each of the position estimates and the errors in determining the position estimates and to emphasize the importance of the positions of the G550E/R553M/R555K mutant, WT, and ΔF508 NBD1-RE proteins with respect to one another.

#8. Suppl Fig. 2 describes the important result that broadening of some signals may reflect ATP binding (and dissociation), which is pertinent to interpretation of all the other results and so should be given more prominence.

All of the experiments conducted, with the exception of Supplementary Figure 2c, were done in the presence of saturating concentrations of ATP. This is now emphasized in the Methods. This statement is now referred to in the text (bottom of page 9) in order to give it more prominence, as suggested.

#9. For readers unfamiliar with NMR, the authors should clarify that the spectra show varying amounts of T1 noise (and explain its source), including a signal band close to the important "sharp resonances, centered at ~8.2 ppm" which could otherwise be misleading.

The source of the T1 noise is due to large concentrations of ATP compared with low concentrations of protein. The noise band is most prominent for the current Figure 5c and Figure 6 spectra. The presence of T1 noise as well as its ATP source is now explained in the legend to Figure 5: "The T1 noise at ~8.2 ppm in the 'H dimension (very small peaks) in spectra of phospho-WT NBD1 and phospho-WT NBD1 + ICL1 is due to 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." It is also mentioned in the legend to Figure 6.

#10. Relaxation data on the triple-mutant at different concentrations are described (p7) as "very similar", whereas in Suppl Table 1 elevated R2 rates for many positions seem consistently higher above uncertainty for the more concentrated sample. The authors should briefly mention the expected result if aggregation rather than, e.g., sample viscosity affected the relaxation rates.
Many of the resonances referred to here had very low signal-to-noise and/or were overlapped, and hence many of the derived $R_2$ rates were not reliable. We made some additional assignments during revision of this manuscript, which made it necessary to reexamine our relaxation data. Any non-reliable $R_2$ rates have now been removed from the table for clarity.

#11. Add references: (on p3) the sentence "Conformational changes associated...open and close" needs a citation; (on p15) "As few studies have probed...." should cite the He et al., 2008 and Serohijos et al., 2008 references listed in the bibliography.


#12. p6: "suggests that portions of the RI and RE are likely disordered"; which aspect of the data implicates the RI and RE segments?

We have rewritten this sentence to state that the number of sharp resonances at 8.2 ppm in the 1H dimension indicate the presence of disordered regions, which likely include the RI and RE. The sentence now reads as follows: "The sharp resonances, centered at ~8.2 ppm (Figure 1b), are due to disordered segments of NBD1-RE and the number of these peaks (~20) suggests that significant segments of the protein are disordered in solution, most likely including the RI and RE, with only transient sampling of ordered conformations."

#13. Inconsistent labeling: "phospho-WT" in Fig 1c denotes the WT NBD1-RE construct, but in Fig 4b "non-phospho-WT" presumably means WT NBD1 lacking the RE.

We have corrected this in the original Figure 4b (now Figure 5c).

#14. p14, top half: the two sentences on EM data add nothing (because of low resolution, as the authors state) and should be deleted; the sentence about "lack of sequence similarity" should also be deleted since the authors negate its message by using homology to model the structure of CFTR.

We agree that the EM data do not provide information regarding specific ICD/NBD interactions but have kept the description of the EM data in order to acknowledge its presence for completeness. We have also acknowledged the lack of sequence similarity, but in accord with the reviewer's point now noting that it is problematic for modeling with the following sentence: "While the lack of sequence similarity in the MSDs and ICDs complicate modeling, structure-based alignment using multiple sequences from the C family of ABC proteins was used to optimize results (see Methods)."

#15. p18, line 8: should be Figure 3c,d instead of "Figure 3b,c"?

This has been corrected.

#16. p18, 2nd para: "to enhance ATP binding and hydrolysis", and "requirement for phosphorylation prior to ATP binding and hydrolysis"; what is the evidence that phosphorylation is required for ATP binding and hydrolysis?

Phosphorylation of CFTR enhances the kinetics of ATP hydrolysis and channel activity. Thus, we have altered this sentence to say "the enhancement of ATP binding and hydrolysis"
in phosphorylated CFTR compared with non-phosphorylated CFTR" and referenced Csanady et al, 2000 and Li et al, 1996.

#17. p22: "Removal of the RI results in CFTR channels with shorter open bursts" is true only in the presence of PKA, not for phosphorylated channels after PKA removal.

We have corrected this sentence to include "in the presence of PKA".

#18. p22: "altered Cl- conductance with a decreased open probability" is misleading, and should be replaced with 'altered whole-cell Cl- conductance due to a decreased open probability'.

We have replaced "altered Cl- conductance with a decreased open probability" with "altered whole-cell Cl- conductance due to a decreased open probability".

#19. p24: It is not clear how the authors determined that phosphorylation of Ser422, Ser660, and Ser670 was "complete", nor what is meant by "almost complete" phosphorylation; if by MS, then the LC-MS data should be included in the supplementary material, since tryptic cleavage sites are sensitive to phosphorylation.

We have now clarified in the text that the determination of the phosphorylation state was performed using mass spectrometry and have included the MS/MS analysis of our tryptic peptides as Supplementary Figure 6. The MS/MS data were collected and analyzed by the Advanced Protein Technology Center at the Hospital for Sick Children.

#20. p30, Fig. 3 legend: "Analogous to Figure 1(c)" should probably refer to "Figure 1(b)" or "Figure 1(d)" but, even then, "analogous" doesn't sound right.

We have replaced "Analogous to" with "As in" and corrected the figure reference. Note that the origin Figure 3 has been replaced with the current Figure 4.

#21. Suppl Table 1 legend: "divided by 5.3 to account for the difference in resolution", should be 'difference in amplification'.

The difference in the digital resolution of the proton and nitrogen dimensions in our spectra is 5.3, which is what we had intended to convey, since significant chemical shift changes require deviations of at least a couple of digital points. As the reviewer was confused, we have further clarified this in the legend to Supplementary Table 1 and the modified phrase is also now included in the legend to Figure 1.

Reviewer 3:

General comments: .... The major accomplishment of the work is the NMR resonance assignments. However, the authors do not appear to have deposited the resonance assignments in the public data base (BioMagResBank) or provided the assignments as supporting information. The authors have not even labeled any of the spectra with assignments. In my view, the paper cannot be published without provision of shift assignments.

Resonance assignments will be released by the BMRB. In the meantime, the assignments have been supplied as Supplementary Materials for the reviewers. In addition, many resonances in the remade figures have now been labeled.

#1. The abstract should mention that the murine protein is used.
The abstract now states that murine CFTR is used.

#2. In addition to mapping shift perturbations onto structures, the authors could provide graphs of perturbations vs. sequence, which aid in quantitative assessment of the effects.

As suggested by this reviewer and noted previously, a number of graphs showing chemical shift changes as a function of residue number have been provided (Figure 1d, Figure 2g, Figure 3b.) In addition, the magnitude of chemical shift changes for various perturbations has been plotted onto the structure of NBD1-RE (Figure 1e, Figure 3d, Figure 4a-f, Figure 5d.

#3. The authors could provide (in supporting information) graphs of 13C secondary shifts. This would help support correctness of assignments (at least relative to known secondary structure) and help highlight regions lacking structure or that are highly dynamic.

In order to support the correctness of our assignments relative to the known structure, we have now added a Supplementary Figure 5 that includes the secondary structure prediction based on the chemical shift index (CSI) analysis of the G550E/R553M/R555K mutant, WT, and AF508 NBD1-RE proteins, taking into account whatever shifts are available. This provides similar structural information to that suggested by the reviewer. As most of the residues that we interpret as dynamic are not able to be assigned (due to broadening from slower motions or overlap due to disorder) and since the available 13C shifts are virtually the same for the G550E/R553M/R555K mutant and WT (and not available for AF508), we do not think that a plot of 13C secondary shifts would provide more useful insights into the dynamic nature of the protein.

2nd Editorial Decision 10 July 2009

Firstly, I would like to pass on the apologies of the referee for the late evaluation of this study but unfortunately the revised manuscript came at the same time as the writing of a major grant proposal. Your revised manuscript has been reviewed once more by one of the original referees and while s/he finds that overall it is greatly improved there are a number of minor changes that need to be incorporated prior to publication. Overall, the referee does recommend publication, however, they state that this is dependent on changes in the terminology in the manuscript, and as strongly stated in the report the unambiguous term coupling helix should be used instead of ICL before s/he can recommend publication.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEEEREE COMMENTS

Referee #2 (Remarks to the Author):

General Comments:
The authors have greatly improved the presentation of their data by revising the figures, and by illustrating quantitative information, as requested by the reviewers. They have also clarified their
interpretation of the results and made some effort to walk the reader through the respective caveats as well as the various controls performed as part of this difficult work.

Now that the wood and trees can be distinguished, the authors are encouraged to address a few remaining concerns.

Specific comments:

p.3: the response concerning the term ICLs is unsatisfactory. Why perpetuate confusing and misleading terminology? The inclusion of "(somewhat confusingly)" just highlights the lunacy. Which are the "frequent" references in the literature that provide the excuse for continuing to use ICL? There is not a single reference to topology or structure in this paragraph. In any case, emulation of admittedly bad behavior by others is not a reasonable excuse: as every mother would say, "if Johnny put his head in the oven, would you do it too?" The references to Seibert et al., who used the term ICL to mean exactly what you call ICD, are perfectly correct. Once the structure of the ICDs (or ICLs) became known, there is no justification whatsoever for using the term ICL to refer to a tiny fragment of the ICD, especially when a heuristically useful and unambiguous alternative name for your 16 residue fragment, "coupling helix", exists.

p6, bottom: "most likely including the RI and RE" is simply speculation unless data are presented to support it, and yet it is an important conclusion repeated several times in the paper (e.g. p10, l.8).

Two pages later (p.8, bottom), data are mentioned: "many of the sharp resonances...are from...the RE and....the RI", but there is no straightforward way for the reader to evaluate that from Figure 1. Given the importance of this point, the resonances from those residues should be identified somehow in the Figure.

p12, l.14: should read "phosphorylation of the RI and RE, or removal of the RE, causes"

p13, l.1: should read "Phosphorylation of NBD lacking the RE and of NBD1-RE"

p15, l.4: omit the first half of the sentence "While the lack of sequence similarity in the MSD and ICDs.....optimize results". First, there is no 'lack of sequence similarity' in general because even evolutionarily unrelated sequences show some similarity just by chance. Second, it is established (see e.g. Dassa & Bougie Res Microbiol 152(3-4): 211-29) that also within the ABC-C family there is homology in the MSDs and ICDs, even if that homology is not as obvious as in the NBDs. Third, without additional information it's probably unwise to make a big deal out of the "structure-based" vs. sequence-based alignment, since the logical assumption that structural information improves alignment accuracy may not be true for all sets of sequences.

p20: "the slower rate of PKA activation in deltaF508 CFTR compared with WT" was first shown by Drumm, 1991, Science 254:1787-9, who should be cited here.

The ppm units should be indicated in Figure 1(d-e), Figure 2(g), Figure 3(b,d), Figure 5(d)

Figure 1: color-coding should be indicated on the spectra as in Figures 2,3,5

Figure 3b: The vertical axes in the two graphs (chemical shift changes) are scaled differently, which precludes straightforward comparison between WT and deltaF508 NBD1-RE. Figure 4: would benefit from an in-figure legend, particularly because there are errors in the legend, which should read "and PDB code 1XMI for b, e)" on l.3; "NBD1-R (d,e)" on l.5; and "in panels (d) and (e)" on l.9.

Figure 6: color-coding should be indicated as in Figures 2,3,5

Figure 7: The authors should consider including the phospho deltaF508 NBD1-RE + ICL1 condition (from Fig. 6) to make this summary figure more complete.

Supplementary Figure 1a: color coding to match Suppl. Fig. 8 might be more appropriate and instructive? Differential coloring of the ICLs according to which NBD they interact with in the homology model, rather than by their order in the primary sequence would be more helpful.

Supplementary Figure 1b: F508 is not highlighted yellow as promised in the figure legend.

Supplementary Figures 2 and 3: spectrum and color identifiers (like in Figures 2,3,5) are needed, and also the letter designations (a,b,c,etc).

Supplementary Figure 6: is unreadable at this resolution; and, from what can be gleaned, it doesn't appear address the degree of phosphorylation, i.e. the relative quantities of phosphorylated vs. non-phosphorylated protein, a quantification which is complicated by differential trypsin cleavage of the two protein states.
Accompanying this letter is our revised manuscript entitled "NMR evidence for differential phosphorylation-dependent interactions in WT and ΔF508 CFTR" by Kanelis, Hudson, Thibodeau, Thomas and Forman-Kay. We very much appreciate the favorable response of Reviewer 2 and his/her useful suggestions. We have addressed all the comments made by Reviewer 2, which we describe below. For clarity, the reviewer's comment is included before our response.

Reviewer 2:

#1. p.3: The response concerning the term ICLs is unsatisfactory. Why perpetuate confusing and misleading terminology? The inclusion of "(somewhat confusingly)" just highlights the lunacy. Which are the "frequent" references in the literature that provide the excuse for continuing to use ICL? There is not a single reference to topology or structure in this paragraph. In any case, emulation of admittedly bad behavior by others is not a reasonable excuse: as every mother would say, "If Johnny put his head in the oven, would you do it too?" The references to Seibert et al., who used the term ICL to mean exactly what you call ICD, are perfectly correct. Once the structure of the ICDs (or ICLs) became known, there is no justification whatsoever for using the term ICL to refer to a tiny fragment of the ICD, especially when a heuristically useful and unambiguous alternative name for your 16-residue fragment, "coupling helix", exists.

We acknowledge the confusion regarding our nomenclature of the intracellular domains and have changed the term ICL to coupling helix in the text and the figures. We continue to refer to the entire region containing the intracellular loops as the intracellular domains (ICDs) to reflect the interaction of the intracellular loops as a structured domain.

#2. p6, bottom: "most likely including the RI and RE" is simply speculation unless data are presented to support it, and yet it is an important conclusion repeated several times in the paper (e.g. p.10, l.8). Two pages later (p.8, bottom), data are mentioned: "many of the sharp resonances...are from....the RE and...the RI", but there is no straightforward way for the reader to evaluate that from Figure 1. Given the importance of this point, the resonances from those residues should be identified somehow in the Figure.

Figure 1b was altered to label residues of the RI and RE that have been assigned. These resonances are very sharp and intense and are located centered about ~8.2 ppm in the H dimension, which indicates that they are located within disordered regions of the protein.

#3. p12, l.14: should read "phosphorylation of the RI and RE, or removal of the RE, causes"

We have altered this sentence in the text as suggested by the reviewer.

#4. p13, l.1: should read "Phosphorylation of NBD lacking the RE and of NBD1-RE"

We have altered this sentence in the text as suggested by the reviewer.

#5. p15, l.4: omit the first half of the sentence "While the lack of sequence similarity in the MSD and ICDs.....optimize results". First, there is no 'lack of sequence similarity' in general because even evolutionarily unrelated sequences show some similarity just by chance. Second, it is established (see e.g. Dassa & Bougie Res Microbiol 152(3-4): 211-29) that also within the ABC-C family there is homology in the MSDs and ICDs, even if that homology is not as obvious as in the NBDs. Third, without additional information it's probably unwise to make a big deal out of the "structure-based" vs. sequence-based alignment, since the logical assumption that structural information improves alignment accuracy may not be true for all sets of sequences.

We appreciate the reviewer's comments regarding the sequence similarity within the MSDs and ICDs and have altered the sentence in the text as suggested by the reviewer.
#6. p20: "the slower rate of PKA activation in deltaF508 CFTR compared with WT" was first shown by Drumm, 1991, Science 254:1787-9, who should be cited here.

We thank the reviewer for observing the missing reference of the Drumm et al, 1991 paper, which has now been added to this section.

#7. The ppm units should be indicated in Figure 1(d-e), Figure 2(g), Figure 3(b,d), Figure 5(d)

The ppm units have been added to Figures 1(d-e), Figure 2(g), Figure 3(b,d), Figure 5(d).

#8. Figure 1: color-coding should be indicated on the spectra as in Figures 2,3,5

The color-coding for WT NBD1-RE and DF508 NBD1-RE has been indicated on Figure 1a.

#9. Figure 3b: The vertical axes in the two graphs (chemical shift changes) are scaled differently, which precludes straightforward comparison between WT and deltaF508 NBD1-RE.

Figures 2g and 3b have been altered so that the vertical axes are identical for a straightforward comparison of WT NBD1-RE and WT NBD1 (Figure 2g), and WT and δF508 NBD1-RE (Figure 3b).

#10. Figure 4: would benefit from an in-figure legend, particularly because there are errors in the legend, which should read "and PDB code 1XMI for b, e)" on l.3; "NBD1-R (d,e)" on l.5; and "in panels (d) and (e)" on l.9.

We thank the reviewer for noticing the mislabeling in our figure legend. We have corrected our figure legend and have included labels in Figure 4 ("non-phospho-WT NBD1-RE vs. phospho-WT NBD1-RE" and "non-phospho-δF508 NBD1-RE vs. phospho-δF508 NBD1-RE") for clarity as suggested.

#11. Figure 6: color-coding should be indicated as in Figures 2,3,5.

Figure 6 has been altered to include the color-coding for non-phospho-ΔF508 NBD1-RE and phospho-ΔF508 NBD1-RE.

#12. Figure 7: The authors should consider including the phospho deltaF508 NBD1-RE + ICL1 condition (from Fig. 6) to make this summary figure more complete.

We have included the phospho deltaF508 NBD1-RE + coupling helix 1 condition in our model.

#13. Supplementary Figure 1a: color coding to match Suppl. Fig. 8 might be more appropriate and instructive? Differential coloring of the ICLs according to which NBD they interact with in the homology model, rather than by their order in the primary sequence would be more helpful.

We have altered Supplementary Figures 1a and 8b so that the ICDs are colored according to their interactions with the NBDs. Orange red is used for coupling helices 1 and 4, which interact with NBD1, and light orange is used for the non-interacting regions of ICD1. Bright purple is used for coupling helices 2 and 3, which interact with NBD2, and pale purple is used for the non-interacting regions of ICD2.
#14. Supplementary Figure 1b: F508 is not highlighted yellow as promised in the figure legend.

We have altered Figure 1b to highlight F508 (left panel) or A508 (right panel).

#15. Supplementary Figures 2 and 3: spectrum and color identifiers (like in Figures 2, 3, 5) are needed, and also the letter designations (a, b, c, etc).

Supplementary Figure 2 has been altered to include the letter designations a, b, and c. Supplementary Figure 3 has been altered to include color identifiers for WT NBD1-RE and G550E/R553M/R555K NBD1-RE. Color identifiers were not included in Figure 2 because all spectra are of non-phospho-WT NBD1-RE, as described in the figure legend.

#16. Supplementary Figure 6: is unreadable at this resolution; and, from what can be gleaned, it doesn't appear address the degree of phosphorylation, i.e. the relative quantities of phosphorylated vs. nonphosphorylated protein, a quantification which is complicated by differential trypsin cleavage of the two protein states.

Supplementary Figure 6 has been enlarged. In addition we have altered the figure legend to better explain how these data indicate the degree of phosphorylation.

Please note that the contact information for the corresponding author for this paper is:
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Based on the changes we have made in our manuscript and figures in response to the reviewer's very useful suggestions, we feel that this revised version is improved. As we have made all the reviewer's requested changes, we are confident that the manuscript will now be suitable for publication in The EMBO Journal. Thank you for your time and consideration of this manuscript.