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Protein Kinase A Regulates ATP Hydrolysis and Dimerization by a CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) Domain

L. Daniel Howell  
*Liberty University, dhowell@liberty.edu*

Roy Borchardt

Jolanta Kole

Andrew M. Kaz

Christoph Randak

*See next page for additional authors*

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Gating of the CFTR Cl\(^-\) channel is associated with ATP hydrolysis at the nucleotide-binding domains (NBD1, NBD2) and requires PKA (protein kinase A) phosphorylation of the R domain. The manner in which the NBD1, NBD2 and R domains of CFTR (cystic fibrosis transmembrane conductance regulator) interact to achieve a properly regulated ion channel is largely unknown. In this study we used bacterially expressed recombinant proteins to examine interactions between these soluble domains of CFTR in vitro. PKA phosphorylated a fusion protein containing NBD1 and R (NBD1–R–GST) on CFTR residues Ser-660, Ser-700, Ser-712, Ser-737, Ser-768, Ser-795 and Ser-813. Phosphorylation of these serine residues regulated ATP hydrolysis by NBD1–R–GST by increasing the apparent \(k\) for ATP (from 70 to 250 \(\mu\)M) and the Hill coefficient (from 1 to 1.7) without changing the \(V_{\text{max}}\). When fusion proteins were photolabelled with 8-azido-\([\alpha^32\text{P}]\)ATP, PKA phosphorylation increased the apparent \(k_d\) for nucleotide binding and it caused binding to become co-operative. PKA phosphorylation also resulted in dimerization of NBD1–R–GST but not of R–GST, a related fusion protein lacking the NBD1 domain. Finally, an MBP (maltose-binding protein) fusion protein containing the NBD2 domain (NBD2–MBP) associated with and regulated the ATPase activity of PKA-phosphorylated NBD1–R–GST. Thus when the R domain in NBD1–R–GST is phosphorylated by PKA, ATP binding and hydrolysis becomes co-operative and NBD dimerization occurs. These findings suggest that during the activation of native CFTR, phosphorylation of the R domain by PKA can control the ability of the NBD1 domain to hydrolyse ATP and to interact with other NBD domains.

**Key words:** ATPase, cystic fibrosis transmembrane conductance regulator, dimerization, protein kinase A, phosphorylation.

## INTRODUCTION

The CFTR (cystic fibrosis transmembrane conductance regulator) is responsible for regulating chloride ion transport in many epithelial cells. CFTR is a member of the ABC (ATP-binding cassette) transporter family of proteins and consists of five domains, including the first and second NBDs (nucleotide-binding domains; NBD1 and NBD2) and the regulatory domain (R). NBD domains are universal among ABC transporters, but the R domain is unique to CFTR.

Many questions remain regarding the role of the R domain in CFTR and the mechanism by which this domain regulates channel activity. The R domain is phosphorylated by several kinases in vitro [1], most notably PKA (protein kinase A). Because PKA phosphorylation of CFTR is normally required for channel activation [2] the R domain has been viewed as a plug that blocks ion traffic until phosphorylated. However, its mechanism of action may be more complex since (i) exogenously added phosphorylated R domain elevates the open probability of mutant ΔR-CFTR channels lacking much of the R domain [3,4], (ii) phosphorylation of some PKA sites in the R domain inhibits channel activity [5,6] and (iii) channel gating may be modulated by incremental phosphorylation of the multiple PKA sites in the R domain [7]. Additionally, the R domain may co-ordinate regulatory interactions with ancillary proteins [8–10].

The mechanism of CFTR channel gating by the NBD domains is controversial. CFTR is a member of the C subfamily of ABC proteins and, like other ABC-C proteins, the NBDs of CFTR appear to be functionally asymmetric [11–13]. Both NBD1 and NBD2 have been shown to possess ATP binding and hydrolase activities [14–16] and these activities were initially thought to mediate channel opening and channel closing, respectively [7]. Subsequent data implicated NBD1 in channel closing [17] and NBD2 in channel opening [18], and more recent studies suggest that CFTR channel activation may actually be driven by the formation of NBD dimers [13,19,20]. Although controversial, these recent physiological findings suggest a critical role for NBD–NBD interactions during CFTR channel opening, and this provides a strong rationale for studying the biochemical basis for NBD dimerization and co-operativity.

For ABC proteins in general, a growing body of evidence suggests that the two NBDs, though functionally asymmetric, interact so as to form complete ATP-binding pockets at the interfacial region where the two domains presumably make contact [22–30]. NBD–NBD interactions are also suggested by the co-operative ATP dependence exhibited by several ABC proteins including PKA-phosphorylated CFTR [31–33]. Nonetheless, dimerization has only been reported for isolated cytoplasmic domains from ABC proteins containing mutations designed to render them catalytically inactive [34].

Numerous attempts have been made to determine how the soluble domains of CFTR interact. In many cases, CFTR protein fragments, recombinant fusion proteins and peptides have proven to be powerful tools for exploring these interactions [27,35–41].

**Abbreviations used:** ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; GST, glutathione S-transferase; MBP, maltose-binding protein; NBD, nucleotide-binding domain; PKA, protein kinase A; TPCK, tosylphenylalanylchloromethane.
Although physical interactions have been detected by these methods, the functional consequences of these interactions have not been studied beyond halide permeability assays, with the exception that nucleotide binding was found to reduce PKA phosphorylation of an NBD1–R peptide [35]. In this study, we used an enzymically active GST (glutathione S-transferase) fusion protein (NBD1–R–GST) to investigate physical interactions among the soluble domains of CFTR and to biochemically examine the functional consequences of these interactions. We found that when PKA phosphorylates a group of R-domain serine residues in NBD1–R–GST, this caused the fusion protein to dimerize, to exhibit co-operative ATP binding and hydrolysis, and to be subject to regulation by NBD2. These findings indicate that a cytoplasmic domain from CFTR (NBD1–R–GST) can exhibit PKA-regulated dimerization and co-operative ATP hydrolysis in the absence of other domains from native CFTR.

EXPERIMENTAL

Materials

Thrombin and Na2ATP were from Sigma; [γ-32P]ATP was from DuPont-New England Nuclear; PKA was from Promega; TPCK (‘Tos-Phe-CH2Cl’; tosylphenylalanylechloromethane)-trypsin was from Worthington Biochemical Corp., Lakewood, NJ, U.S.A. Other reagents were purchased from Fisher Scientific.

Production of recombinant fusion proteins

NBD1–R–GST (a GST fusion protein with CFTR residues 417–830) and R–GST (a GST fusion protein with CFTR residues 830) and R–GST (a GST fusion protein with CFTR residues 417–830; wild-type sequence or containing specific Ser substitutions) fusion proteins were expressed in E. coli and purified as described in [14]. The proteins migrated on SDS/polyacrylamide gels with the expected molecular masses of 70 kDa for NBD1–R–GST and 55 kDa for R–GST. Western blots confirmed that each fusion protein was recognized by G450, an antibody directed against the R domain [14,42]. NBD2–MBP (CFTR residues 1208–1399 expressed as a fusion protein with MBP (maltose-binding protein) was produced as described previously [16]. For some studies, the GST moiety was removed from fusion proteins by incubation with 5 NIH units of thrombin for 5 min at 30 °C in a solution containing 100 mM Tris (pH 7.5), 5 mM MgCl₂ and 100 mM NaCl.

ATPase assays

Enzyme assays were performed as described previously [14]. Briefly, hydrolase activity was measured in 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1–5 μCi [γ-32P]ATP and 70 μM Na₂ATP unless otherwise indicated. The reaction (50 μl) was initiated by adding 100 ng of NBD1–R–GST protein. After 2 h at 30 °C it was terminated by adding 10 μl of ice-cold 100 % tri-chloroacetic acid and the reaction mix was centrifuged at 1000 g for 15 min at 5 °C. 32PO₄ was recovered from the supernatant by extraction with a molybdate complex into toluene/butanol (1:1, v/v). A portion of the extracted 32PO₄ was mixed with 1 ml of scintillation fluid and quantified by scintillation counting.

Although the NBD2–MBP fusion protein has been reported to possess low intrinsic ATPase activity [16], this activity was not detectable under the assay conditions used here for measuring hydrolysis by NBD1–R–GST (i.e. micromolar ATP concentrations and short assay times). The ATPase activity of NBD2–MBP could be detected, however, using longer assay times and millimolar ATP concentrations (results not shown), confirming that the NBD2 domain was in the native conformation throughout these assays.

8-Azido-ATP labelling of recombinant proteins

Photolabelling reactions were performed in 20 μl volumes containing 50 mM Tris (pH 7.5), 2.5 mM MgCl₂, 50 mM NaCl, 1 μCi of 8-azido-[α-32P]ATP and 1 μg of fusion protein. Mixtures were incubated on ice for 1 min and then irradiated for 24 s with UV light (254 nm) in a CL-1000 Ultraviolet Crosslinker oven (UVP, San Gabriel, CA, U.S.A.). Binding reactions were quenched by the addition of Laemmli buffer plus 100 mM dithiothreitol. The samples were then heated to 95 °C for 2 min and resolved by SDS/PAGE on a 10 % polyacrylamide gel. Proteins were detected by Coomassie Blue staining and radiolabelled proteins were detected by exposing dried gels to film (Kodak X-OMAT AR) or a Phosphorimager screen. Fusion proteins were dialysed to remove dithiothreitol and were usually digested with thrombin prior to nucleotide-binding assays because GST also binds 8-azido-ATP. About 10 % of the total photolabelling was non-specific in that it could not be displaced by 100 mM MgATP or by 25 mM 8-azido-ATP (results not shown). The stoichiometry of photolabelling was up to 9 %. No photolabelling occurred in the absence of UV irradiation or in samples quenched with dithiothreitol prior to UV irradiation, nor did photolabelling increase with longer incubation times or increased UV exposure.

Sucrose-density-gradient ultracentrifugation

Sucrose gradients (2–21 %) were prepared in Beckman polyanallomer centrifuge tubes (14 mm × 89 mm) using a Radian-117 gradient maker (BioComp Instruments) according to the manufacturer’s instructions. Gradients were formed in a buffer containing 50 mM Tris, pH 7.5, and 50 mM NaCl at room temperature and then chilled to 5 °C prior to use. We mixed 100 μl of 20 mg/ml protein standards solution (containing catalase, haemoglobin and cytochrome c) with 5 μl of sample and applied the mixture to the top of the gradient. The samples were subjected to centrifugation (210 000 g in a Beckman SW41 rotor) for 16 h at 5 °C. Following centrifugation, fractions were collected by carefully withdrawing aliquots from the top of the gradient.

Phosphorylation of fusion proteins by PKA

To achieve maximal phosphorylation, fusion proteins were incubated for 15 min at 30 °C with 20 units of PKA catalytic subunit in a buffer containing 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1–5 μCi [γ-32P]ATP and 10 μM Na₂ATP. Photolabelling reactions (30 μl) were terminated by the addition of protein kinase inhibitor to 1 μM or by the addition of 15 μl of Laemmli buffer (100 mM Tris, pH 6.8, 50 % sucrose, 10 % SDS, 3 % β-mercaptoethanol and 0.025 % Bromophenol Blue) followed by boiling for 2 min.

Tryptic peptide mapping of phosphorylated fusion proteins

Tryptic peptide maps were generated as described [1,43,44]. Briefly, gel pieces containing the phosphorylated fusion proteins were excised from a dried SDS/PAGE gel, washed four times with 2.5 ml of 10 % acetic acid/50 % methanol followed by four changes of 50 % methanol (2.5 ml each), and dried under vacuum. The dried gel pieces were resuspended in 150 μl of 50 mM NH₄HCO₃ (pH 8.0) and digested at 37 °C with 20 μg of TPCK-trypsin for 24 h. Fresh trypsin (20 μg) was added 1.5 h before the end of the digestion period. Gel pieces were removed by centrifugation (12 000 g for 5 min) and the supernatant containing
Figure 1  Identification of NBD1–R–GST residues phosphorylated by PKA

Wild-type NBD1–R–GST (I), wild-type R–GST (H) and R–GST proteins containing specific Ser → Ala mutations (A–G) were PKA phosphorylated, digested with TPCK-trypsin, and mapped as described in the Experimental section. The loss of specific R-domain Ser residues resulted in the loss of specific radiolabelled peptides in the maps (e.g. the loss of Ser-737 in D resulted in the loss of peptide 10), allowing the identification of R-domain residues phosphorylated by PKA in vitro. A total of 12 peptides were observed for wild-type R–GST and were associated with the following seven Ser residues: Ser-660 (peptide 6), Ser-700 (peptide 1), Ser-712 (peptide 4), Ser-737 (peptide 10), Ser-768 (peptides 2, 3 and 8), Ser-795 (peptides 7, 9 and 11) and Ser-813 (peptide 12). Note that peptide 12 has been cropped from most of the panels. Maps obtained with wild-type NBD1–R–GST were identical to those obtained with wild-type R–GST.

We identified the amino acids phosphorylated by PKA by mapping multiple R–GST fusion proteins containing individual Ser → Ala substitutions (Figure 1). As shown in Figure 1(H), 12 radiolabelled peptides were resolved from wild-type R–GST. Single Ser → Ala substitutions resulted in the elimination of specific radiolabelled peptides, allowing each peptide to be identified with a unique phosphorylation site in the fusion protein. For example, replacement of Ser-737 eliminated phosphopeptide 10, indicating that peptide 10 contains Ser-737 (Figure 1D). Similarly, the substitution of Ala for Ser-768 resulted in the elimination of specific radiolabelled peptides, allowing each peptide to be identified with a unique phosphorylation site in the fusion protein. For example, replacement of Ser-737 eliminated phosphopeptide 10, indicating that peptide 10 contains Ser-737 (Figure 1D). Similarly, the substitution of Ala for Ser-768 resulted in the elimination of specific radiolabelled peptides, allowing each peptide to be identified with a unique phosphorylation site in the fusion protein. For example, replacement of Ser-737 eliminated phosphopeptide 10, indicating that peptide 10 contains Ser-737 (Figure 1D).

RESULTS

Phosphorylation of NBD1–R–GST by PKA

When NBD1–R–GST or R–GST were incubated with [γ−32P]ATP and PKA, they became radiolabelled at multiple sites. Two-dimensional tryptic phosphopeptide mapping showed that the same sites were phosphorylated on both proteins (Figures 1H and 1I), confirming that no NBD1 residues are phosphorylated and demonstrating that for each fusion protein the same R-domain residues are modified by PKA in vitro.

PKA converts NBD1–R–GST monomers to dimers

Pilot experiments using filtration chromatography suggested that unphosphorylated NBD1–R–GST and R–GST existed as
monomers (results not shown). Using sucrose-density-gradient ultracentrifugation, we sought to confirm those preliminary findings and examine the effect of PKA phosphorylation on the quaternary structure of these fusion proteins (Figure 2). Unphosphorylated NBD1–R–GST fusion proteins migrated through sucrose gradients with a molecular mass of $\approx 70$ kDa (Figure 2A) and R–GST migrated with a mass of 50 kDa (Figure 2B), the expected monomeric sizes for these fusion proteins. By contrast, as shown in Figure 2(A), maximally phosphorylated NBD1–R–GST migrated through sucrose gradients with an apparent molecular mass of $\approx 150$ kDa, about twice the monomeric size of the fusion protein. A similar shift in migration was observed with thrombin-cleaved NBD1–R (Figure 2C), indicating that the GST moiety is not required for this dimerization. Phosphorylation did not convert R–GST monomers to dimers (Figure 2B), indicating that the R domain is not sufficient for dimerization and that NBD1 residues (CFTR amino acids 417–620) are necessary. Since the R domain is the main site of phosphorylation in NBD1–R–GST (Figure 1), these results provide convincing biochemical evidence that PKA-regulated dimerization involves an interaction between NBD1 and the R domain.

PKA regulates the ATPase activity of NBD1–R–GST

As described above, PKA phosphorylation led to a physical interaction (i.e. dimerization) between NBD1–R–GST fusion proteins. To determine whether this interaction influences the ATPase activity of NBD1–R–GST we compared ATP hydrolysis by NBD1–R–GST with and without PKA pretreatment over a range of ATP concentrations (Figure 3). The unphosphorylated fusion protein hydrolysed ATP with a $K_\text{m}$ of 70 $\mu$M, a $V_{\text{max}}$ of $\approx 20$ min$^{-1}$ and a Hill coefficient of 1. PKA phosphorylation of NBD1–R–GST altered the kinetics of hydrolysis dramatically. The rate of ATP hydrolysis by PKA-phosphorylated NBD1–R–GST as a function of ATP concentration was plotted using the Hill equation ($v = \frac{S\cdot V_{\text{max}}}{S + K_\text{m}}$, where $v$ is the reaction velocity, $S$ is the ATP concentration, $V_{\text{max}}$ is the maximal reaction velocity, $K_\text{m}$ is the Michaelis–Menten constant and $m$ is the Hill coefficient; $n = 2–4$ determinations for each data point; error bars represent the range). The fitted curve yielded an apparent $K_\text{m}$ of 250 $\mu$M and a Hill coefficient of 1.7. The increased Hill coefficient is consistent with the observed dimerization of NBD1–R–GST following PKA phosphorylation shown in Figure 2(A). The increased Hill coefficient is also consistent with the reported
PKA regulates 8-azido-ATP binding to NBD1

To independently examine how PKA phosphorylation affects nucleotide binding by NBD1, we used 8-azido-[γ-32P]ATP for photolabelling. NBD1–R–GST was shown to bind 8-azido-ATP based on its molecular mass and the shift in its molecular mass after digestion by thrombin (Figure 4A). Western blotting with an R-domain antibody detected the same bands before and after digestion by thrombin (results not shown). As reported for other proteins containing the R domain [1,38], NBD1–R–GST showed a gel shift (to an isofrom with a higher apparent molecular mass) after PKA phosphorylation; this caused the expected changes in position for the signals detected by photolabelling, Western blotting and protein staining, providing further evidence that each of these signals was due to the same protein (results not shown).

Properties of full-length CFTR; by contrast, the fusion protein shows an increase in the $K_a$ for ATP hydrolysis after PKA phosphorylation, whereas full-length CFTR shows a decrease [31].

DISCUSSION

In CFTR, the NBD1 and R domains have been implicated in channel regulation and activation. Here, we used a recombinant fusion protein (NBD1–R–GST) to examine whether PKA phosphorylation of the R domain can control the ability of the NBD1 domain to bind and hydrolyse ATP. The main findings of this study are (i) that PKA acts on the R domain of this recombinant protein to directly regulate the intrinsic ATPase activity of its NBD1 domain, (ii) that phosphorylation causes NBD1–R–GST to associate with itself and to thereby exhibit co-operative ATP

NBD2 domain interacts with PKA-phosphorylated NBD1–R–GST

Lu and Pedersen [37] have shown that an unphosphorylated recombinant NBD1–R protein physically associates with a recombinant NBD2 protein. Using the 32P radioisotope as a probe, we examined whether PKA-phosphorylated NBD1–R–GST could interact with NBD2–MBP. NBD1–R–GST was radiolabelled by incubation with [γ-32P]ATP and PKA and then loaded on to a column of amylose resin with or without bound NBD2–MBP. After washing the column, bound proteins were eluted with 10 mM maltose. Most of the 32P was recovered in the initial wash fractions as [32P]ATP. However, an easily detectable amount of radioactivity was retained on the amylose column containing NBD2–MBP and was specifically eluted with maltose (Figure 5A); no radioactivity was specifically eluted by maltose from a control amylose column lacking the NBD2 fusion protein. As this signal could have been obtained by the elution of NBD2–MBP with bound [32P]ATP in its binding site rather than 32P-labelled NBD1–R–GST, we confirmed the specific elution of phosphorylated NBD1–R–GST by SDS/PAGE and autoradiography (Figures 5B and 5C). When a similar experiment was performed using R–GST in lieu of NBD1–R–GST, no GST fusion protein was retained on the amylose column (Figure 5D). These results suggest that the R domain is not the site of association between NBD1–R and NBD2.

We tested the hypothesis that association between NBD1 and NBD2 influences the ATPase function of NBD1–R–GST. The NBD2–MBP protein had little effect on the ATPase activity of PKA-phosphorylated NBD1–R–GST in buffer lacking NBD2–MBP. In buffer containing NBD2–MBP, ATPase activity increased in the absence of PKA phosphorylation, but was not further increased by PKA phosphorylation.
binding and hydrolysis and (iii) that phosphorylated NBD1–R–
GST also associates with and is regulated by a fusion protein
containing NBD2 (NBD2–MBP).

To establish that the NBD1 ATPase of CFTR is regulated by
phosphorylation of the R domain (rather than by phosphorylation
of the NBD1 moiety itself) we first identified which residues of
NBD1–R–GST are phosphorylated by PKA. This was achieved
by comparing two-dimensional tryptic peptide maps of PKA-
phosphorylated NBD1–R–GST with those of PKA-phosphoryl-
ated R–GST, a fusion protein lacking the NBD1 domain. Identical
maps were obtained from both phosphoproteins indicating that
the NBD1 domain is not a significant substrate for PKA. Using
site-directed Ser → Ala substitutions, the phosphorylated residues
were identified as Ser-660, Ser-700, Ser-712, Ser-737, Ser-768,
Ser-795 and Ser-813. All occur in the R domain and six of these
seven residues (all but Ser-712) were previously observed to be
phosphorylated on full-length CFTR in vitro [1]; however, not
all phosphorylated CFTR residues may have been identified in
that study, as acknowledged by the authors.

It has been challenging to determine which CFTR residues
are phosphorylated in vivo due to the low levels of CFTR in
cells and the high degree of radiolabelling required to identify
individual phosphopeptides. Nonetheless, using two-dimensional
phosphopeptide mapping, Cheng et al. [43,45] determined that
CFTR residues Ser-660, Ser-737, Ser-795 and Ser-813 are phos-
phorylated in response to forskolin in CFTR-transfected cells,
but they also acknowledged that some phosphorylated residues
remained unidentified. Two-dimensional peptide mapping also
revealed a number of radiolabelled peptides derived from CFTR
in forskolin-treated T84 cells, but only two residues were unequi-
vocally identified: Ser-660 and Ser-700 [1]. Thus all phospho-
serines previously detected on CFTR during in vivo labelling
were also detected in this study on NBD1–R–GST.

One potential pitfall of this study’s general approach is the
risk that a contaminating protein in the NBD1–R–GST prep-
paration might account for the detected ATPase activity. For the
preparation used in this study, two findings argue against this
possibility. First, kinetic studies showed that the G551D mu-
tation changed the K_m but not the V_max of the NBD1–R–GST
preparation [14]. Second, when the fusion protein preparation was
photalabelled using 8-azido-ATP, the labelled protein occurred in
the same position (by PAGE) as the signals for NBD1–R–GST
detected by protein staining and by Western blot, and it showed
the expected gel shifts when NBD1–R–GST was digested by throm-
bin and when it was phosphorylated by PKA. Thus even though
a contaminating ATPase could have led to differences in the
total measured ATPase activity of the G551D versus wild-type
fusion protein preparations (e.g. if the G551D mutation affected
the tendency of contaminants to associate with NBD1–R–GST during its purification), these findings indicate that NBD1–R–GST is the protein responsible for the observed ATP binding and ATP hydrolysis properties of the preparation.

To test whether PKA phosphorylation of the R domain directly affects the ATPase activity of NBD1, we compared hydrolysis of ATP by untreated and PKA-treated NBD1–R–GST. Surprisingly, the apparent \( K_m \) for ATP was increased 3.5-fold following treatment of NBD1–R–GST with PKA. This observation was surprising because we expected PKA to lower the \( K_m \) based on two considerations. (i) ATP hydrolysis accompanies CFTR activation by PKA and (ii) in native CFTR, PKA acts to reduce ATPase activity of CFTR, their study could not discriminate between the NBD1 and NBD2 ATPases, nor could PKA alters the ATPase activity of CFTR, their study could not discriminate between the NBD1 and NBD2 ATPases, nor could it exclude the possibility that other CFTR domains are needed for PKA phosphorylation of the R domain. Since a similar fusion protein lacking that domain (i.e. R–GST) did not associate with NBD2 despite extensive PKA treatment. We did not examine whether our unphosphorylated fusion proteins associate with NBD2–MBP, but the observations of Lu and Pedersen [37], together with the findings presented here, indicate that NBD1–R and NBD2 might physically interact prior to phosphorylation although the interaction only has a functional consequence on the ATPase activity of NBD1 when the R domain is phosphorylated.

The spontaneous dimerization of GST has been utilized to promote the homodimerization of fusion protein partners [53]. However, GST is probably not responsible for the dimerization of NBD1–R–GST for the following reasons: first, most of the NBD1–R–GST migrated through sucrose gradients as monomers until phosphorylated by PKA. Although small amounts of the unphosphorylated fusion protein did migrate as dimers or larger aggregates (Figure 2A), we attribute this to (i) the technical difficulties of obtaining clearly resolved proteins using the sucrose gradient technique and/or (ii) a low degree of NBD1–R mediated oligomerization occurring in the absence of phosphorylation. Second, phosphorylated NBD1–R migrated through sucrose gradients as a dimer even though the GST moiety had been cleaved by thrombin (Figure 2C). Third, the R–GST protein appeared only as monomers despite having the GST attached. Finally, in the cases where GST has been used to facilitate fusion protein dimerization, the GST moiety was attached to the C-terminus of its fusion partner. In most other fusion proteins, including those used in this study, the GST is attached to the N-terminus of the fusion partner. This difference may explain why NBD1–R–GST and many other GST fusion proteins do not spontaneously dimerize via the GST moieties.

NBD–NBD interactions are emerging as pivotal events during the activation of ABC transporters [35,36,38,41,54]. For CFTR in particular, very recent physiological studies highlight how ATP-dependent NBD dimerization may directly control channel activation [13,19,20]. Since CFTR activation depends on phosphorylation of the R domain by PKA, these concepts provide a compelling reason for studying the biochemical mechanisms by which the phosphorylation state of the R domain controls NBD dimerization and co-operativity. The present study begins to define these mechanisms by using CFTR cytoplasmic domain fusion proteins to show that PKA phosphorylation of the R domain can control NBD1 ATP hydrolysis by regulating the affinity and co-operativity of ATP binding. In addition, this study shows that phosphorylation of the R domain promotes dimerization of NBD1–R fusion proteins and it alters the interaction between NBD1–R and NBD2. One implication of these findings is that PKA phosphorylation may have similar effects on the function and co-operative interactions of the R, NBD1 and NBD2 domains.
during physiological activation of native CFTR. Thus a priority for further studies will be to determine whether these effects are promoted or disrupted by mutations of the CFTR gene or by drugs targeting the CFTR protein.

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