Activation mechanisms for the cystic fibrosis transmembrane conductance regulator protein involve direct binding of cAMP

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The CFTR [CF (cystic fibrosis) transmembrane conductance regulator] chloride channel is activated by cyclic nucleotide-dependent phosphorylation and ATP binding, but also by non-phosphorylation-dependent mechanisms. Other CFTR functions such as regulation of exocytotic protein secretion are also activated by cyclic nucleotide elevating agents. A soluble protein comprising the first NBD (nucleotide-binding domain) and R-domain of CFTR (NBD1–R) was synthesized to determine directly whether CFTR binds cAMP. An equilibrium radioligand-binding assay was developed, firstly to show that, as for full-length CFTR, the NBD1–R protein bound ATP. Half-maximal displacement of [³H]ATP by non-radioactive ATP at 3.5 μ M and 3.1 mM was demonstrated. [³H]cAMP bound to the protein with different affinities from ATP (half-maximal displacement by cAMP at 2.6

and 167 μ M). Introduction of a mutation (T421A) in a motif predicted to be important for cyclic nucleotide binding decreased the higher affinity binding of cAMP to 9.2 μ M. The anti-CFTR antibody (MPNB) that inhibits CFTR-mediated protein secretion also inhibited cAMP binding. Thus binding of cAMP to CFTR is consistent with a role in activation of protein secretion, a process defective in CF gland cells. Furthermore, the binding site may be important in the mechanism by which drugs activate mutant CFTR and correct defective Δ F508-CFTR trafficking.

Key words: cAMP, cyclic nucleotide, cystic fibrosis transmembrane conductance regulator, ligand binding, mutation, protein secretion.

INTRODUCTION

CF (cystic fibrosis), one of the most common single gene disorders of Caucasian populations, is caused by mutations in the CFTR (CF transmembrane conductance regulator) [1]. CFTR is an integral membrane protein, normally located in the apical membrane of polarized epithelial cells [2]. Although originally characterized as a cAMP-activated Cl[−] channel [3], CFTR also regulates other ion channels [4] and exocytotic protein secretion [5]. It is not known which of these functions of CFTR is most important in the pathophysiology of CF, in particular the chronic lung disease that is the main cause of death. CFTR is highly expressed in the submucosal gland cells of the airways [6], which secrete the primary airways fluid that contains ions, serous and mucous proteins, the balance of which is abnormal in CF airways [5,7]. Evidence that CFTR is a key regulator of exocytotic protein secretion is based on demonstration of severe reduction in cAMP-mediated protein secretion from native exocrine gland cells from CF individuals [5,8–10], inhibition of β -adrenergic-stimulated mucin secretion by an anti-CFTR antibody, introduced into living rat submandibular acinar cells [11,12], demonstration that cells transfected with CFTR showed increased cAMP-dependent exocytosis [13] and protein secretion [14,15].

Activation of the Cl[−] channel function of CFTR requires phosphorylation by PKA (protein kinase A; also known as cAMP-dependent protein kinase) followed by ATP binding and hydrolysis [3,16]. CFTR Cl[−] channel activity in excised membrane patches, activated by cAMP in the presence of ATP, was inhibited by PKA-I (PKA inhibitor peptide) as a result of functional

coupling of CFTR with the membrane-associated isoenzyme of PKA (PKA-II) via a PKA anchoring protein [17]. However, in intact cells expressing CFTR, stimulation of Cl[−] channel activity by injection of cAMP was only inhibited by 60–70% by PKA inhibitors [18], suggesting direct activation by cAMP. Since CFTR-mediated protein secretion from exocrine acinar cells such as rat submandibular acini is also stimulated by cAMP-elevating agonists [11,19], the question arises whether this process is also activated in part by cAMP binding. There is evidence for a PKA-independent mechanism of regulation of exocytotic protein secretion in some cell types that involves cAMP binding to the exchange factor activated by cAMP known as Epac2 (exchange protein directly activated by cAMP 2) or cAMP-GEFII (guanine exchange factor II) [20,21]. Thus CFTR might play an analogous role in exocrine gland cells whose function is severely defective in CF [5,7–10].

On the basis of sequence comparison with known cyclic nucleotide-binding proteins and the effect of mutation of residues predicted to be important in cyclic nucleotide binding, Sullivan et al. [18] suggested the presence of a cyclic nucleotide-binding site in the region between amino acid residues 394–426 [N-terminal to NBD1 (nucleotide-binding domain 1)] of CFTR. However, the presence of cyclic NBD sequences does not necessarily predict that a protein binds cyclic nucleotides [22]. In order to investigate directly whether CFTR is a cyclic nucleotide-binding protein, we have synthesized a protein that encompasses the first NBD, NBD1, and R-domain (amino acid residues 357–856 of CFTR), as well as the putative cyclic NBD. We demonstrate directly that cyclic nucleotides bind to the NBD1–R domain of CFTR.

Abbreviations used: BCA, bicinechonic acid; 8-cpt, 8-(4-chlorophenyl)thio; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; Epac, exchange protein directly activated by cAMP; GEF, guanine exchange factor; IPTG, isopropyl β -D-thiogalactoside; NBD, nucleotide-binding domain; Ni-NTA, Ni²⁺-nitrilotriacetate; PDE, cyclic nucleotide phosphodiesterase; PKA, protein kinase A (or cAMP-dependent protein kinase); PKA-I, PKA inhibitor peptide; PKA-R, regulatory subunit of PKA; TNP-ATP, 2'-O-(trinitrophenyl)-ATP.

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EXPERIMENTAL

Materials and antibodies

pGEM-T vector and Western Blue stabilized substrate for alkaline phosphatase were from Promega, and pRSETB vector, SOB medium (20 g of Tryptone, 5 g of yeast extract, 0.5 g of NaCl, 186 mg of KCl and 2.4 g of anhydrous MgSO_4), IPTG (isopropyl β -D-thiogalactoside) and antiXpress antibody were from Invitrogen. PRISM BigDye Terminator cycle sequencing ready reaction kit was from Applied Biosystems and Micro BCA (bicinchoninic acid) assay and Gelcode[®] Blue Stain reagent were from Pierce. Ni-NTA (Ni^{2+} -nitrilotriacetate)-agarose was from Qiagen and TNP-ATP [$2'$ -*o*-(trinitrophenyl)-ATP] was from Molecular Probes. All radiolabelled nucleotides were from Amersham Biosciences and Optiphase 'HiSafe'2 liquid scintillant cocktail from Wallac. Microcon centrifugal filters were from Millipore. All other reagents were from Sigma. The anti-CFTR antibodies directed against NBD1 (MPNB) and the C-terminus (MPCT1) were raised in rabbits against peptides coupled with KLH [keyhole-limpet (*Diodora aspera*) haemocyanin], affinity-purified using the appropriate peptide linked to Sepharose and have been previously characterized [11,12,23]. The MATG 1104 antibody against the R-domain (amino acid residues 722–734) of CFTR was supplied by Transgene. Oligonucleotide primers were synthesized by Eurogentec.

Expression and purification of NBD1-R domain proteins

cDNA corresponding to amino acids 357–856 of CFTR [1], cloned into pGEM-T vector as previously described [24], was excised by digestion with BglII and ligated into the BglII site of the pRSETB vector. The T421A mutation was introduced into the pRSETB vector using Stratagene's QuikChange[®] II Site-directed Mutagenesis kit and oligonucleotide primers (forward and reverse) containing the mutation amidst a 21 base-pair 5'- and 3'-flanking region. Clones were identified by resistance to carbenicillin and correct orientation in positive colonies confirmed by PCR. Plasmids were transformed into TOP10F' *Escherichia coli* cells for propagation and maintenance and clones sequenced using the PRISM BigDye Terminator cycle sequencing ready reaction kit to confirm fidelity of the sequence and that the cDNA was inserted in-frame with the purification tag.

NBD1-R domain proteins were expressed by transformation into BL21(DE3)pLysS *E. coli* and selection of carbenicillin- and chloramphenicol-resistant transformants as follows. SOB medium (50 ml) containing carbenicillin (50 $\mu\text{g/ml}$) and chloramphenicol (34 $\mu\text{g/ml}$) was inoculated with transformed cells and grown overnight. The overnight culture [D_{600} (attenuance) ~ 0.7] was diluted 10-fold in 500 ml of SOB and the cells grown until the D_{600} was 0.4–0.6 (~ 1.5 h). Recombinant protein synthesis was induced by addition of 1 mM IPTG for 4 h. Cells were harvested by centrifugation at 7000 g for 20 min at 4°C and stored frozen at -20°C . Thawed cells were lysed in Tris/EDTA buffer (10 mM Tris/HCl, pH 8.0, and 1 mM EDTA) containing 2 mg/ml lysozyme at 4°C for 30 min. After addition of MgCl_2 (10 mM) and DNaseI (67 m-units/ml), further incubation at 4°C for 30 min was followed by sonication. Inclusion bodies were harvested by centrifugation at 7000 g for 20 min at 4°C, washed in Tris/EDTA buffer containing 1% (v/v) Triton X-100 for 10 min at 4°C and centrifuged at 10000 g for 20 min at 4°C. The pellet of inclusion bodies was solubilized in 0.1 M NaH_2PO_4 (pH 8.0) containing 8 M urea and 10 mM 2-mercaptoethanol. Insoluble material was removed by centrifugation at 3000 g for 3 min.

Solubilized inclusion bodies were added to Ni-NTA-agarose, pre-equilibrated in 0.1 M NaH_2PO_4 (pH 8.0) containing 8 M urea

(buffer A) and incubated at 4°C for 1 h on a rotary shaker. The mixture was transferred to a column and washed with 10–15 vol. of buffer A containing 10 mM 2-mercaptoethanol, followed by 5 vol. of buffer A containing 10 mM 2-mercaptoethanol at pH 6.3 (until the eluant A_{280} was <0.001). Bound protein was eluted in fractions with 3 vol. of buffer A at pH 4.5. Fractions were solubilized in SDS-solubilizing buffer [0.125 M Tris/HCl (pH 6.8) containing 5% (w/v) SDS, 25% (w/v) sucrose and 0.5% (w/v) dithiothreitol] for 10 min at 50°C and separated by SDS/PAGE using 10% (w/v) acrylamide gels as previously described [12]. Following electrophoresis, the gel was washed three times for 5 min in deionized water, Gelcode[®] stain added for 30–60 min and the gel washed three times for 10–20 min in deionized water. Eluted fractions containing purified protein were pooled, separated by SDS/PAGE and blotted on to nitrocellulose in 25 mM Tris (pH 8.3) containing 192 mM glycine and 20% (v/v) methanol for 1.5 h at 90 V. Unbound protein binding sites were blocked by incubation in 5% (w/v) skimmed milk powder in 20 mM Tris/HCl (pH 7.4) containing 0.5 M NaCl and 0.05% (v/v) Tween 20 (buffer B) for 1 h at 21°C. Blots were then incubated overnight at 4°C with the primary antibody (see the Results section) in buffer B containing 5% (w/v) milk powder. After extensive washing in buffer B, blots were incubated with secondary alkaline phosphatase-conjugated antibody (1:3000) in buffer B for 30 min at room temperature, washed and protein bands visualized by incubation for up to 1 h in Western Blue stabilized substrate for alkaline phosphatase.

Renaturation of NBD1-R domain fusion proteins

Dialysis and rapid dilution were investigated as methods to reduce denaturant concentration and induce refolding of the protein. Concentration of protein before and after renaturation was determined using the Micro BCA assay and refolding efficiency initially assessed as recovery of soluble protein. Purified NBD1-R domain protein (0.1 mg/ml) was dialysed at 4°C for periods of 2 h in 100 vol. of buffers containing 3 M urea, 1 M urea and no urea respectively or for periods of 3 h in buffers containing 6 M urea, 4 M urea, 2 M urea and no urea respectively. In some experiments the final dialysis was extended to 18 h. Buffers tested were: 50 mM sodium phosphate (pH 7.5) containing 1 mM dithiothreitol and 1 mM EDTA; 0.1 M NaH_2PO_4 (pH 8.0) containing 50 mM glycine, 1 mM EDTA and 0.005% Tween 20 (buffer C) and 5 mM sodium acetate (pH 5.0). For rapid dilution, NBD1-R domain protein solutions (2 mg/ml) were adjusted to pH 5.0 or 8.0 and diluted 10-fold into 5 mM sodium acetate (pH 5.0) or buffer C respectively. Samples were incubated for 18 h at 4°C and insoluble material removed by centrifugation at 100000 g for 80 min at 4°C. Under all dialysis conditions tested, the yield of soluble protein was 10–15%. Rapid dilution into 5 mM sodium acetate (pH 5.0) also yielded approx. 15% of soluble protein, whereas dilution into buffer C resulted in yields of 80–100%. Thus rapid dilution into buffer C was used for refolding in all experiments described. Following refolding, proteins were concentrated to 200–250 $\mu\text{g/ml}$ using YM50 centrifugal filters, subjected to SDS/PAGE and stained with Coomassie Blue. It was found that further concentration of refolded protein usually resulted in precipitation. Thus, in all assays except for TNP-ATP binding (see below), protein solutions were diluted by approx. 100-fold.

In vitro phosphorylation

Refolded NBD1-R domain protein was diluted to $\sim 3 \mu\text{g/ml}$ in 100 mM Tris/HCl (pH 7.4) containing 20 mM MgCl_2 , 4 mM EDTA and 200 $\mu\text{g/ml}$ of BSA and phosphorylated with PKA (catalytic subunit, 100 units/ml) and [γ - ^{32}P]ATP (0.5 $\mu\text{Ci}/15 \mu\text{l}$

reaction volume) for 1 h at 30 °C. Samples were mixed with SDS-solubilizing buffer, heated at 50 °C for 10 min and analysed by SDS/PAGE and autoradiography as previously described [12]. Control reactions in the absence of either PKA or NBD1–R domain protein were negative.

TNP-ATP binding

ATP binding was assayed by fluorescent enhancement of TNP-ATP. Fluorescence was measured at λ_{ex} and λ_{em} of 410 and 540 nm (10 nm slit widths) respectively (LS-5B fluorimeter; PerkinElmer). Preliminary experiments showed that for sufficient sensitivity to make TNP-ATP binding measurements, an NBD1–R protein concentration of 200 $\mu\text{g/ml}$ was required. As described above, this was close to the maximum concentration at which refolded protein would stay in solution. Thus the solution of refolded NBD1–R domain protein (200 $\mu\text{g/ml}$ in buffer C) was adjusted to pH 7.5 and 4 mM MgCl_2 added (3 mM final). Protein was incubated with TNP-ATP (0–10 μM) by the sequential addition of aliquots of 100 μM TNP-ATP (in buffer C, pH 7.5, containing 4 mM MgCl_2). Urea (0.8 M) was also added to the TNP-ATP stock solution to avoid effects of dilution of the urea present in buffer C following refolding by dilution. Fluorescence enhancement due to protein binding was calculated by subtraction of fluorescence with no added protein at each TNP-ATP concentration.

Cyclic nucleotide binding

Initially, binding of [^3H]cAMP was investigated using filtration to separate bound from free cAMP. NBD1–R domain protein, diluted in Tris/NaCl buffer [50 mM Tris/HCl (pH 7.5) containing 50 mM NaCl] (1–10 $\mu\text{g/ml}$, equivalent to 0.015–0.15 μM assuming a molecular mass of 65 kDa), was mixed with 0.1 μM [^3H]cAMP (14 μCi) and incubated for periods of up to 1 h at 4 or 37 °C. Aliquots were then filtered on polycarbonate filters (0.45 μm), washed with 10 vol. of Tris/NaCl buffer, mixed with liquid scintillant cocktail and left for 18 h in the dark before assay of radioactivity. At either temperature, [^3H]cAMP binding was saturated within 15 min and approx. 50 % displacement by excess non-radioactive cAMP (1 mM) was observed under all conditions (results not shown). The amount of bound [^3H]cAMP was also low (1500–2000 c.p.m.) unless relatively large concentrations of protein were used. Thus, because the assay was both lacking in sensitivity and showed high non-specific binding, a more sensitive assay was developed.

Nucleotide binding by equilibrium radioligand binding

In order to produce an assay that was more sensitive and could be used for direct comparison of ATP and cyclic nucleotide binding, an equilibrium binding method, based on the work of Dremier et al. [22], was developed. NBD1–R domain protein, diluted in Tris/NaCl/Mg buffer containing (Tris/NaCl buffer 10 mM MgCl_2) (1–6.5 $\mu\text{g/ml}$, equivalent to 15–100 nM, assuming a molecular mass of 65 kDa), was mixed with 0.1 μM [^3H]ATP or 0.1 μM [^3H]cAMP (both 14 μCi) and incubated for periods of up to 1 h at 4 °C. The mixture was applied to the top sample chamber of a Microcon (YM30) centrifugal filter. An aliquot was removed for radioactive counting before the filters were spun in a microcentrifuge for 10 min at 4 °C when the volume in the upper chamber (where the protein is retained) had decreased by 10-fold. Aliquots of the buffer were removed from the upper and lower (flow-through) chambers. All samples were mixed with liquid scintillant cocktail, left for 18 h in the dark and radioactivity assayed.

Figure 1 Characterization of NBD1–R domain fusion protein

NBD1–R domain protein was expressed in BL21(DE3)pLysS *E. coli*, extracted from inclusion bodies and purified by Ni-NTA-agarose affinity chromatography as described in the Experimental section. (A) Affinity-purified protein was refolded by rapid dilution as described in the Experimental section and concentrated to 200–250 $\mu\text{g/ml}$ using YM50 centrifugal filters. Then, 200 ng of protein was subjected to SDS/PAGE and stained with Coomassie Blue. Lane 1: molecular mass markers (numbers denote molecular masses in kDa); lane 2: NBD1–R domain protein. (B) Three hundred nanograms of protein were separated by SDS/PAGE, blotted on to nitrocellulose and probed with the following antibodies, as described in the Experimental section: lane 1: MPNB1 (against NBD1); lane 2: MATG 1104 (against the R-domain); lane 3: anti-Xpress; lane 4: MPCT1 (against the C-terminus). Numbers denote molecular masses in kDa. Arrowheads denote position of major band at 65 kDa.

RESULTS

In order to determine directly whether CFTR binds cAMP we have developed a model system, using a soluble NBD1–R domain protein that encompasses amino acid residues 357–856 of CFTR, which includes the putative cyclic NBD.

Characterization of soluble NBD1–R domain fusion protein

Affinity-purified protein was refolded by rapid dilution, concentrated to 200–250 $\mu\text{g/ml}$ using YM50 centrifugal filters, subjected to SDS/PAGE and stained with Coomassie Blue. As shown in Figure 1(A), the fraction showed a single predominant band of approx. 65 kDa. The protein was also characterized by Western blotting using antibodies against various domains of the fusion protein: the fusion tag (anti-Xpress), NBD1 (MPNB), the R-domain (MATG) and, as a negative control, the C-terminus of CFTR (MPCT1). As shown in Figure 1(B), the anti-C-terminus antibody did not detect any protein, whereas the other three antibodies detected a band of approx. 65 kDa.

ATP binding and phosphorylation of NBD1–R domain fusion protein

If successfully refolded, the NBD1–R domain protein should bind ATP and be a substrate for phosphorylation by PKA [3]. The fluorescence enhancement of TNP-ATP has been the most commonly used method to demonstrate ATP binding to the NBDs of CFTR [25–28]. As shown in Figure 2(A), addition of TNP-ATP to the refolded protein resulted in enhancement in fluorescence at 540 nm, demonstrating ATP binding. The fluorescence enhancement curves fitted hyperbolic curves for 1-site ligand binding, yielding a value for half-maximal ATP binding at pH 7.5 of $2.7 \pm 0.7 \mu\text{M}$ ($n = 5$). Subsequent addition of unlabelled ATP reversed the fluorescent enhancement by approx. 70 % at 25 mM ATP (results not shown). Other measurements of the affinity of NBD1 for TNP-ATP have been made using similar constructs (NBD1–R as a His-tagged protein expressed in *E. coli* [25,26] or NBD1 alone as a fusion protein [27,28]). However,

Figure 2 ATP binding (A) and phosphorylation (B) of NBD1-R domain protein

(A) Refolded NBD1-R domain protein (0.2 mg/ml) was incubated with TNP-ATP (0–10 μ M) as described in the Experimental section. Fluorescence enhancement due to protein binding was calculated by subtraction of fluorescence with no added protein at each TNP-ATP concentration. Curve shown is representative of five experiments. (B) Refolded NBD1-R domain protein (3 μ g/ml) was phosphorylated with PKA and [γ - 32 P]ATP as described in the Experimental section. Numbers denote molecular masses in kDa.

all used different assay conditions (pH 7.4/7.5, no magnesium [26–28]; pH 5, 2 mM MgSO_4 and 0.4 M urea [25]). Nevertheless, apparent half-maximum binding values published (0.2 μ M [26], 0.8 μ M [25], 1.8 μ M [27] and 3.1 μ M [28]) were in the same range as those obtained in the present study. As shown in Figure 2(B), NBD1-R domain protein was phosphorylated by PKA. Thus, on the basis of recovery of soluble protein, TNP-ATP binding and phosphorylation by PKA, the results show that milligram quantities of purified, functional NBD1-R domain protein was produced.

Nucleotide binding by equilibrium radioligand binding

In order to produce an assay that could be used for direct comparison of ATP and cyclic nucleotide binding, an equilibrium binding method, based on the work of Dremier et al. [22] was investigated.

Demonstration and characteristics of ATP binding to NBD1-R domain protein

NBD1-R domain protein (30 nM) was incubated with 0.1 μ M [^3H]ATP, in the presence or absence of 1 mM non-radioactive ATP for 15 min at 4°C before concentration of the protein by centrifugation in the upper chamber of a Microcon centrifugal filter. As shown in Figure 3(A), the concentration of [^3H]ATP radioactivity increased in the upper chamber when the protein was concentrated, thus demonstrating binding. There was a concomitant decrease in concentration of [^3H]ATP radioactivity in the lower chamber. Displacement of [^3H]ATP binding to the NBD1-R domain protein by unlabelled ATP (1 mM) was also demonstrated

Figure 3 Demonstration of [^3H]ATP binding to NBD1-R domain protein by an equilibrium binding method

(A) NBD1-R domain protein (30 nM) in Tris/NaCl/Mg buffer was incubated with [^3H]ATP for 15 min at 4°C in the presence or absence of unlabelled ATP (10 mM). Samples were applied to the top chamber of a Microcon (YM30) centrifugal filter and concentrated by centrifugation as described in the Experimental section. Radioactivity was assayed in 20 μ l aliquots removed before centrifugation (open bars) and from the upper (filled bars) and lower (hatched bars) chambers after centrifugation. Results are means $\pm$ S.E.M. for $n=4$. *, $P < 0.05$, **, $P < 0.001$ for difference from radioactive counts added at the start. †, $P < 0.001$ for difference from radioactive counts in the equivalent chamber at 0.1 μ M ATP. All differences were assessed by Student's paired t test. (B) Concentration dependence of ATP binding. Results are expressed as the ratio of radioactive counts in the upper chamber after centrifugation to counts in the starting mixture and are means $\pm$ S.E.M. for $n=4$.

(Figure 3A). There was no difference in the degree of binding after incubation for 30 or 60 min (results not shown), indicating that 15 min was sufficient time to attain equilibrium. In order to compare the extent of nucleotide binding between experiments, the data were expressed as the ratio of radioactive counts in the upper or lower chambers to that in the starting mixture before centrifugation to concentrate the protein. Thus a ratio significantly >1 in the upper chamber and significantly <1 in the lower chamber demonstrates binding. The ratio of counts in the upper chamber was 3.59 ± 0.11 ($n=4$) and in the lower chamber 0.37 ± 0.02 ($n=4$) for binding in the presence of 0.1 μ M ATP. When ATP binding to the NBD1-R domain protein was measured in the presence of increasing concentrations of unlabelled ATP, displacement of [^3H]ATP from the protein was maximal at 10 mM ATP (Figure 3B) and followed a curve for which the best fit was a biexponential curve of the form: $y = Pe^{-ax} + Qe^{-bx} + \text{residual}$. Half-maximal displacement of [^3H]ATP from the two sites was calculated at $3.5 \pm 0.2 \mu\text{M}$ ($n=3$) and $3.1 \pm 0.4 \text{ mM}$ ($n=3$). Since complete displacement of [^3H]ATP counts would give a ratio of 1.0, the calculated residual of 1.45 gives non-specific binding of 17%.

Figure 4 Demonstration of [^3H]cAMP binding to NBD1-R domain protein by an equilibrium binding method

(A) NBD1-R domain protein or BSA (15 nM) in Tris/NaCl buffer was incubated with [^3H]cAMP for 15 min at 4°C in the presence or absence of unlabelled cAMP (1 mM). Samples were applied to the top chamber of a Microcon (YM30) centrifugal filter and concentrated by centrifugation as described in the Experimental section. Radioactivity was assayed in 20 μl aliquots removed before centrifugation (open bars) and from the upper (filled bars) and lower (hatched bars) chambers after centrifugation. Results are means $\pm$ S.E.M. for $n = 9$ (NBD1-R domain) and $n = 3$ (BSA). *, $P < 0.01$, **, $P < 0.001$ for difference from radioactive counts added at the start. !, $P < 0.001$ for difference from radioactive counts in the equivalent chamber at 0.1 μM cAMP. All differences were assessed by Student's paired t test. (B) NBD1-R domain protein (0.015–0.1 μM), PKA or BSA (both 0.1 μM) were assayed for [^3H]cAMP binding as in (A). Results are expressed as the ratio of radioactive counts per 20 μl in the upper (filled bars) or lower (hatched bars) chambers to those in the starting mixture before centrifugation to concentrate the protein (open bars). Results are means $\pm$ S.E.M. for $n = 4$ –9 (NBD1-R domain); $n = 5$ (PKA), $n = 3$ (BSA). *, $P < 0.01$, **, $P < 0.001$ for difference from starting ratio of 1.00. All differences were assessed by Student's paired t test.

Demonstration of cAMP binding to NBD1-R domain protein

We next used the equilibrium-binding assay to investigate whether CFTR also binds cAMP. Thus NBD1-R domain protein (15 nM) was incubated with 0.1 μM [^3H]cAMP, in the presence or absence of 1 mM non-radioactive cAMP for 15 min at 4°C before concentration of the protein in a Microcon centrifugal filter. As shown in Figure 4(A), the concentration of [^3H]cAMP radioactivity increased in the upper chamber, when the protein was concentrated, demonstrating binding as shown for ATP (Figure 3). There was also a concomitant decrease in concentration of [^3H]cAMP radioactivity in the lower chamber. A protein such as BSA that does not bind cAMP [22] showed no concentration of radioactivity in the upper chamber (Figure 4A). Displacement of [^3H]cAMP binding to the NBD1-R domain protein by unlabelled cAMP (1 mM) was also demonstrated (Figure 4A). As shown in Figure 4(B), when expressed as the ratio of radioactive counts in the upper or lower chambers to that in the starting mixture, the

ratio of counts in the upper chamber increased with increasing NBD1-R domain protein concentration from 2.15 at 0.015 μM to 3.83 at 0.1 μM . PKA at 0.1 μM gave a ratio of 4.86 and BSA, a ratio of 1.06. At all concentrations of NBD1-R domain protein or PKA, the ratio of counts in the lower chamber was < 1 .

Affinity of NBD1-R domain proteins for cAMP

When [^3H]cAMP binding to NBD1-R domain proteins was measured in the presence of increasing concentrations of unlabelled cAMP, displacement of [^3H]cAMP showed maximum displacement at 1 mM cAMP (Figure 5A), an order of magnitude lower than that for ATP. However, as for ATP binding, cAMP displacement followed a biexponential curve, yielding values for half-maximal displacement of [^3H]cAMP of 2.6 and 167 μM .

Sullivan et al. [18] compared the whole-cell chloride channel activity elicited by injection of 50 μM cAMP into cells expressing wild-type CFTR or the mutant form, T421A. They showed that the mutation caused an approx. 80 % reduction in channel activity [18]. We introduced the T421A mutation into CFTR cDNA (see the Experimental section) and expressed an NBD1-R domain protein to investigate the effect on cAMP binding. As shown in Figure 5(B), the T421A mutant protein bound [^3H]cAMP but showed half-maximal displacement by unlabelled cAMP at 9.2 and 201 μM . Thus the results suggest that the T421A mutation results in decreased affinity of the higher affinity binding, which is responsible for activation of CFTR.

Displacement of cAMP binding by analogues

[^3H]cAMP binding to NBD1-R domain protein was displaced by dibutyryl- ($N^6,2'$ - O -dibutyryl-) and 8-cpt-[8-(4-chlorophenyl)-thio-] analogues of cAMP, to the same extent as by cAMP at a concentration of 100 μM (Table 1). By contrast, cGMP and, in particular, 8-cpt-cGMP were less effective than cAMP or its analogues in competing with [^3H]cAMP binding (Table 1). While 100 μM cAMP was 80–90 % as effective as 1 mM cAMP at displacement of [^3H]cAMP, 100 μM cGMP was only 60–65 % as effective and 8-cpt-cGMP (100 μM) did not significantly displace [^3H]cAMP (Table 1). We also tested the effect of unlabelled ATP (up to 10 mM) on [^3H]cAMP binding. As shown in Figure 5(C), displacement of [^3H]cAMP was maximal at 1 mM ATP, the range of the physiological concentration of ATP and followed a biexponential curve, showing half-maximal displacement at 10.4 and 198 μM . Compared with cAMP, ATP was less effective at displacing [^3H]cAMP. Thus 1 mM ATP competed out 55.2 ± 1.5 % ($n = 5$) of the [^3H]cAMP counts, whereas cAMP competed out 80.6 ± 3.1 % ($n = 7$; $P < 0.001$ for difference from ATP). Further demonstration that cAMP competed effectively with ATP for binding is shown in Figure 5(D). Thus, in the presence of 1 mM ATP, addition of 1–10 μM cAMP increased [^3H]cAMP binding to the same level as for 0.1 μM [^3H]cAMP alone. Addition of higher concentrations (0.1–10 mM) of unlabelled cAMP displaced [^3H]cAMP counts with a single exponential decay giving a half-maximal concentration of approx. 80 μM .

Effect of CFTR antibody on nucleotide binding

Since isoprenaline stimulation of mucin secretion from rat submandibular acinar cells is inhibited by the MPNB1 anti-CFTR antibody [11,12,19], we next investigated if the antibody affected nucleotide binding to the NBD1-R domain protein. As shown in Figure 6, the MPNB antibody inhibited both cAMP and ATP binding in a concentration-dependent manner. Inhibition of

Figure 5 Concentration dependence of [^3H]cAMP displacement from NBD1-R domain protein by unlabelled cAMP or ATP

NBD1-R domain protein (30 nM) in Tris/NaCl buffer was incubated with [^3H]cAMP for 15 min at 4 °C in the presence or absence of unlabelled cAMP (**A, B**), ATP (**C**) or both (**D**). Samples were applied to the top chamber of a Microcon (YM30) centrifugal filter and concentrated by centrifugation as described in the Experimental section. Radioactivity was assayed in 20 μl aliquots removed before centrifugation and from the upper and lower chambers after centrifugation. In (**A, C, D**), results are expressed as the ratio of radioactive counts in the upper chamber after centrifugation to counts in the starting mixture. In (**B**), results are expressed as % displacement, taking binding at 0.1 μM [^3H]cAMP as zero; a ratio of 1.0 would be 100% displacement. All the results are means $\pm$ S.E.M.; (**A**) $n = 7$ for displacement by cAMP; (**B**) displacement by cAMP from wild-type (closed circles, $n = 5$) or T421A (open squares, $n = 5$) NBD1-R domain protein. *, $P < 0.05$ for difference from wild-type; (**C**) $n = 6$ for displacement by ATP; (**D**) displacement by cAMP in the presence of 1 mM ATP (closed circles) compared with binding of 0.1 μM [^3H]cAMP alone (star). Results are means for two experiments.

Table 1 [^3H]cAMP displacement by cAMP and cGMP analogues

NBD1-R domain protein (30 nM) in Tris/NaCl buffer was incubated with [^3H]cAMP for 15 min at 4 °C in the presence or absence of unlabelled cAMP or cGMP analogues as indicated. Samples were applied to the top chamber of a Microcon (YM30) centrifugal filter and concentrated by centrifugation as described in the Experimental section. Radioactivity was assayed in 20 μl aliquots removed before centrifugation and from the upper and lower chambers after centrifugation. Results are expressed as the ratio of radioactive counts in the upper chamber after centrifugation to counts in the starting mixture ('Ratio') and as percentage of the displacement effected by 1 mM unlabelled cAMP (% 1 mM). Results are means $\pm$ S.E.M. for the number of experiments shown in parentheses. *, $P < 0.01$; **, $P < 0.001$ for difference from ratio obtained in the absence of unlabelled cAMP (0.1 μM). †, $P < 0.001$ for difference from ratio obtained in the presence of 1 mM unlabelled cAMP. ‡, $P < 0.05$, §§, $P < 0.001$ for difference from percentage of 1 mM obtained by 100 μM unlabelled cAMP. All differences were assessed by Student's unpaired t test.

Analogue	Concentration	Ratio	% 1 mM
cAMP	0.1 μM	3.19 ± 0.27 (14)	
	1 mM	$1.32 \pm 0.06^{**}$ (14)	
	100 μM	$1.54 \pm 0.16^*$ (4)	88.3 ± 2.6 (4)
Dibutyl- γ -cAMP	100 μM	$1.34 \pm 0.07^*$ (3)	98.3 ± 7.1 (3)
8-cpt-cAMP	100 μM	$1.47 \pm 0.09^*$ (3)	93.7 ± 9.9 (3)
cGMP	100 μM	$1.73 \pm 0.07^{*†}$ (5)	$65.6 \pm 8.0^{\ddagger}$ (5)
8-cpt-cGMP	100 μM	$3.37 \pm 0.10^{\dagger}$ (5)	$22.9 \pm 3.5^{\ddagger\ddagger}$ (5)

both nucleotides was maximally inhibited by MPNB antibody at 100 $\mu\text{g/ml}$ giving displacement of radiotracer that was of the same magnitude as 1 mM unlabelled nucleotide. The MPCT-1 antibody, which does not bind to the NBD-R domain protein, had no effect on nucleotide binding except that, at 100 $\mu\text{g/ml}$, there

was inhibition of cAMP binding that was of the same magnitude as 1 $\mu\text{g/ml}$ of MPNB antibody. In order to compare the effect of MPNB antibody on ATP and cAMP binding, the data were expressed as percentage displacement of radiotracer. As shown in Figure 6(C), the effect of antibody was greater on cAMP binding except at the maximally effective concentration (100 $\mu\text{g/ml}$).

DISCUSSION

Cyclic nucleotide binding to the NBD1-R domain fusion protein

It had previously been suggested that CFTR might possess a cyclic nucleotide-binding site in the region at the N-terminal end of NBD1, in part on the basis of sequence similarities to the cyclic nucleotide-gated ion channels [18], a well-characterized family of channels in which gating is cyclic nucleotide- but not phosphorylation-dependent [29]. However, proteins identified from searches for cyclic nucleotide-binding motifs often fail to exhibit cyclic nucleotide binding [22]. Thus, in the present study, we measured cyclic nucleotide-binding directly, using a His-tagged fusion protein that encompasses the entire first cytoplasmic domain of wild-type CFTR [24], including the putative cyclic nucleotide-binding region. The soluble NBD1-R domain protein has the advantage that it can be synthesized and purified in milligram quantities and is readily amenable to ligand binding assays. Our evidence that the refolded protein is functionally the same as when part of full-length CFTR is based on the demonstration that it binds ATP and is phosphorylated by PKA (Figures 2 and 3). Similar constructs have been synthesized by others [25,26] and we found, as did Neville et al. [25], that the most

Figure 6 Effect of anti-CFTR antibodies on nucleotide binding to NBD1-R domain protein

(A, B) NBD1-R domain protein (30 nM) in Tris/NaCl buffer was incubated for 18 h at 4°C with either MPNB (closed squares) or MPCT1 (open squares) antibody at the concentration shown. [3 H]cAMP (A) or [3 H]ATP (B) was added and incubation continued for 15 min at 4°C. Samples were applied to the top chamber of a Microcon (YM30) centrifugal filter and concentrated by centrifugation as described in the Experimental section. Radioactivity was assayed in 20 μ l aliquots removed before centrifugation and from the upper and lower chambers after centrifugation. Results are expressed as the ratio of radioactive counts in the upper chamber after centrifugation to counts in the starting mixture and are means $\pm$ S.E.M.; (A) $n = 3$ for effect of antibody. *, $P < 0.05$, **, $P < 0.005$, for difference from binding in the absence of antibody. Displacement by 1 mM cAMP (star) is shown for comparison. (B) $n = 4$ for effect of antibody. *, $P < 0.01$ for difference from binding in the absence of antibody. Displacement by 1 mM ATP (star) is shown for comparison. (C) Results from (A) and (B) for MPNB antibody, expressed as % displacement of [3 H]cAMP (open circles) or [3 H]ATP (closed circles). *, $P < 0.05$, **, $P < 0.001$ for difference from ATP binding at the same antibody concentration. All differences were assessed by Student's unpaired t test.

successful refolding procedure in terms of recovery of soluble protein and ability to bind ATP was rapid dilution, as described in the Experimental section. The value for affinity for TNP-ATP

(2.7 μ M) is consistent with values previously reported for various constructs containing NBD1 [25–28]. As in most other studies [25–28], displacement of TNP-ATP by ATP required concentrations in the millimolar range. Higher values for ATP binding (10–100 μ M) have been obtained using other ATP analogues and different reporting methods [30–32]. Values for half-maximal concentration of ATP required for opening of CFTR chloride channels also vary widely, from 10^{-6} to 10^{-4} M [33–35]. Our data using an equilibrium-binding assay may help to explain these discrepancies since we demonstrated two affinities for ATP binding, one in the micromolar range consistent with the value obtained using TNP-ATP and one in the millimolar range. Using the functional NBD1-R domain protein, we have now directly demonstrated cAMP binding. As for ATP, two different binding affinities were observed, with half-maximum concentrations in the 10^{-6} and 10^{-4} M range (Figure 5A). Sullivan et al. [18] reported half-maximal activation of CFTR by cAMP in *Xenopus* oocytes of 4 μ M for cAMP, similar to the higher affinity binding (2.6 μ M) shown here.

Relationship to other cyclic nucleotide-binding proteins

The binding of cAMP is consistent with sequence alignments that can be made for the region of CFTR (392–438) and other known cyclic nucleotide-binding proteins (see [18]). The most highly conserved feature of the cAMP binding region of PKA-R (regulatory subunit of PKA) and CAP (catabolite activator protein) is the phosphate-binding cassette, which contains the FGEL(I) and PRAAT motifs that are essential for cAMP binding [36]. The FGEL(I) motif is present in CFTR (405–408) but the RA residues of the PRAAT motif are present as KT (420–421) in CFTR. An R to K mutation in the PRAAT motif caused an approx. 10-fold decrease in affinity for cAMP in PKA-R [37], consistent with the relatively low affinity (micromolar) of CFTR for cAMP. The presence of KT (420–421) in CFTR resembles more the cGMP-binding motif of the rod photoreceptor cGMP-gated channel [38] and PKG (also known as cyclic-GMP-dependent protein kinase) [39]. This might explain why cGMP is relatively efficient at displacing cAMP binding (Table 1). We synthesized a T421A mutant form of the NBD1-R domain protein and showed a decreased affinity for cAMP binding (Figure 5B), consistent with the demonstration that the full-length T421A CFTR showed reduced chloride channel activity in whole cells [18]. The results also serve to strengthen the hypothesis that the 392–428 region of CFTR is the site of cAMP binding and that the NBD1-R domain fusion protein model reflects the function of full-length CFTR.

Relationship to NBD1 structure

The region encompassing the cyclic nucleotide-binding motifs is within the crystal structures solved for mouse and human CFTRs [31,40]. The FGEL motif of CFTR is present in a short helical region with the KTS motif present in the adjacent flexible linker or regulatory insert region that is unique to CFTR [31,40]. Although residues such as Trp⁴⁰¹ that are highly conserved in ABC transporter NBDs are present within the putative cyclic NBD, our results suggest that cAMP can bind effectively to CFTR in the presence of millimolar ATP (Figure 5D). It is possible that the relative action of cAMP and ATP binding may determine which function of CFTR predominates.

Role of cyclic nucleotide binding in normal and mutant CFTR function

The present results demonstrating cAMP binding to CFTR strengthen the hypothesis of direct activation of CFTR chloride

channels by cyclic nucleotides [18]. In addition, in exocrine gland cells whose function is defective in CF, CFTR might play an analogous role to the exchange factor activated by cAMP, known as Epac2 or cAMP-GEFII, which has been suggested to be important in regulation of exocytotic protein secretion [20,21]. Thus we suggest that CFTR-mediated protein secretion results from a combination of phosphorylation of CFTR by activation of PKA and direct binding of cAMP to CFTR. The evidence presented in support of this hypothesis is, first, the direct demonstration that CFTR binds cAMP (Figures 4 and 5) and, secondly, the MPNB antibody that inhibits CFTR-mediated protein secretion in rat submandibular acini also inhibits cAMP binding (Figure 6). In addition, we previously showed that inhibition of isoprenaline-stimulated mucin secretion by MPNB antibody is partially corrected by phosphodiesterase inhibitors or 8-cpt-cAMP [11,19] and we suggested that this might be due to production of supra-physiological levels of cyclic nucleotides. The results in the present study support this hypothesis since, when present at high concentration within cells, cAMP or its analogues could compete with the antibody and provide a mechanism for reversal of antibody inhibition. Since cGMP also competes at the cAMP-binding site (Table 1), this might also explain why PDE5 (cyclic nucleotide phosphodiesterase 5) inhibitors that increase cGMP in rat submandibular acini [19] also partially correct the antibody inhibition. It is interesting that the binding of cAMP to proteins such as PKA-R results in conformational change affecting protein-protein interactions [36]. Thus cyclic nucleotide binding to CFTR may affect interaction of CFTR with proteins involved in exocytosis such as syntaxin-1A and SNAP-23 (23 kDa synaptosome-associated protein) that are already known to interact with CFTR [41,42].

A number of small molecules, including the benzo(c)quinolizinium (MPB) compounds [43] and genistein [44], have been shown to activate wild-type and mutant CFTR without increasing cAMP levels. We have shown that benzo(c)quinolizinium compounds and the PDE5 inhibitor, sildenafil, are selective stimulators of trafficking of $\Delta F508$ -CFTR [23,45], by far the most common mutant form, which is incorrectly processed and less efficiently trafficked to the apical membrane than wild-type CFTR [23,46,47]. Our results indicate that they act by direct binding to the first cytoplasmic domain of CFTR or by excess production of cyclic nucleotides [19,24]. It will be important for the development of specific $\Delta F508$ -CFTR trafficking drugs to determine if they bind to the cyclic nucleotide-binding site.

In conclusion, we suggest that the cyclic nucleotide-binding site demonstrated in the present study is an important regulatory site in CFTR function and should also be investigated as a mechanism for correction by drugs of $\Delta F508$ -CFTR trafficking.

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