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# Activation Mechanisms for the Cystic Fibrosis Transmembrane Conductance Regulator Protein Involve Direct Binding of Cyclic AMP

Malcolm M.C. Pereira, Jody Parker, Fiona L.L. Stratford, Margaret McPherson and Robert L. Dormer<sup>1</sup>

Department of Medical Biochemistry & Immunology, School of Medicine, Cardiff University, Cardiff CF14 4XN, UK.

Running title: Cyclic nucleotide binding to CFTR

Abbreviations used: 8-cpt-cyclic AMP, 8-(4-chlorophenyl)thio-cyclic AMP; 8-cpt-cyclic GMP, 8-(4-chlorophenyl)thio-cyclic GMP; BSA, bovine serum albumin; CAP, catabolite activator protein; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; cyclic AMP, adenosine-3',5'-cyclic monophosphate; cyclic GMP, guanosine-3',5'-cyclic monophosphate; dibutyryl cyclic AMP, N<sup>6</sup>,2'-O-dibutyryl cyclic AMP; Epac, exchange protein directly activated by cyclic AMP; GEF, guanine exchange factor; H8, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; H89, N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; KLH, keyhole limpet haemocyanin; NBD, nucleotide-binding domain; PDE, cyclic nucleotide phosphodiesterase; PKA, cyclic-AMP-dependent protein kinase; PKA-I, cyclic-AMP-dependent protein kinase inhibitor peptide; PKA-R, regulatory subunit of cyclic-AMP-dependent protein kinase; PKG, cyclic-GMP-dependent protein kinase; TNP-ATP, 2'-o-(trinitrophenyl)-adenosine 5'-triphosphate.

<sup>1</sup> To whom correspondence should be addressed (email [dormer@cardiff.ac.uk](mailto:dormer@cardiff.ac.uk)).

The cystic fibrosis transmembrane conductance regulator (CFTR) 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 nucleotide binding- and R- domains of CFTR (NBD1-R) was synthesised to determine directly whether CFTR binds cyclic AMP. 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 [<sup>3</sup>H]-ATP by non-radioactive ATP at 3.5 μM and 3.1 mM was demonstrated. [<sup>3</sup>H]-cyclic AMP bound to the protein with different affinities than for ATP (half-maximal displacement by cyclic AMP at 2.6 μM and 167 μM). Introduction of a mutation (T421A) in a motif predicted to be important for cyclic nucleotide binding decreased the higher affinity binding of cyclic AMP to 9.2 μM. The anti-CFTR antibody (MPNB) that inhibits CFTR-mediated protein secretion also inhibited cyclic AMP binding. Thus, binding of cyclic AMP to CFTR is consistent with a role in activation of protein secretion, a process defective in cystic fibrosis gland cells. Furthermore, the binding site may be important in the mechanism by which drugs activate mutant CFTR and correct defective ΔF508-CFTR trafficking.

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

## INTRODUCTION

Cystic fibrosis (CF), one of the most common single gene disorders of Caucasian populations, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. CFTR is an integral membrane protein, normally located in the apical membrane of polarised epithelial cells [2]. Although originally characterised as a cyclic AMP-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 cyclic AMP-mediated protein secretion from native exocrine gland cells from CF individuals [5,8-10], inhibition of  $\beta$ -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 cyclic AMP dependent exocytosis [13] and protein secretion [14,15].

Activation of the Cl channel function of CFTR requires phosphorylation by PKA followed by ATP binding and hydrolysis [3,16]. CFTR Cl channel activity in excised membrane patches, activated by cyclic AMP in the presence of ATP, was inhibited by PKA inhibitor peptide as a result of functional coupling of CFTR to PKA-II via a PKA anchoring protein [17]. However, in intact cells expressing CFTR, stimulation of Cl channel activity by injection of cyclic AMP was only inhibited by 60-70% by PKA inhibitors [18], suggesting direct activation by cyclic AMP. Since CFTR-mediated protein secretion from exocrine acinar cells such as rat submandibular acini is also stimulated by cyclic AMP elevating agonists [11,19], the question arises whether this process is also activated in part by cyclic AMP binding. There is evidence for a PKA-independent mechanism of regulation of exocytotic protein secretion in some cell types that involves cyclic AMP binding to the exchange factor activated by cyclic AMP known as Epac2 or cAMP-GEFII [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 and colleagues suggested the presence of a cyclic nucleotide-binding site in the region between amino acid residues 394-426 [N-terminal to NBD1) of CFTR [18]. However, the presence of cyclic nucleotide binding domain 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 nucleotide binding-domain, NBD1 and R- domain (amino acids residues 357-856 of CFTR), as well as the putative cyclic nucleotide-binding domain. We demonstrate directly that cyclic nucleotides bind to the NBD1-R domain of CFTR.

## **EXPERIMENTAL**

### **Materials and Antibodies**

pGEM-T vector, Western Blue stabilised substrate for alkaline phosphatase were from Promega and pRSETB vector, SOB medium, IPTG, antiXpress antibody from Invitrogen. PRISM BigDye Terminator cycle sequencing ready reaction kit was from Applied Biosystems and Micro BCA assay, Gelcode® Blue Stain reagent from Pierce. Ni-NTA agarose was from Qiagen and TNP-ATP 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 to KLH, affinity purified using the appropriate peptide linked to Sepharose and have been previously characterised [11,12,23]. The MATG 1104 antibody against the R-domain (amino acid residues 722-734) of CFTR was supplied by Transgene. Oligonucleotides primers were synthesised by Eurogentec Ltd.

### **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 *Bgl*II and ligated into the *Bgl*II 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' *E. 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. 50ml SOB medium containing carbenicillin (50µg/ml) and chloramphenicol (34µg/ml) was inoculated with transformed cells and grown overnight. The overnight culture (OD<sub>600</sub> ~0.7) was diluted 10-fold into 500ml SOB and the cells grown until the OD<sub>600</sub> was 0.4-0.6 (~1.5h). Recombinant protein synthesis was induced by addition of 1mM IPTG for 4h. Cells were harvested by centrifugation at 7000g for 20min at 4°C and stored frozen at -20°C. Thawed cells were lysed in Tris/EDTA buffer (10mM Tris-HCl, pH8.0, 1mM EDTA) containing 2mg/ml lysozyme at 4°C for 30min. After addition of MgCl<sub>2</sub> (10mM) and DNase1 (67mU/ml), further incubation at 4°C for 30 min was followed by sonication. Inclusion bodies were harvested by centrifugation at 7000g for 20 min at 4°C, washed in Tris/EDTA buffer containing 1% (v/v) Triton X-100 for 10min at 4°C and centrifuged at 10,000g for 20min at 4°C. The pellet of inclusion bodies was solubilised in 0.1M NaH<sub>2</sub>PO<sub>4</sub> pH 8.0 containing 8M urea, 10mM β-mercaptoethanol. Insoluble material was removed by centrifugation at 3000g for 3min.

Solubilised inclusion bodies were added to Ni<sup>2+</sup>-nitrilotriacetic acid (NiNTA)-agarose, pre-equilibrated in 0.1M NaH<sub>2</sub>PO<sub>4</sub> pH 8.0 containing 8M urea (buffer A) and incubated at 4°C for 1h on a rotary shaker. The mixture was transferred to a column and washed with 10-15 volumes of buffer A containing 10mM β-mercaptoethanol, followed by 5 volumes of buffer A containing 10mM β-mercaptoethanol at pH 6.3 (until the eluant OD<sub>280</sub> was <0.001). Bound protein was eluted in fractions with 3

volumes of buffer A at pH 4.5. Fractions were solubilised in 0.125M Tris-HCl, pH 6.8, containing 5% (w/v) SDS, 25% (w/v) sucrose, 0.5% (w/v) dithiothreitol) for 10min at 50°C and separated by SDS-polyacrylamide gel electrophoresis using 10% acrylamide gels as previously described [12]. Following electrophoresis the gel was washed three times for 5min in deionised water, Gelcode stain added for 30-60min and the gel washed three times for 10-20 min in deionised water. Eluted fractions containing purified protein were pooled, separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose in 25mM Tris, pH 8.3, containing 192mM glycine, 20% (v/v) methanol for 1.5 h at 90V. Unbound protein binding sites were blocked by incubation in 5% (w/v) skimmed milk powder in 20 mM Tris-HCl, pH 7.4, containing 0.5M NaCl, 0.05% (v/v) Tween-20 (buffer B) for 1h at room temperature. Blots were then incubated overnight at 4°C with the primary antibody (see Results) 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 30min at room temperature, washed and protein bands visualised by incubation for up to 1h in Western Blue stabilised 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 (0.1mg/ml) was dialysed at 4°C for periods of 2h in 100 volumes of buffers containing 3M urea, 1M urea and no urea respectively or for periods of 3h in buffers containing 6M urea, 4M urea, 2M urea and no urea respectively. In some experiments the final dialysis was extended to 18h. Buffers tested were: 50mM Na-phosphate, pH 7.5, containing 1mM dithiothreitol, 1mM EDTA; 0.1M NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0 containing 50mM glycine, 1mM EDTA, 0.005% (v/v) Tween-20 (buffer C) and 5mM sodium acetate, pH 5.0. For rapid dilution, NBD1-R domain protein solutions (2mg/ml) were adjusted to pH 5.0 or 8.0 and diluted 10-fold into 5mM sodium acetate, pH 5.0 or buffer C respectively. Samples were incubated for 18h at 4°C and insoluble material

removed by centrifugation at 100,000g for 80min at 4°C. Under all dialysis conditions tested, the yield of soluble protein was 10-15%. Rapid dilution into 5mM sodium acetate, pH 5.0 also yielded approximately 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 200-250µg/ml using YM50 centrifugal filters, subjected to SDS polyacrylamide gel electrophoresis 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 approximately 100-fold.

### **In vitro Phosphorylation**

Refolded NBD1-R domain protein was diluted to ~3µg/ml in 100mM Tris-HCl, pH 7.4 containing 20mM MgCl<sub>2</sub>, 4mM EDTA, 200µg/ml of BSA and phosphorylated with PKA (catalytic subunit, 100units/ml), [ $\gamma$ -<sup>32</sup>P]-ATP (0.5µCi/15µl reaction volume) for 1 h at 30°C. Samples were mixed with SDS solubilising buffer, heated at 50°C for 10min and analysed by SDS-polyacrylamide gel electrophoresis 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 excitation and emission wavelengths of 410nm and 540nm (10nm slit widths) respectively (LS-5B fluorimeter, Perkin-Elmer). Preliminary experiments showed that for sufficient sensitivity to make TNP-ATP binding measurements, an NBD1-R protein concentration of 200µg/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 (200µg/ml in buffer C) was adjusted to pH 7.5 and 4mM MgCl<sub>2</sub> added (3mM final). Protein was incubated with TNP-ATP (0-10µM) by the sequential addition of aliquots of 100µM TNP-ATP (in buffer C, pH7.5 containing 4mM MgCl<sub>2</sub>). 0.8M urea 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 [<sup>3</sup>H]-cyclic AMP was investigated using filtration to separate bound from free cyclic AMP. NBD1-R domain protein, diluted in 50mM Tris-HCl, pH7.5 containing 50mM NaCl buffer (1-10μg/ml, equivalent to 0.015-0.15μM assuming a molecular weight of 65kDa) was mixed with 0.1μM [<sup>3</sup>H]-cyclic AMP (14μCi) and incubated for periods of up to 1h at 4°C or 37°C. Aliquots were then filtered on polycarbonate filters (0.45μm), washed with 10 volumes of Tris/NaCl buffer, mixed with liquid scintillant cocktail and left for 18h in the dark before assay of radioactivity. At either temperature, [<sup>3</sup>H]-cyclic AMP binding was saturated within 15min and approximately 50% displacement by excess non-radioactive cyclic AMP (1mM) was observed under all conditions (data not shown). The amount of bound [<sup>3</sup>H]-cyclic AMP was also low (1500-2000cpm) 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 50mM Tris-HCl, pH7.5 containing 50mM NaCl, 10mM MgCl<sub>2</sub> (1-6.5μg/ml, equivalent to 15-100nM, assuming a molecular weight of 65kDa) was mixed with 0.1μM [<sup>3</sup>H]-ATP or 0.1μM [<sup>3</sup>H]-cyclic AMP (both 14μCi) and incubated for periods of up to 1h 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 reduced by 10-fold.

Aliquots of buffer were removed from the upper and lower (flow-through) chambers. All samples were mixed with liquid scintillant cocktail, left for 18h in the dark and radioactivity assayed.

## RESULTS

In order to determine directly whether CFTR binds cyclic AMP 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 nucleotide-binding domain.

### Characterisation of soluble NBD1-R domain fusion protein

Affinity-purified protein was refolded by rapid dilution, concentrated to 200-250 $\mu$ g/ml using YM50 centrifugal filters, subjected to SDS polyacrylamide gel electrophoresis and stained with Coomassie Blue. As shown in Fig.1A, the fraction showed a single predominant band of approximately 65kDa. The protein was also characterised 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 Fig.1B, the anti-C-terminus antibody did not detect any protein whereas the other three antibodies detected a band of approximately 65kDa.

### 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 nucleotide binding domains of CFTR [25-28]. As shown in Fig.2A, addition of TNP-ATP to the refolded protein resulted in enhancement in fluorescence at 540nm, demonstrating ATP binding. The fluorescence enhancement curves fitted hyperbolic curves for 1-site ligand binding, yielding a value for half-maximal ATP binding at pH7.5 of  $2.7\pm 0.7\mu$ M (n=5). Subsequent addition of unlabelled ATP reversed the

fluorescent enhancement by approximately 70% at 25mM ATP (data 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, all used different assay conditions (pH 7.4/7.5, no magnesium [26-28]; pH 5, 2mM MgSO<sub>4</sub>, 0.4M urea [25]). Nevertheless, apparent half-maximum binding values published (0.2μM [26], 0.8μM [25], 1.8μM [27], 3.1μM [28]) were in the same range as those obtained in the present study. As shown in Fig.2B, 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 data show that mg 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 (30nM) was incubated with 0.1μM [<sup>3</sup>H]-ATP, in the presence or absence of 1mM non-radioactive ATP for 15min at 4°C before concentration of the protein by centrifugation in the upper chamber of a Microcon centrifugal filter. As shown in Fig.3A, the concentration of [<sup>3</sup>H]-ATP radioactivity increased in the upper chamber, when the protein was concentrated, thus demonstrating binding. There was a concomitant decrease in concentration of [<sup>3</sup>H]-ATP radioactivity in the lower chamber. Displacement of [<sup>3</sup>H]-ATP binding to the NBD1-R domain protein by unlabelled ATP (1mM) was also demonstrated (Fig.3A). There was no difference in the degree of binding after incubation for 30min or 60 min (data not shown), indicating that 15min was sufficient time to attain equilibrium. In order to compare the extent of nucleotide binding between experiments, the data was 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  $3.59 \pm 0.11$  ( $n=4$ ) and in the lower chamber  $0.37 \pm 0.02$  ( $n=4$ ) for binding in the presence of  $0.1\mu\text{M}$  ATP. When ATP binding to the NBD1-R domain protein was measured in the presence of increasing concentrations of unlabelled ATP, displacement of [ $^3\text{H}$ ]-ATP from the protein was maximal at  $10\text{mM}$  ATP (Fig.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 [ $^3\text{H}$ ]-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 [ $^3\text{H}$ ]-ATP counts would give a ratio of 1.0, the calculated residual of 1.45 gives non-specific binding of 17%.

### **Demonstration of cyclic AMP binding to NBD1-R domain protein**

We next used the equilibrium-binding assay to investigate whether CFTR also binds cyclic AMP. Thus, NBD1-R domain protein ( $15\text{nM}$ ) was incubated with  $0.1\mu\text{M}$  [ $^3\text{H}$ ]-cyclic AMP, in the presence or absence of  $1\text{mM}$  non-radioactive cyclic AMP for 15min at  $4^\circ\text{C}$  before concentration of the protein in a Microcon centrifugal filter. As shown in Fig.4A, the concentration of [ $^3\text{H}$ ]-cyclic AMP radioactivity increased in the upper chamber, when the protein was concentrated, demonstrating binding as shown for ATP (Fig.3). There was also a concomitant decrease in concentration of [ $^3\text{H}$ ]-cyclic AMP radioactivity in the lower chamber. A protein such as BSA that does not bind cyclic AMP [20], showed no concentration of radioactivity in the upper chamber (Fig.4A). Displacement of [ $^3\text{H}$ ]-cyclic AMP binding to the NBD1-R domain protein by unlabelled cyclic AMP ( $1\text{mM}$ ) was also demonstrated (Fig.4A). As shown in Fig.4B, 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  $0.015\mu\text{M}$  to 3.83 at  $0.1\mu\text{M}$ . PKA at  $0.1\mu\text{M}$  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 cyclic AMP**

When [ $^3\text{H}$ ]-cyclic AMP binding to NBD1-R domain proteins was measured in the presence of increasing concentrations of unlabelled cyclic AMP, displacement of [ $^3\text{H}$ ]-cyclic AMP showed maximum displacement at 1mM cyclic AMP (Fig.5A), an order of magnitude lower than for ATP. However, as for ATP binding, cyclic AMP displacement followed a biexponential curve, yielding values for half-maximal displacement of [ $^3\text{H}$ ]-cyclic AMP of 2.6 $\mu\text{M}$  and 167 $\mu\text{M}$ .

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

### **Displacement of cyclic AMP binding by analogues**

[ $^3\text{H}$ ]-cyclic AMP binding to NBD1-R domain protein was displaced by dibutyryl- and 8-cpt- analogues of cyclic AMP, to the same extent as by cyclic AMP at a concentration of 100 $\mu\text{M}$  (Table 1). By contrast, cyclic GMP and, in particular 8-cpt-cyclic GMP, were less effective than cyclic AMP or its analogues in competing with [ $^3\text{H}$ ]-cyclic AMP binding (Table 1). Whereas 100 $\mu\text{M}$  cyclic AMP was 80-90% as effective as 1mM cyclic AMP at displacement of [ $^3\text{H}$ ]-cyclic AMP, 100 $\mu\text{M}$  cyclic GMP was only 60-65% as effective and 8-cpt-cyclic GMP (100 $\mu\text{M}$ ) did not significantly displace [ $^3\text{H}$ ]-cyclic AMP. (Table 1). We also tested the effect of unlabelled ATP (up to 10mM) on [ $^3\text{H}$ ]-cyclic AMP binding. As shown in Fig.5C, displacement of [ $^3\text{H}$ ]-cyclic AMP was maximal at 1mM ATP, the range of the physiological concentration of ATP and followed a biexponential curve, showing half-maximal displacement at 10.4 $\mu\text{M}$  and 198 $\mu\text{M}$ . Compared to cyclic AMP, ATP was less effective at displacing [ $^3\text{H}$ ]-cyclic AMP. Thus, 1mM ATP competed out 55.2  $\pm$  1.5% (n=5) of the [ $^3\text{H}$ ]-cyclic AMP counts whereas cyclic AMP competed out 80.6

$\pm 3.1\%$  ( $n=7$ ;  $p<0.001$  for difference from ATP). Further demonstration that cyclic AMP competed effectively with ATP for binding is shown in Fig.5D. Thus, in the presence of 1mM ATP, addition of 1-10 $\mu$ M cyclic AMP increased [ $^3$ H]-cyclic AMP binding to the same level as for 0.1 $\mu$ M [ $^3$ H]-cyclic AMP alone. Addition of higher concentrations (0.1-10mM) of unlabelled cyclic AMP displaced [ $^3$ H]-cyclic AMP counts with a single exponential decay giving a half-maximal concentration of approximately 80 $\mu$ M.

### **Effect of CFTR antibody on nucleotide binding**

Since isoproterenol stimulation of mucin secretion from rat submandibular acinar cells is inhibited by the MP-NB1 anti-CFTR antibody [11,12,19], we next investigated if the antibody affected nucleotide binding to the NBD1-R domain protein. As shown in Fig.6, the MPNB antibody inhibited both cyclic AMP and ATP binding in a concentration-dependent manner. Inhibition of both nucleotides was maximally inhibited by MPNB antibody at 100 $\mu$ g/ml giving displacement of radiotracer that was of the same magnitude as 1mM 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 $\mu$ g/ml there was inhibition of cyclic AMP binding that was of the same magnitude as 1 $\mu$ g/ml of MPNB antibody. In order to compare the effect of MPNB antibody on ATP and cyclic AMP binding, the data was expressed as percentage displacement of radiotracer. As shown in Fig.6C, the effect of antibody was greater on cyclic AMP binding except at the maximally effective concentration (100 $\mu$ 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 with 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 (Figs.2 & 3). Similar constructs have been synthesized by others [25,26] and we found, as did Neville et al [25], that the most successful refolding procedure in terms of recovery of soluble protein and ability to bind ATP was rapid dilution, as described in the Methods. The value for affinity for TNP-ATP (2.7 $\mu$ 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 mM range. Higher values for ATP binding (10-100 $\mu$ 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<sup>-6</sup>M to 10<sup>-4</sup>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  $\mu$ M range consistent with the value obtained using TNP-ATP and one in the mM range. Using the functional NBD1-R domain protein, we have now directly demonstrated cyclic AMP binding. As for ATP, two different binding affinities were observed, with half maximum concentrations in the 10<sup>-6</sup>M and 10<sup>-4</sup>M range (Fig.5A). Sullivan et al [18] reported half-maximal activation of CFTR by cyclic AMP in *Xenopus* oocytes of 4 $\mu$ M for cyclic AMP, similar to the higher affinity binding (2.6 $\mu$ M) shown here.

### **Relationship to other cyclic nucleotide binding proteins**

The binding of cyclic AMP 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 cyclic AMP binding region of

PKA-R and CAP is the phosphate-binding cassette, which contains the FGEL(I) and PRAAT motifs that are essential for cyclic AMP binding [36]. The FGEL(I) motif is present in CFTR (405-408) but the RA residues of the PRAAT motif is present as KT (420-421) in CFTR. An R to K mutation in the PRAAT motif caused an approximately 10-fold decrease in affinity for cyclic AMP in PKA-R [37], consistent with the relatively low affinity ( $\mu\text{M}$ ) of CFTR for cyclic AMP. The presence of KT (420-421) in CFTR resembles more the cyclic GMP binding motif of the rod photoreceptor cyclic GMP-gated channel [38] and PKG [39]. This might explain why cyclic GMP is relatively efficient at displacing cyclic AMP binding (Table 1). We synthesised a T421A mutant form of the NBD1-R domain protein and showed an decreased affinity for cyclic AMP binding (Fig.5B), consistent with the demonstration that the full-length T421A CFTR showed reduced chloride channel activity in whole cells [18]. The data also serve to strengthen the hypothesis that the 392-428 region of CFTR is the site of cyclic AMP 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 CFTR [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 W401 that are highly conserved in ABC transporter NBD's, are present within the putative cyclic nucleotide binding domain, our data suggest that cyclic AMP can bind effectively to CFTR in the presence of mM ATP (Fig.5D). It is possible that the relative action of cyclic AMP and ATP binding may determine which function of CFTR predominates.

### **Role of cyclic nucleotide binding in normal and mutant CFTR function**

The present data demonstrating cyclic AMP binding to CFTR strengthens 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 cyclic AMP known as

Epac2 or cAMP-GEFII that have 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 cyclic AMP to CFTR. The evidence presented in support of this hypothesis is firstly, the direct demonstration that CFTR binds cyclic AMP (Figs. 4 and 5) and secondly, the MPNB antibody that inhibits CFTR-mediated protein secretion in rat submandibular acini also inhibits cyclic AMP binding (Fig.6). In addition, we previously showed that inhibition of isoproterenol stimulated mucin secretion by MPNB antibody is partially corrected by phosphodiesterase inhibitors or 8-cpt-cyclic AMP [11,19] and suggested that this might be due to production of supra-physiological levels of cyclic nucleotides. The data in the present study supports this hypothesis since, when present at high concentration within cells, cyclic AMP or its analogues would compete with the antibody and provide a mechanism for reversal of antibody inhibition. Since cyclic GMP also competes at the cyclic AMP binding site (Table 1), this might also explain why PDE5 inhibitors that raise cyclic GMP in rat submandibular acini [19], also partially correct the antibody inhibition. It is interesting that the binding of cyclic AMP 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 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 cyclic AMP 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 data 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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## FIGURE LEGENDS

### 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 Methods. **(A)** Affinity-purified protein was refolded by rapid dilution as described in the Methods and concentrated to 200-250µg/ml using YM50 centrifugal filters. 200ng of protein was subjected to SDS polyacrylamide gel electrophoresis and stained with Coomassie Blue. Lanes 1: molecular weight markers (numbers denote molecular weights in kDa); lane 2: NBD1-R domain protein. **(B)** 300ng of protein was separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose and probed with the following antibodies, as described in the Methods: lane 1: MPNB1 (against NBD1); lane 2: MATG 1104 (against the R-domain); lanes 3: anti-Xpress; lane 4: MPCT1 (against the C-terminus). Numbers denote molecular weights in kDa. Arrowheads denote position of major band at 65kDa.

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

**(A)** Refolded NBD1-R domain protein (0.2mg/ml) was incubated with TNP-ATP (0-10µM) as described in the Methods. 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 5 experiments. **(B)** Refolded NBD1-R domain protein (3µg/ml) was phosphorylated with PKA and [ $\gamma$ -<sup>32</sup>P]-ATP as described in the Methods. Numbers denote molecular weights in kDa.

### Figure 3 Demonstration of [<sup>3</sup>H]-ATP binding to NBD1-R domain protein by an equilibrium binding method

**(A)** NBD1-R domain protein (30nM) in Tris/NaCl/Mg buffer was incubated with [<sup>3</sup>H]-ATP for 15min at 4°C in the presence or absence of unlabelled ATP (10mM). Samples were applied to the top chamber of a Microcon (YM 30) centrifugal filter and concentrated by centrifugation as described in the Methods. Radioactivity was assayed in 20µl aliquots removed before centrifugation (open bars) and from the upper (filled bars) and lower (hatched bars) chambers after centrifugation. Data are mean ± SEM 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 0.1µM ATP. All differences assessed by Student's paired t-test. **(B)** Concentration dependence of ATP binding. Data are expressed as the ratio of radioactive counts in the upper chamber after centrifugation to counts in the starting mixture and are mean ± SEM for n=4.

### Figure 4 Demonstration of [<sup>3</sup>H]-cyclic AMP binding to NBD1-R domain protein by an equilibrium binding method.

**(A)** NBD1-R domain protein or BSA (15nM) in Tris/NaCl buffer was incubated with [<sup>3</sup>H]-cyclic AMP for 15min at 4°C in the presence or absence of unlabelled cyclic AMP (1mM). Samples were applied to the top chamber of a Microcon (YM 30) centrifugal filter and concentrated by centrifugation as described in the Methods. Radioactivity was assayed in 20µl aliquots removed before centrifugation (open bars) and from the upper (filled bars) and lower (hatched bars) chambers after centrifugation. Data are mean ± SEM for n=9 (NBD1-R domain); 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  $\mu$ M cyclic AMP. All differences assessed by Student's paired t-test. **(B)** NBD1-R domain protein (0.015 to 0.1  $\mu$ M), PKA or BSA (both 0.1  $\mu$ M) were assayed for [ $^3$ H]-cyclic AMP binding as in A. Data is expressed as the ratio of radioactive counts per 20  $\mu$ l in the upper (filled bars) or lower (hatched bars) chambers to that in the starting mixture before centrifugation to concentrate the protein (open bars). Data are mean  $\pm$  SEM 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 assessed by Student's paired t-test.

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

NBD1-R domain protein (30nM) in Tris/NaCl buffer was incubated with [ $^3$ H]-cyclic AMP for 15min at 4°C in the presence or absence of unlabelled cyclic AMP **(A, B)**, ATP **(C)** or both **(D)**. Samples were applied to the top chamber of a Microcon (YM 30) centrifugal filter and concentrated by centrifugation as described in the Methods. Radioactivity was assayed in 20  $\mu$ l aliquots removed before centrifugation and from the upper and lower chambers after centrifugation. In **A,C,D**, data are expressed as the ratio of radioactive counts in the upper chamber after centrifugation to counts in the starting mixture. In **B**, data are expressed as % displacement, taking binding at 0.1  $\mu$ M [ $^3$ H]-cyclic AMP as zero; a ratio of 1.0 would be 100% displacement. All data are mean  $\pm$  SEM. **(A)** n=7 for displacement by cyclic AMP; **(B)** Displacement by cyclic AMP 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 cyclic AMP in the presence of 1mM ATP (closed circles) compared to binding of 0.1  $\mu$ M [ $^3$ H]-cyclic AMP alone (star). Mean of 2 experiments.

### FIGURE 6: Effect of anti-CFTR antibodies on nucleotide binding to NBD1-R domain protein

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

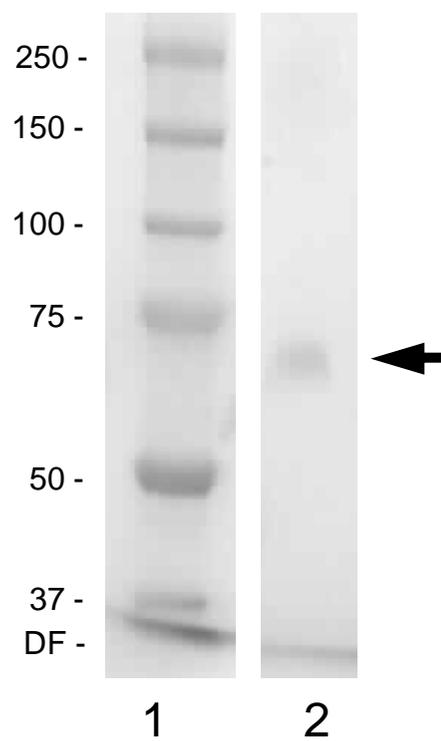
**Table 1. [<sup>3</sup>H]-cyclic AMP displacement by cyclic AMP and cyclic GMP analogues.**

NBD1-R domain protein (30nM) in Tris/NaCl buffer was incubated with [<sup>3</sup>H]-cyclic AMP for 15min at 4°C in the presence or absence of unlabelled cyclic AMP or cyclic GMP analogues as indicated. Samples were applied to the top chamber of a Microcon (YM 30) centrifugal filter and concentrated by centrifugation as described in the Methods. Radioactivity was assayed in 20µl aliquots removed before centrifugation and from the upper and lower chambers after centrifugation. Data 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 1mM unlabelled cyclic AMP (% 1mM). Data are mean ± SEM for the number of experiments shown in parentheses. \*, p<0.01, \*\*, p<0.001 for difference from ratio obtained in the absence of unlabelled cyclic AMP (0.1µM). Ψ, p<0.001 for difference from ratio obtained in the presence of 1mM unlabelled cyclic AMP. #, p<0.05, ##, p<0.001 for difference from percentage of 1mM obtained by 100µM unlabelled cyclic AMP. All differences assessed by Student's unpaired t-test.

Analogue	Concentration	Ratio	% 1mM
Cyclic AMP	0.1µM	3.19 ± 0.27 (14)	
	1mM	1.32 ± 0.06** (14)	
	100µM	1.54 ± 0.16* (4)	88.3 ± 2.6 (4)
Dibutyryl-cyclic AMP	100µM	1.34 ± 0.07* (3)	98.3 ± 7.1 (3)
8-cpt- cyclic AMP	100µM	1.47 ± 0.09* (3)	93.7 ± 9.9 (3)
Cyclic GMP	100µM	1.73 ± 0.07*·Ψ (5)	65.6 ± 8.0# (5)
8-cpt- cyclic GMP	100µM	3.37 ± 0.10 <sup>Ψ</sup> (5)	22.9 ± 3.5## (5)

Figure 1

**A**



**B**

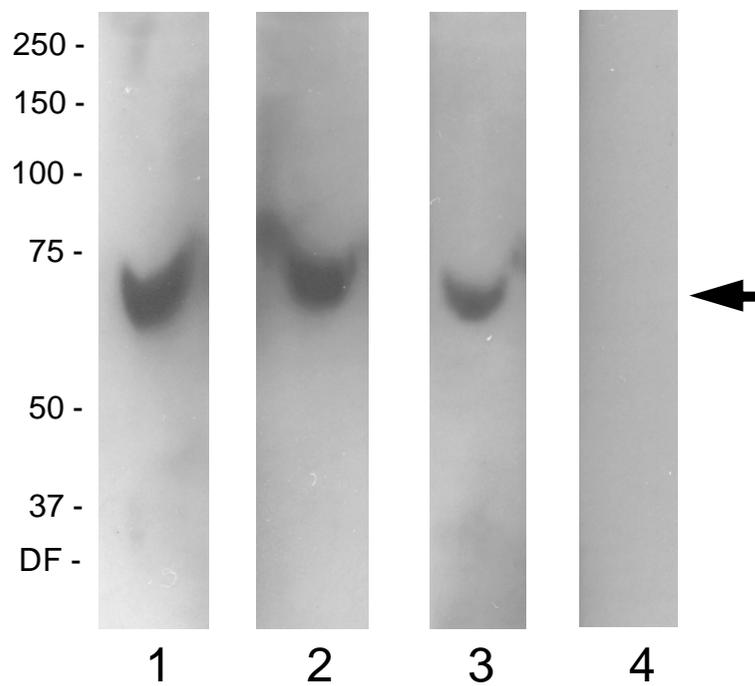


Figure 2

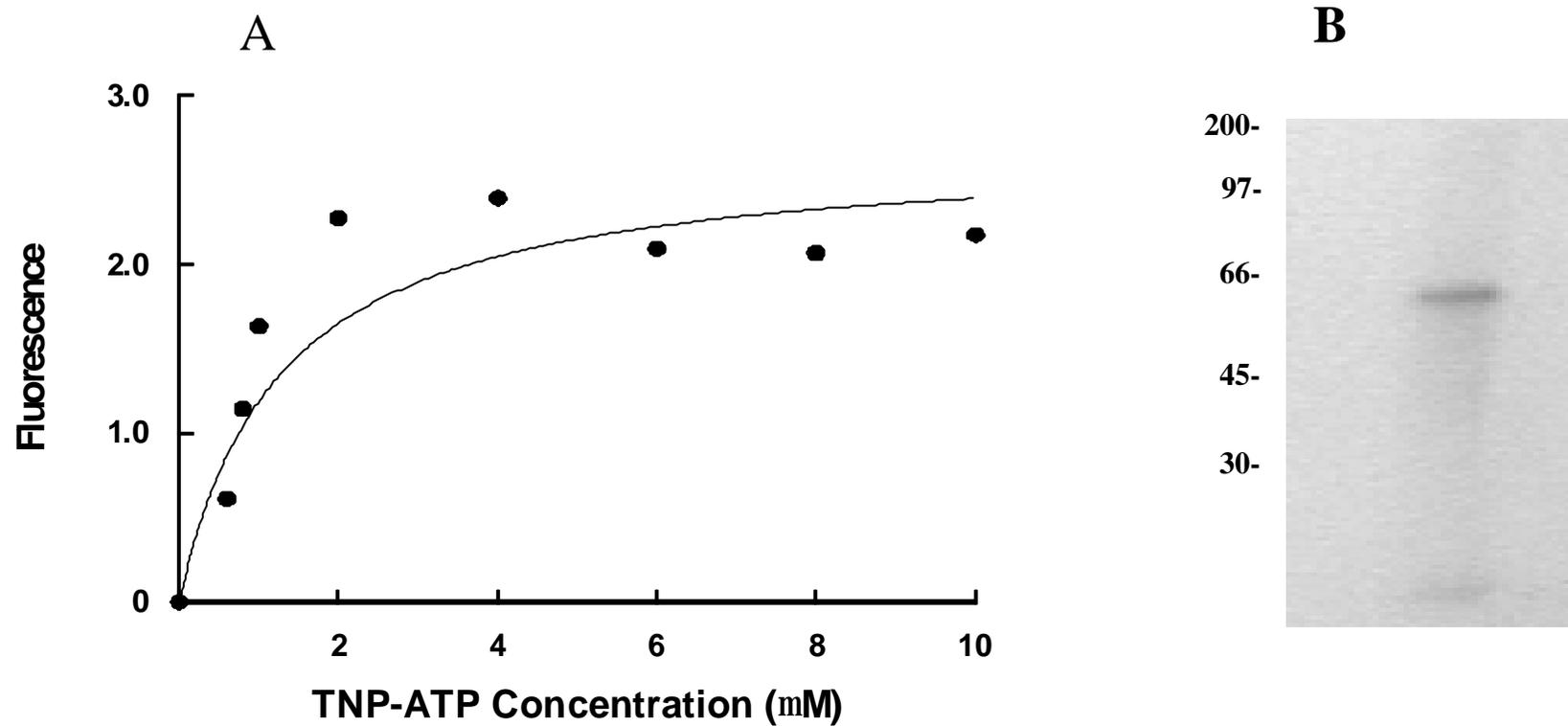


Figure 3

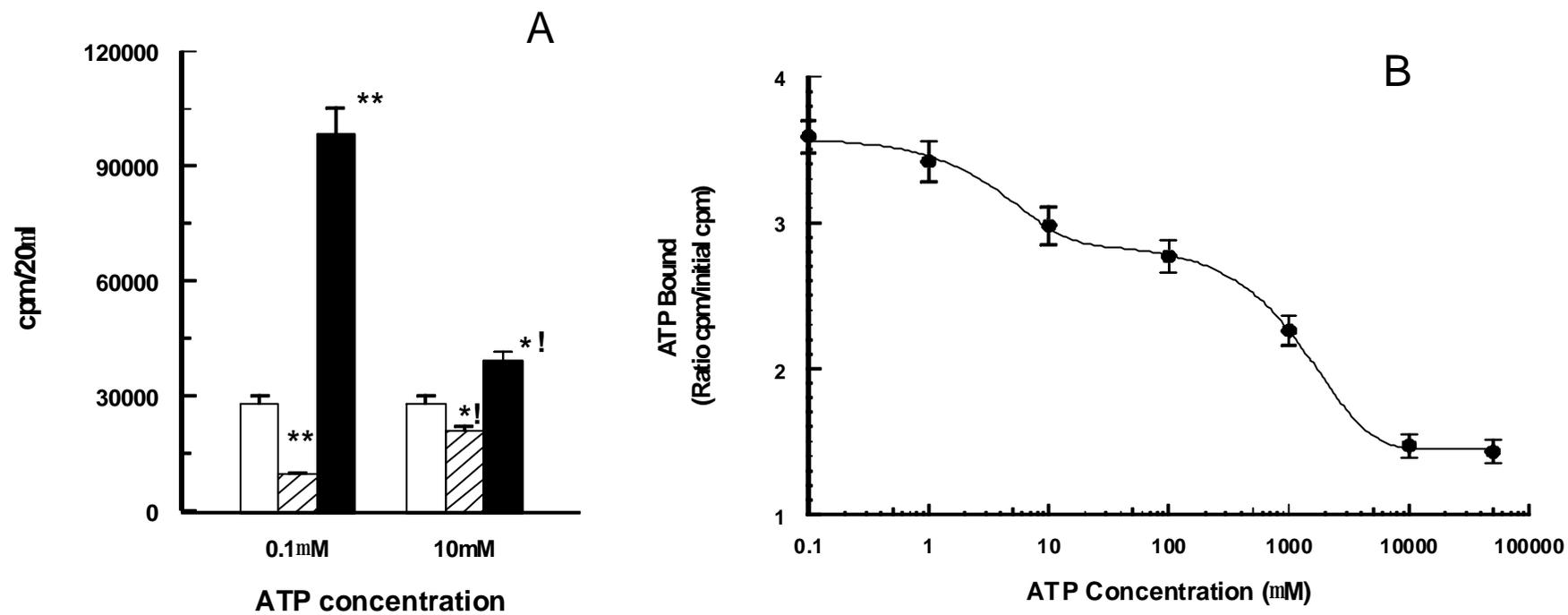


Figure 4

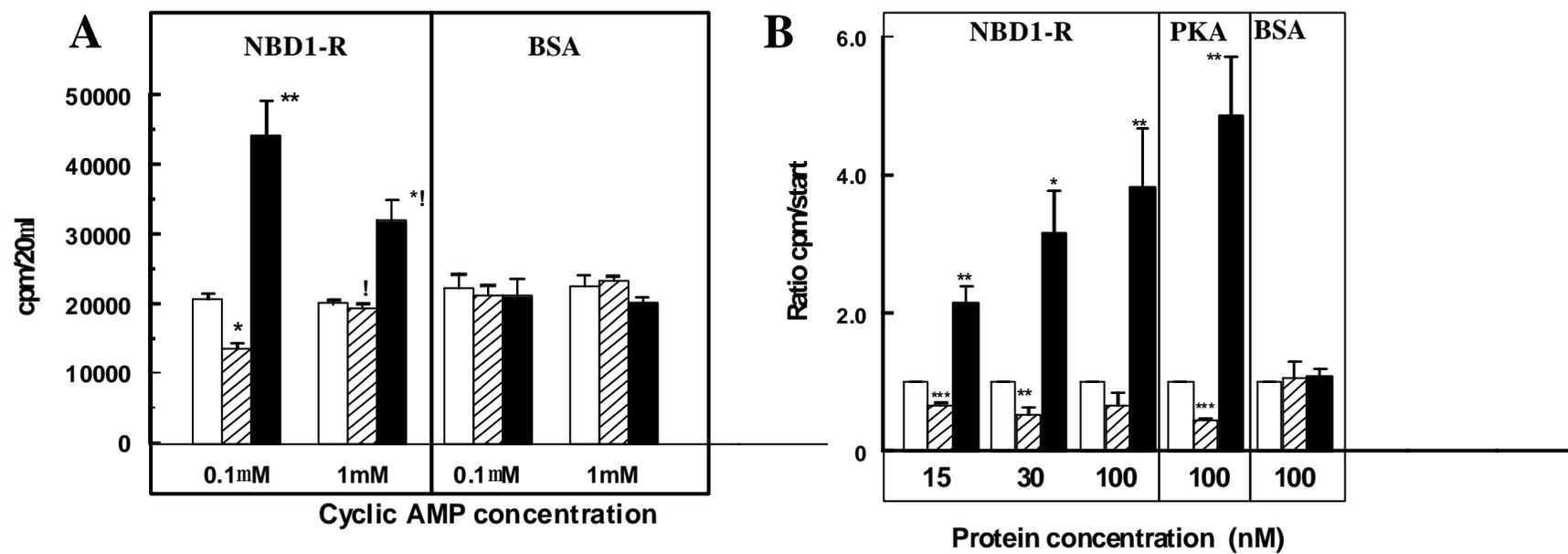


Figure 5

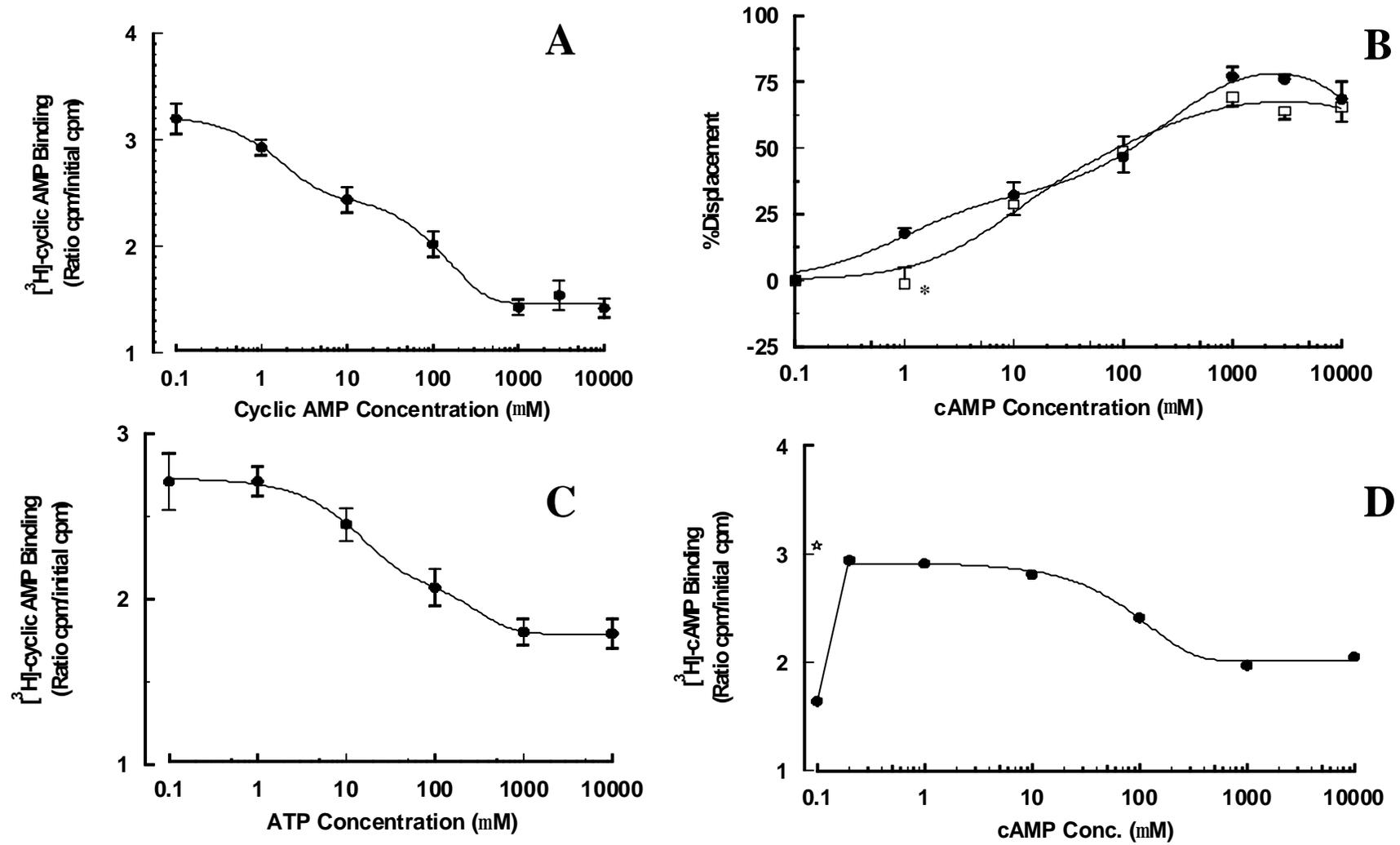


Figure 6

