The cystic fibrosis transmembrane conductance regulator (CFTR) is an anion channel in the ATP-binding cassette (ABC) transporter family. CFTR consists of two transmembrane domains, two nucleotide-binding domains (NBD1 and NBD2), and a regulatory domain. Previous biochemical reports suggest NBD1 is a site of stable nucleotide interaction with low ATPase activity, whereas NBD2 is the site of active ATP hydrolysis. It has also been reported that NBD2 additionally possessed adenylate kinase (AK) activity. Knowledge about the intrinsic biochemical activities of the NBDs is essential to understanding the CI− ion gating mechanism. We find that purified mouse NBD1, human NBD1, and human NBD2 function as adenylate kinases but not as ATPases. AK activity is strictly dependent on the addition of the adenosine monophosphate (AMP) substrate. No liberation of [32P]phosphate is observed from the γ32P-labeled ATP substrate in the presence or absence of AMP. AK activity is intrinsic to both human NBDs, as the Walker A box lysine mutations abolish this activity. At low protein concentration, the NBDs display an initial slower nonlinear phase in AK activity, suggesting that the activity results from homodimerization. Interestingly, the G551D gating mutation has an exaggerated nonlinear phase compared with the wild type and may indicate this mutation affects the ability of NBD1 to dimerize. hNBD1 and hNBD2 mixing experiments resulted in an 8–57-fold synergistic enhancement in AK activity suggesting heterodimer formation, which supports a common theme in ABC transporter models. A CFTR gating mechanism model based on adenylate kinase activity is proposed.

Cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP-binding cassette (ABC) transporter that functions as a chloride channel. The ABC transporter superfamily has a large number of functionally diverse transmembrane proteins that transport a variety of substrates (from ions to proteins) within, into, and out of the cell (1). The typical ABC transporter consists of two transmembrane domains and two cytoplasmic nucleotide-binding domains (NBDs). The NBDs are thought to utilize energy from the hydrolysis of ATP to transport substrates across the membrane. Some ABC transporters function actively against a concentration gradient, and others are passive transporters (e.g. CFTR). In the human genome sequencing efforts, 51 ABC transporter genes have been identified, 13 of which have been linked to genetic diseases (2). The most common and intensely studied of these diseases is cystic fibrosis.

Primary amino acid sequence analysis suggests CFTR is composed of homologous halves. Each half contains six membrane-spanning segments and an NBD. The two CFTR halves are linked by a cytoplasmic regulatory domain (R-domain) which is unique and not shared with other ABC transporters. The R-domain contains a number of protein kinase A (PKA) phosphorylation sites (3). PKA phosphorylation at multiple sites is a prerequisite for the activation of CFTR chloride channel by ATP (4). To date, over 1300 disease-causing mutations have been identified in the CFTR gene (www.genet.sickkids.on.ca/cftr/). These mutations are located throughout the coding sequence and include deletions and nonsense mutations. Most mutations are rare in the cystic fibrosis (CF) population. The exception is the deletion of a phenylalanine residue at position 508 (∆F508) that accounts for about 70% of CF mutations worldwide (5). The ∆F508 mutation is located in NBD1 and results in a CFTR protein that has a dual defect. First, the protein is improperly processed from the endoplasmic reticulum to the cell plasma membrane. Second, the mutant protein that reaches the membrane is reported to have only ~30% of wild-type channel function (5). In contrast, the second most common CF mutation G551D is only defective in channel function (6) and has a prevalence of 2–7% in the CF population (7).

Schemes of ATP binding and hydrolysis by CFTR have been proposed to govern channel gating responses (8). In CFTR, the NBDs are thought not to be functionally equivalent because of the lack of sequence conservation in three of the ABC transporter signature motifs (9). Similarly, biochemical reports suggest that NBD1 is a site of stable nucleotide interaction (10), whereas NBD2 has been reported to be the site of ATP hydrolysis activity (11). In contrast, other biochemical studies where the NBD1 construct includes the regulatory (R) domain (NBD1-R) suggest that the protein had the ability to function as an ATPase (12, 13). Most interesting were biochemical reports by Randak et al. (14, 15) that indicated the NBD2 possessed both adenylate kinase as well as ATPase activity. Adenylate kinases catalyze the phosphotransfer reaction (ATP + AMP ⇌ ADP + ADP). In experiments using patch-clamp analysis together with the Walker A lysine mutations in the NBDs, Randak and Welsh (16) concluded that NBD2, not NBD1, was responsible for adenylate kinase activity. The structural similarity of the CFTR NBDs to the adenylate kinases was reported (17) soon after the CFTR gene was cloned in 1989. However, this observation was never investigated experimentally until 1997 (14). The crystal structure of mouse NBD1 (mNBD1) was recently determined (18). Comparing the
mNBD1 and the adenylyl kinase structures, we saw resemblances that raised the possibility that both NBDs may have adenylyl kinase activity.

To gain insight into the CFTR functional mechanism (Cl- ion gating) we have pursued a detailed biochemical examination of highly purified soluble and refolded cytoplasmic domains (i.e. NBD1, NBD1-R, and NBD2). Knowledge about the individual domains and their biochemical activities is essential for the proper understanding of the CFTR gating mechanism. Insight into the CFTR gating mechanism would be extremely useful in designing functional assays to both find and understand how small molecules function to restore CFTR channel activity.

In this report, we provide evidence that the mouse NBD1, human NBD1, and human NBD2 all have the ability to function as an adenylyl kinase (AK). Careful separation of all nucleotide products by thin layer chromatography (TLC) reveals that, in contrast to extant reports in the CFTR literature, the isolated CFTR NBDs do not catalyze ATP hydrolysis because no liberation of [32P]phosphate is observed from γ-[32P]ATP. Each NBD at low protein concentration displayed an initial slower nonlinear phase in AK activity, which suggests that homodimerization may be important for activity. The G551D gating mutation has an exaggerated nonlinear phase as compared with wild type, and may indicate this mutation affects NBD1 ability to form dimers. In hNBD2 immunoprecipitation experiments, hNBD1 co-precipitates suggesting a physical association between the two NBDs. Mixing experiments with hNBD1 and hNBD2 at low protein concentration resulted in a significant synergistic enhancement in AK activity suggesting heterodimer formation. From our biochemical findings we therefore propose a CFTR gating mechanism based on AK activity.

EXPERIMENTAL PROCEDURES

Cloning of the Mouse and Human CFTR NBDs—The NBDs were amplified by PCR from mouse and human cDNA libraries using Pfu/Taq polymerases (Stratagene). Oligonucleotide primers complementary to the 5’- and 3’-ends of the open reading frames were designed to introduce unique restriction sites. The PCR products were digested with appropriate restriction endonuclease and ligated to an Escherichia coli expression vector pET28b (Novagen). The resulting human NBD plasmids, pET28b-hNBD1 and pET28b-hNBD2, produced both human NBDs with an N-terminal hexahistidine (His6) tag hNBD1 (Thr389-Leu671) and hNBD2 (Lys1200-Leu1480). The mouse NBD1 fragment (Thr389–Asp673) was fused to the C terminus of the His6-tagged Smt3 fusion protein as described (19) and was identical to the construct that yielded the NBD1 crystal structure (18). The mouse and human CFTR gene fragments were sequenced to confirm the integrity and were identical to the GenBankTM sequence, accession numbers (NM_021050) and (NM_000492), respectively. All mutant clones were sequenced to confirm the presence of the mutations and the absence of unwanted mutations.

Expression, Purification, and Refolding of the CFTR NBD Proteins—Mouse and human CFTR NBD clones were expressed in E. coli using BL21 DE3 pLysS cells induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside at 25 °C. Soluble mouse NBD1 was initially purified by nickel ion affinity chromatography, followed by a Sepharose S200 (16/60 mm column) sizing chromatography step; the Smt3 tag then was removed using the Ulp1 protease (19). Subsequently, a second nickel affinity step was employed to separate the tag from the cleaved mNBD1 protein.

The human NBD proteins were found in the insoluble fraction. The cell pellets (30 g) were resuspended in 600 ml of buffer 1 (25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Tween-20, and 10 mM 2-mercaptoethanol) followed by the addition of protease inhibitors (E-64, pepstatin, and leupeptin at 2 μg/ml, DFP at 50 μM). The cell pellets were dounce-homogenized and incubated for 1 h at 4 °C. Samples were spun at 54,000 × g for 1 h at 4 °C. Supernatants were removed, and the pellets were resuspended in 600 ml of buffer 2 (25 mM Tris-HCl, pH 7.5, 6 M guanidine hydrochloride, and 10 mM 2-mercaptoethanol) overnight on a magnetic stirrer at 4 °C. Samples were spun at 54,000 × g for 1 h at 4 °C. To the supernatants, 30 ml of Nickel Hi-Selects resin (Sigma) were added and incubated overnight at 4 °C. Samples were centrifuged to pellet resin and resuspended in buffer 2 and poured into a column. The packed column was washed with buffer 3 (25 mM Tris-HCl, pH 7.5, 8 M urea, and 10 mM 2-mercaptoethanol) containing sequentially increasing amounts of imidazole. Fractions were analyzed using SDS-PAGE and Coomassie Blue staining. Fractions containing protein were concentrated to 1 mg/ml. Protein concentrations were determined from A280 using calculated extinction coefficients (20).

The initial “in house” NDSB-256 (dimethyl benzyl ammonium propane sulfonate) (Antrace Inc.) refolding buffer was (25 mM Tris-HCl, pH 7.0, 1 mM EDTA, 10 mM α-cyclodextrin, 0.1 mM GSH/0.01 mM GSSG, and 0.5 mM NDSB-256). The refolding screen for the denatured proteins has been described (21). Larger batches (10 mg) of purified proteins were refolded (hNBD1 100 μg/ml, hNBD2 50 μg/ml) in either condition 10 (21), condition 10 plus 0.5 M NDSB-256 or an NDSB-256 buffer from above. After 24 h incubation at 4 °C, samples were concentrated, filtered and sized on a Superdex 26/10 30/30 mm column. Sizing fractions that yielded monomeric/dimeric NBD proteins were pooled. Protein concentrations of the refolded samples were determined by quantitative Westerns using specific monoclonal antibodies (hNBD1 clone L12B4) and (hNBD2 clone M3A7) (Chemicon Inc.) and known quantities of denatured hNBD proteins as a standard curve. Western detection was accomplished using infrared fluorescence-labeled secondary antibody and the Odyssey system (LI-COR Bioscience) (22).

Adenylate Kinase and ATPase Assay—The forward adenylate kinase rates (ADP formation) were followed using labeled [γ-32P]ATP and TLC followed by autoradiography with a Fuji phosphorimaging analyzer. The reactions (30 μl) contained 50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, 500 μM [γ-32P]ATP, 400 μM AMP, and varying amounts of NBD were incubated at 37 °C for 60 min. The reverse adenylate kinase rates (ATP and AMP formation) were followed using [8-14C]ADP (PerkinElmer Life Sciences). The reactions contained 50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, 400 μM [8-14C]ADP, and varying amounts of NBD were incubated at 37 °C for 60 min. The reactions were terminated by addition of 2 μl of 1 M formic acid. Aliquots (1–2 μl) were spotted onto poly(ethylene) imine (PEI)-cellulose TLC plates (catalog number 5579/7, EMD Chemicals Inc, Gibbstown, NJ), which were developed with a solution of 0.75M LiCl and 1 M formic acid.

Determination of Kinetic Parameters and Data Analysis—The apparent Km and Vmax values for ATP, AMP, and ADP were determined by fitting the rectangular hyperbolic plot of linear rates of product (μM/min) versus increasing substrate concentration (μM) to the Michaelis-
CFTR NBDs Possess Adenylate Kinase Activity

Wang et al. described by Wang et al. (23) were unsuccessful. BSA removal after the refolding resulted in protein that reformed soluble aggregates and lacked adenylyl kinase or ATPase activity. We conclude that BSA is not an effective chemical chaperone and instead acts as a dispersant. The other two methods relied on 0.5M arginine (24) or NDSB-256 as chemical chaperones. For hNBD1, the refolding method employing 0.5M arginine or NDSB-256 yielded active protein. Because active hNBD2 protein was obtained with the NDSB-256 procedure, the arginine method was not pursued. For both these methods, NBD protein recovery yields were <1%.

In an effort to increase protein recovery yields we used a fractional factorial refolding screen (21). Each protein was refolded at a 400-μl scale and after a 24-h, 4 °C incubation period, each screening condition was directly assayed for activity. For the hNBD1 protein, the best refolding conditions were condition 10 (50 mM Tris, pH 8.2, 0.5 mM Tween-80, 5 mM TCEP, 550 mM GdnHCl, and 2 mM MgCl2) and condition 30 (50 mM MES pH 6.5, 0.5 mM Tween-80, 5 mM TCEP, polyethylene glycol 3350, 440 mM sucrose) with the hNBD1 protein screened at 100 μg/ml (not shown). Tween-80 and TCEP additives appeared important in the refolding of the hNBD1 proteins (Fig. 2A, left). A prominent dimeric band at 70 kDa.

RESULTS

Expression and Purification of CFTR NBD Proteins—To understand the CFTR gating mechanism we have pursued a detailed biochemical examination of highly purified soluble and refolded cytoplasmic domains (i.e. NBD1 and NBD2) of the CFTR protein. The CFTR literature has demonstrated that the CFTR domains are extremely challenging to study because of their poor levels of expression and insolubility in most recombinant protein expression systems. The mNBD1, being the most tractable, was pursued as described by others (18) with modification to the protein purification procedures (see “Experimental Procedures”). The mNBD1 proteins (wild type and G551D) were purified to homogeneity as judged by a Coomassie-stained SDS-PAGE gel (Fig. 1A). A total of nine mNBD1 wild type and four mNBD1 G551D independent protein samples were purified for this study. Western blotting confirmed the protein identity as it cross-reacted with the human NBD1 monoclonal antibody (clone L12B4, Chemicon) (Fig. 1B). The identity of the mNBD1 proteins was also confirmed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and N-terminal sequencing (not shown).

The six histidine-tagged versions of hNBD1 and hNBD2 were over-expressed as insoluble proteins and purified under denaturing conditions to homogeneity (Fig. 1B). Four independently denatured wild-type hNBD1 and four hNBD2 samples were purified for this study. To obtain active, soluble hNBD proteins, we worked out conditions to refold these proteins. We initially pursued three methods of protein refolding; the first two have been described (23, 24) and the third was developed in-house (see “Experimental Procedures”). Multiple attempts of refolding both hNBD1 and hNBD2 using the bovine serum albumin (BSA) method described by Wang et al. (23) were unsuccessful. BSA removal after the refolding resulted in protein that reformed soluble aggregates and lacked adenylyl kinase or ATPase activity. We conclude that BSA is not an effective chemical chaperone and instead acts as a dispersant. The other two methods relied on 0.5 M arginine (24) or NDSB-256 as chemical chaperones. For hNBD1, the refolding method employing 0.5 M arginine or NDSB-256 yielded active protein. Because active hNBD2 protein was obtained with the NDSB-256 procedure, the arginine method was not pursued. For both these methods, NBD protein recovery yields were <1%.

In an effort to increase protein recovery yields we used a fractional factorial refolding screen (21). Each protein was refolded at a 400-μl scale and after a 24-h, 4 °C incubation period, each screening condition was directly assayed for activity. The hNBD1 protein, the best refolding conditions were condition 10 (50 mM Tris, pH 8.2, 0.5 mM Tween-80, 5 mM TCEP, 550 mM GdnHCl, and 2 mM MgCl2) and condition 30 (50 mM MES pH 6.5, 0.5 mM Tween-80, 5 mM TCEP, polyethylene glycol 3350, 440 mM sucrose) with the hNBD1 protein screened at 100 μg/ml (not shown). Tween-80 and TCEP additives appeared important in hNBD1 refolding as these two components were shared between the two conditions. For hNBD2 protein (screened at 50 μg/ml), the two best conditions were also conditions 10 and 30. Whereas condition 10 for hNBD2 appeared to be superior (in terms of activity) to the NDSB-256 condition, the overall protein recovery yields did not improve dramatically. Large batches (10 mg) of hNBD1 and 2 were refolded three ways: condition 10, condition 10 plus 0.5 M NDSB-256 and the “in-house” NDSB-256 buffer. After the incubation period, samples were concentrated and sized on a 10-30 Superdex S200 column to remove aggregated (unfolded) material. Fractions corresponding to monomeric material were assayed and pooled. Protein concentrations of the pooled fractions were determined by quantitative Westerns using A280 determined amounts (20) of the denatured NBD proteins for a standard curve. Each pooled fraction that was used for further study contained a known amount of hNBD1 (Fig. 1D) or NBD2 (Fig. 1, E and F). During the purification and by Western analysis we saw that hNBD1 and 2 proteins had the propensity to form homodimers even under SDS-PAGE conditions. This was most apparent with hNBD2 (Fig. 1F) as higher concentrations of refolded protein were more readily obtained. With developed procedures in hand to purify mNBD1 protein and, having refolded the hNBD1 and 2 proteins we proceeded to characterize their biochemical activities.
The CFTR NBDs Function as Adenylate Kinases and Not as ATPases—Previous biochemical reports on the CFTR NBDs have suggested low catalytic rates (1–10 turnovers per minute) and the possibility of multiple enzymatic activities residing in a single domain (14). These observations indicated a need for a biochemical assay system that is highly sensitive to and capable of discriminating between multiple enzymatic activities (e.g. adenylate kinase and ATPase activity). We chose a PEI-cellulose TLC system and a radiolabeled $[^{33}P]p$-phosphate ATP substrate because of its sensitivity and ability to follow reactants and products directly. The TLC system also had a solvent (0.75 M LiCl and 1 M formic acid) that is able to separate multiple reaction products that can result from enzyme action on an ATP substrate (ADP, AMP, $P_i$, and pyrophosphate ($P_{i}$)). The importance of a sensitive and discriminatory assay system became obvious as we evaluated CFTR NBD affinity-purified samples.
CFTR NBDs Possess Adenylate Kinase Activity

TABLE 1
Wild-type and mutant protein kinetic parameters for AK activity

| Enzyme* | Mutation | ATP | AMP | ADP |
|---------|----------|-----|-----|-----|
|         |          | $K_m$ (µM) | $V_{max}$ (µmol/min) | $K_m$ (µM) | $V_{max}$ (µmol/min) | $K_m$ (µM) | $V_{max}$ (µmol/min) |
| mNBD1   | WT       | 30 ± 2 | 1.2 ± 0.01 | 30 ± 4 | 0.9 ± 0.02 | 450 ± 60 | 1.4 ± 0.06 |
| mNBD1   | G551D    | 80 ± 5 | 0.4 ± 0.02 | 70 ± 7 | 0.4 ± 0.02 | 360 ± 50 | 1.0 ± 0.05 |
| hNBD1   | WT       | 80 ± 5 | 0.7 ± 0.02 | 60 ± 7 | 0.7 ± 0.03 | 300 ± 20 | 1.0 ± 0.02 |
| hNBD2   | WT       | 70 ± 3 | 0.6 ± 0.01 | 40 ± 5 | 0.7 ± 0.03 | 280 ± 20 | 0.7 ± 0.02 |

*Enzyme concentrations are mNBD1 WT (900 nM), G551D (1800 nM), hNBD1 (4.7 nM), hNBD2 (30 nM).

**$K_m$ and $V_{max}$ values are apparent.

***SEM.

In the presence of $^{33}$P γ-labeled ATP and cold AMP substrates, all mNBD1 samples purified through a His tag affinity column displayed two activities (not shown): the formation of labeled ADP (i.e. adenylate kinase activity) and the formation of labeled pyrophosphate (i.e. ATP diphosphatase activity (EC 3.6.1.8)). The other product of the latter reaction would be unlabeled AMP from the $^{33}$P diphosphatase activity (EC 3.6.1.8)). Both denatured proteins were refolded in parallel in condition 30 at 100 µg/ml protein. After 24 h at 4 °C the samples were assayed for activity without further purification. The 490, 980, 1960 nM reflects total mNBD1 protein of the refolding reaction, 8, thin layer chromatography plate showing the AK activity of the wild-type hNBD2 protein and the K1250A mutant protein. Both denatured proteins were refolded in parallel in condition 10 at 50 µg/ml protein. After 24 h at 4 °C the samples were assayed for activity without further purification. The 98, 196, 440 nM reflects total hNBD2 protein of the refolding reaction. With each image, the arrow indicates the relative position of ADP. Positive mNBD1 and negative No enzyme controls are to the right.

A mNBD1 time course showed that AK activity was linear and stable at 37 °C for up to 2.5 h (Fig. 2D). At 900 nM enzyme, the kinetic parameters of mNBD1 AK activity were determined (Table 1); at saturating ATP the apparent $K_m$ was 30 µM and $V_{max}$ was 0.9 µmol/min whereas, at saturating AMP (400 µM) the apparent ATP $K_m$ was 30 µM and $V_{max}$ was 1.2 µmol/min. At concentrations above 500 µM AMP, the mNBD1 AK activity clearly demonstrated substrate inhibition (not shown). No substrate inhibition was detected up to 5 mM ATP.

For the refolded human NBDs, the AK activity associated with both the hNBD1 and hNBD2 (Fig. 2A) and was dependent on added AMP (Fig. 2A). All purified hNBD1 and hNBD2 samples displayed AK and not ATPase activity. For the hNBDs, GMP could not be substituted for AMP as a substrate (not shown). The mNBD1 AK activity clearly demonstrated substrate inhibition (not shown). No substrate inhibition was detected up to 5 mM ATP.

Similarly, Ap$G$ was also a significantly poorer inhibitor ($IC_{50} = 50 µM$) consistent with the observation that GMP could not substitute for AMP as a substrate (not shown).
hNBD2 IC₅₀ = 160 nM ± 6 nM (Fig. 2C) when ATP and AMP substrates were fixed at 80 μM.

NBD Active Site Mutations and AK Substrate-labeling Studies Provide Further Evidence That the CFTR NBD Catalyzes AK Activity—In an effort to provide additional evidence that the CFTR NBD catalyzes AK activity, we generated a lysine to alanine substitution in the Walker A motifs of both hNBD constructs (hNBD1:K464A and hNBD2:K1250A). The conserved lysines were chosen because they hydrogen bond with the phosphates of the ATP substrate. We expressed and purified these mutant proteins in an identical manner to the wild-type samples. Denatured wild-type and mutant samples were refolded simultaneously at a 400-μl scale using same condition for both. After the 24 h, 4°C incubation period samples were assayed directly for AK activity. The wild-type NBD proteins possessed AK activity, whereas the mutant proteins lacked AK activity (Fig. 3) demonstrating that the AK activity is intrinsic to the hNBD1 and -2 and not a contaminant activity that co-purifies with the NBD proteins. These proteins were each independently refolded three times, and each time the wild-type protein possessed activity whereas, the mutant proteins did not. The hNBD2 (K1250A) mutant result is in agreement with the results reported by Randak and Welsh (16).

In addition to the active site mutant studies, we were interested in providing additional evidence that the AK activity was intrinsic to the purified proteins. Another approach is to show that the AK-specific substrate AMP actually binds/labels the purified NBD proteins. To accomplish this, we exposed the purified mNBD1 protein (60 nM) to photoactivated [³²P]-labeled 8-azido-AMP (7.5 μM) (ALT Inc. Lexington, KY) in the absence of ATP (no substrate conversion to ADP occurs) and resolved the protein samples on a SDS-PAGE gel to show that the mNBD1 protein became radiolabeled (Fig. 4, A and B). When AMP competitive substrates (Ap₅A, AMP, and ATP) were added, the 8-azido-AMP labeling efficiency decreased, showing that the labeling is specific for the nucleotide-binding site(s) of the mNBD1 (Fig. 4, A and B, lanes 2, 3, 5, 6). In contrast, when 400 μM GMP were added, no decrease in labeling was noted (Fig. 4, A and B, lane 4). This 8-azido-AMP labeling experiment was repeated twice at these ligand concentrations with a similar outcome each time. We also exposed the purified mNBD1 protein (60 nM) to photoactivated [³²P]-labeled 8-azido-AMP (7.5 μM) (Fig. 4, C and D) in the absence of AMP to discern whether the competitive substrate profile was different from 8-azido-AMP (Fig. 4, A and B). The 8-azido-AMP labeling experiment was repeated twice at these ligand concentrations with a similar outcome both times. We found that AMP substrate did not compete effectively with the labeling by 8-azido-AMP (Fig. 4C, lane 3), suggesting possible separate binding sites for both AMP and ATP. We noted that ATP was competitive with the azido-AMP substrate but that this observation could be explained by noting that in other adenylate kinase enzymes the specificity determinant of the AMP site is the adenine ring whereas, in the ATP site the triphosphate component is the determinant of specificity (27). To our knowledge, all known adenylate kinases have separate ATP- and AMP-binding sites; even known multimeric AK enzymes (28) have two substrate-binding sites per subunit. Taken together, the labeling of a protein corresponding to the molecular weight of mNBD1 with 8-azido-[³²P]AMP provides further evidence that mNBD1 of CFTR catalyzes the AK activity. An attempt was made to identify the 8-azido-[³²P]AMP labeling site by proteolysis, but because of very low labeling efficiency this was unsuccessful.

Reversibility of the CFTR NBDs Adenylate Kinase Activity—We have demonstrated that mNBD1, hNBD1, and hNBD2 proteins have the ability to catalyze AK activity. All known adenylate kinases also have ability to catalyze the reverse reaction in the presence of just the ADP substrate (i.e. the conversion of two ADP molecules into ATP and AMP). Using [¹³C]-labeled ADP, we examined the mouse and human NBD1 and hNBD2 preparations and demonstrated each protein is capable of catalyzing the reverse reaction (Fig. 5). Three independent purified mNBD1, hNBD1, and hNBD2 protein samples were tested; all displayed the ability to catalyze the reverse adenylate kinase reaction (not shown).

For each NBD sample, equal amounts of ATP and AMP were generated (not shown). The reverse reaction kinetic parameters for each enzyme were determined (Table 1) and they were as follows: mNBD1 ADP Ki = 450 μM, Vmax = 1.4 μM/min; hNBD1 ADP Ki = 300 μM, Vmax = 1.0 μM/min; hNBD2 ADP Ki = 280 μM, Vmax = 0.7 μM/min. For all three proteins the ADP Ki were 4–15-fold higher than the ATP and AMP Ki (Table 1) suggesting the NBDs function within a narrow nucleotide concentration range.

Evidence for Homodimer Formation in the CFTR NBDs—Western blot analysis of the CFTR NBDs indicated to us early on that purified NBDs could dimerize under the appropriate conditions (Fig. 1F). If homodimerization was necessary for AK activity, an initial slower nonlinear rate of activity might be evident at low NBD concentrations. This appears to be the case for mNBD1 protein. When AK activity measured as a function of mNBD1 protein concentration, a slower nonlinear rate is observable at concentrations below 100 nM followed by a faster linear relationship from 100 nM to 800 nM (Fig. 6A) suggesting homodimerization may have occurred between the 50–100 nM of protein. This initial slow nonlinear rate was seen with all four mNBD1 samples tested. It is unclear from this data whether the AK activity below 100 nM results from a monomer NBD or transient NBD dimer. To further support the
hypothesis, we also examined the mNBD1 protein samples at 5 mg/ml (~160 μM) by static light scattering and found that the mNBD1 protein does indeed form entirely homodimers at this concentration (not shown).

Next, we wanted to examine three key CFTR residues in mNBD1 (K464A, G551D, and ΔF508) to determine what effect these mutations had on their ability to form homodimers or alter AK activity. All three mutant proteins overexpressed, however, during their purifications two mutants (ΔF508 and K464A) were problematic. The majority of protein from these two mutants was found in the soluble aggregate fractions. The remaining monomeric mutant protein co-purified with GroEL, an E. coli chaperone, in a 1:1 ratio (not shown). Because of the chaperone contamination we were forced to abandon our analysis of the K464A and ΔF508 proteins. In contrast, the mNBD1 G551D mutant protein purified with the same characteristics as the wild-type protein. The AK activity of G551D mutant was measured as a function of protein concentration (Fig. 6A). From this data, we find the G551D gating mutation lacks AK activity at low protein concentrations (<250 nM) when compared with the wild type protein, suggesting the mutation affects the ability of the NBD1 to dimerize. This was confirmed with two independently purified mNBD1 G551D samples. The kinetic parameters of mNBD1 G551D protein (1800 nM) were determined (Table 1) at saturating ATP (2 mM). The apparent AMP $K_{m}$ was 70 μM and $V_{max}$ was 0.4 μM/min. At high AMP (400 μM) the apparent ATP $K_{m}$ was 80 μM and $V_{max}$ was 0.4 μM/min. At high protein concentrations, the G551D mutation results in only a modest decrease in catalysis and 2-fold decrease in substrate affinity when compared with wild type protein. These data suggest the G551D mutation does not significantly disrupt catalytic function directly but rather the mutation may reduce the affinity between the two NBDs in the intact CFTR protein. Experimentally, this putative association defect can be overcome in vitro by increasing the protein concentration whereas, in vivo this is apparently not possible as this mutation causes a severe CFTR gating defect. We surmise that the G551D mutation renders NBD1 unable to associate or rearrange productively with NBD2 upon nucleotide substrate binding and that this subsequently leads to a defect in Cl⁻ ion transport. Interestingly, in two other ABC transporter proteins the corresponding glycine has also been proposed to function in NBD dimerization (29, 30). However, we cannot formally rule out the possibility that the CFTR G551D fold less efficiently than the wild-type protein (i.e. that there are a lower percentage of active molecules in G551D preparation as compared with wild type preparation) as a good active site titrating compound is not available.

Similiar to the mNBD1, both hNBD1 and hNBD2 when assayed individually demonstrated a slower nonlinear AK rate at low protein concentrations suggesting homodimer formation might be important for stimulated AK activity (Fig. 6, B and C). These results were true for four independently refolded hNBD1 and hNBD2 samples (not shown). Consistently, there was a noticeable exaggerated nonlinear phase when hNBD1 and hNBD2 proteins were refolded with NSDB-256 procedure and less so when the proteins were refolded using condition 10 (Fig. 6C, hNBD2 plots). If hNBD1 protein was refolded in condition 10 without NSDB-256 (Fig. 6B, squares) and in condition 10 buffer with 0.5 mM NSDB-256 (Fig. 6B, circles) a slower nonlinear was also more apparent when NDSB-256 was included. This observation suggests that the addition of NSDB-256 (in otherwise identical buffer) alters the monomer-dimer equilibrium of the refolded hNBD proteins such that a greater
CFTR NBDs Possess Adenylate Kinase Activity

Evidence for Heterodimer Formation in the Human CFTR NBDs—
The functional unit of the full-length CFTR chloride channel is suggested to be a monomeric CFTR protein (31, 32). Within each CFTR protein there are two NBDs (NBD1 and NBD2) that are believed to govern channel gating. Channel gating models in CFTR and in other ABC transporters invoke association of the two NBDs. Nucleotide binding may drive association or rearrangement of the NBDs. Subsequent association or conformational changes within the NBDs is hypothesized to enhance catalysis, which may in turn lead to opening of the transport channel. We were interested in determining (a) if the isolated CFTR NBDs could form heterodimers, (b) whether nucleotide binding drives association between NBDs, and (c) whether NBD association enhances AK activity. Light scattering and other biophysical experiments examining human NBD association were not easily achievable as refolded NBD protein supply was limiting and the separation of homo- and heterodimers would be a challenge as the calculated molecular masses of the hNBD1 and hNBD2 are very similar (~34 kDa). In addition the monomer to dimer transition most likely occur at the nanomolar concentration range, which is beyond the limits of many biophysical techniques. However, the hNBD proteins do not run at the same position on SDS-PAGE gel (Fig. 1B). Using commercially available specific monoclonal antibodies for hNBD1 and hNBD2 we were able to conduct co-immunoprecipitation studies. Using a hNBD2 specific antibody, we immunoprecipitated 25 nM of hNBD2 protein which was mixed with 4 nM hNBD1 for 30 min under heterodimer activity reaction conditions (see below) with or without nucleotide substrates/inhibitor. The precipitated material was then separated on SDS-PAGE gel, Western blotted, and probed with a specific hNBD1 antibody. The Odyssey secondary detection system revealed that the 4 nM hNBD1 protein in the reaction was quantitatively immunoprecipitated by the hNBD2 antibody (Fig. 7) with very little remaining in post-immunoprecipitated supernatant (not shown). Having ATP/AMP or ApA present during the incubation and wash steps of the experiment did not block the immunoprecipitation of hNBD1 (Fig. 7) at these protein concentrations. This pull-down experiment demonstrates that hNBD2 and hNBD1 proteins physically associate and form a stable complex in vitro.

Finally, as NBD heterodimerization to nucleotide and inhibitor. This work was supported by National Institutes of Health grant DK-24241 to R. H. S.

Percentage of Monomer is Present When the Additive is Included—
Taken together, these results suggest that all three NBD proteins examined appear to have the ability to form homodimers, which leads to AK or stimulated AK activity.

**Kinase Activity**

Kinase activity increases in a linear relationship with increasing amounts of hNBD2 (Fig. 8D). This linear increase in AK activity suggests that only a small fraction of the 2 nM hNBD1 is complexed with each addition of hNBD2 protein in this titration. This mixing experiments and the converse mixing experiment were repeated three times with a synergistic increase in adenylate kinase easily apparent in all experiments (not shown). Our data convincingly demonstrate that when hNBD1 and hNBD2 are combined a significant synergistic increase in AK activity occurs suggesting association of hNBD1 with hNBD2 enhances catalytic activity of one or both NBDs. These observations give credence to ABC transporter gating models that hypothesize that the proper association of two NBDs leads to enhanced catalytic activity which may be the molecular mechanism for opening and closing the transport channel.

**NBD1-R C-terminal Domain Has an Inhibitory Role on the Adenylate Kinase Activity**—We believe that our experiments have clearly demonstrated that the NBDs catalyze AK activity, but the possibility remains that larger CFTR protein fragments may gain extra functionality (e.g. ATPase) from additional contiguous residues. Two CFTR biochemical reports suggested this might be a possibility (12, 13). To examine this, pure denatured hNBD1-R protein (Thr390-Ile840) with an N-terminal His tag was put through the refolding screen at 100 μM multiple times. However, unlike the hNBDs, no detectable ATPase or adenylate kinase activity was detected when assayed four ways (ATP alone, ATP and AMP, and with and without the presence of PKA. Sufficient PKA was added to phosphorylate the NBD1-R fragment completely in the first 10 min of the 1 h activity assay as judged by SDS-PAGE and autoradiography. Based on observations that the R-domain phosphorylation is a prerequisite to nucleotide-stimulated Cl− ion transport (4), we believed assaying the phosphorylated NBD1-R protein for enzymatic activity was critical. The lack of activity in the refolded NBD1-R protein may be attributed to the possibility that this protein may fold less efficiently than hNBD1. Alternatively, the phosphorylated R-domain may still prevent activity in the NBD1-R construct if the preferred binding site for the phosphorylated R-domain is only present in the full-length CFTR protein (33) (i.e. in the absence of the phosphorylated R-domain-binding site, rearrangement does not occur and, as a result, the NBD inhibitory property is not relieved). We also repeated the NBD1-R
CFTR NBDs Possess Adenylate Kinase Activity

FIGURE 8. CFTR heterodimer NBD AK activity studies. A, image of a thin layer chromatography plates showing the AK activity when hNBD1 is titrated (from 0 to 2.3 nM) into a fixed concentration of hNBD2 (13 nM). The left side shows the AK activity of hNBD1 titration alone. The far right lane shows the AK activity of hNBD2 alone. B, AK activity in A was quantified by the phosphorimager and graphed as a function of hNBD1 protein concentration. The filled triangles represent the AK activity of hNBD1 alone. The filled squares represent the total AK activity of the heterodimer reaction. The filled circles represent the synergistic AK activity of the heterodimer reaction and are determined by the subtraction of the fixed NBD control reaction and corresponding variable NBD reaction from the heterodimer reaction value. C, image of a thin layer chromatography plates showing the AK activity when hNBD2 is titrated (from 0 to 25 nM) into a fixed concentration of hNBD1 (2 nM). D, AK activity in C was quantified by the phosphorimager and graphed as a function of hNBD2 protein concentration. The triangles represent the AK activity of hNBD2 alone. The squares represent the total AK activity of the heterodimer reaction. The circles represent the synergistic AK activity of the heterodimer reaction.

refolding condition described in Annereau et al. (13) without success. In this experiment the only difference was the NBD1-R constructs (Annereau: Gly404–Lys830 and ours Thr390–Ile840). As our construct encompassed theirs, this reason for failure seemed less probable.

We chose to pursue the NBD1-R study further by expressing a truncated version of the construct (Thr390–Ile708) to test the hypothesis that R-domain contains an inhibitory domain. Studies have been reported (4, 34) that the removal of residues between 708 and 835 in the full-length CFTR protein results in chloride channel that no longer requires activation by PKA. We reasoned that similar truncation would restore NBD1-R activity. In a refolding screen (21) we observed that this refolded truncated NBD1-R construct possessed AK activity, which was comparable to hNBD1 but not ATPase activity (not shown) and supports the possibility that C-terminal R-domain possesses inhibitory properties regardless of its phosphorylation state. This also suggests that a more complete biochemical picture on the regulation of the CFTR protein activities requires analysis of the purified full-length protein.

DISCUSSION

CFTR Gating Model Based on NBD1 and NBD2 Adenylate Kinase Activity—We have demonstrated that isolated NBD1 and NBD2 fragments of CFTR function as adenylate kinases both individual and together, but not as ATPases. CFTR patch-clamp studies by Randak and Welsh (16, 35, 36) suggest that CFTR gating is regulated by AK activity in vivo. Using the patch-clamp technique a number of observations are made, which support an AK gating hypothesis including: 1) They showed that at intermediate ATP concentrations AMP can reversibly increase the CFTR current 20–30% and GMP cannot substitute (16). 2) Randak and Welsh demonstrate that in the presence of AMP the rate of channel opening increases whereas, the adenylate kinase inhibitor Ap5A has the opposite effect (16). 3) The transfer of a phosphate between ATP and AMP was required for normal current generation and positive cooperativity (16). 4) They noticed that the ADP ligand induces positive cooperativity, which supports a conclusion that AK activity gates the CFTR channel rather than ATPase (16). 5) It was found the Ap5A also attenuated the ADP inhibition of CFTR current (16, 35) suggesting that ADP is functioning through an AK reaction. Together, their CFTR gating data and our own characterization of the CFTR NBDs biochemical properties are consistent with a CFTR gating model based on adenylate kinase activity. In such a model, after R-domain phosphorylation by PKA, and in the presence of ATP and/or AMP, the NBD1 and NBD2 domains associate or conformationally rearrange such that they become active in or stimulate AK activity. Nucleotide(s) binding to the NBDs causes the proper interaction of the two membrane spanning domains (MSD) that in turn permits Cl⁻ ions to move through the channel. In fact, others have shown that purified dimeric MSD2 from CFTR can indeed mediate Cl⁻ ion flux whereas, monomeric forms of MSD2 cannot (37). This model could also explain a well known observation that when ADP is added to patch-clamped CFTR channels the current is inhibited (16, 35). In the presence of a threshold concentration of ADP it is possible that the heterodimer AK activity functions in reverse driving the disassociation or nonproductive rearrangement of the heterodimer or ADP simply obstructs the mechanism. This would suggest that Cl⁻ ions are transported through the channel when the heterodimer is in the ATP- and/or AMP-bound state.

How and why might adenylate kinase activity be needed in a CFTR mechanism to transport Cl⁻ ions? It is known that AK activity generates very little free energy (38) and may be an insufficient amount of energy to drive conventional energy-dependent transport. However, CFTR is known to passively transport Cl⁻ ions (i.e. not against a Cl⁻ gradient) and may not need a large input of energy. Indeed, ATP and/or AMP binding maybe all that is required by the CFTR protein to rearrange itself in a productive Cl⁻ ion transport conformation after R-domain phosphorylation has occurred. The role of the adenylate kinase activity in CFTR may be to convert the inducing ligand(s) (ATP and/or AMP) to another form (ADP), which dissociates more quickly from the protein and thereby provides the CFTR containing cell with more rapid tempo-
ral control of Cl\textsuperscript{−} ion transport. For a passive transporter this seems the simplest model. In this ligand-induced model, the stoichiometry of Cl\textsuperscript{−} ion transport with respect to adenylate kinase activity is not required to be fixed and may be dependent on the electrochemical (Cl\textsuperscript{−}) gradient that is applied. Brownian movement of the Cl\textsuperscript{−} ion provides the kinetic energy for the movement across the membrane and may be assisted by the chemical attributes of the channel.

**Nucleotide Stoichiometry of the CFTR NBDs and Implications for Full-length CFTR Protein**—Currently, it is unclear whether a monomer of either hNBD1 or hNBD2 is capable of AK activity on its own or whether dimerization is necessary for activity. Comparing the structures of mNBD1 with the adenylate kinases, it seems possible that an AMP substrate could bind adjacent to the ATP site in a \(\alpha\)-helical region (18), suggesting a monomer may be an active species. If NBD monomers are an active species then a minimum of four nucleotide-binding sites are present in the heterodimer. However, we favor that NBD dimers are the only AK active species since nucleotide substrate sites are partially solvent exposed in the monomer (18) and the AK catalytic machinery cannot easily be protected from hydrolysis by predicted monomeric conformational changes. In contrast, in the NBD dimer models, water molecules are more easily excluded from interfering with the AK catalysis. Based on our protein labeling studies and strict requirement for AMP (GMP will not substitute), we speculate that there must be at least two nucleotide-binding sites per NBD monomer. Future efforts should determine the nucleotide stoichiometry and whether the dimer is the only active species. These activities will further refine the proposed gating model. An interesting observation with AP\(_A\) in the patch-clamp studies by Randak and Welsh (16) shows that the maximum current inhibition is 50%, suggesting it may block only two of four suspected nucleotide substrates sites at a time.

Although we were unable to show any ATPase or AK activity with a longer hNBD1-R construct there remains the possibility that the full-length CFTR protein may encode both AK and ATPase activities. Ramjeesingh et al. (39) have purified the full-length CFTR protein and examined it in vitro. The Bear and other laboratories ((11, 12, 13, 32, 39, 40) have presented both biochemical and functional studies that indicate that CFTR could function as an ATPase and that this activity may be responsible for gating the channel.

This study suggests four important considerations that are relevant to future biochemical investigation of full-length CFTR or other ABC transporters. 1) The possibility of both AK and ATPase activity in CFTR requires methods that can distinguish between two activities (note: \(\alpha\)-phosphate-labeled ATP cannot distinguish between the two activities). 2) AK enzymes generally have low ATP and AMP \(K_m\) values (<50 \(\mu\text{M}\)); impure ATP stocks could unintentionally provide the AMP substrate. 3) Contaminant enzymatic activities can confound assay results (e.g. our initial ATP diphosphatase contaminant). Abundant membrane bound and extracellular enzymes exist that manipulate ATP (41) and the chemical attributes of the channel.

**REFERENCES**

1. Hollinger, B., and Blight, M. A. (1999) *J. Biol. Chem.* 274, 381–389
2. Stolzova, J., Polepov, R., and Hubacek, J. A. (2004) *Physiol. Rev.* 83, 315–324
3. Ostergaard, L. S. Balduison, O., and Welsh, M. J. (2001) *J. Biol. Chem.* 276, 5699–5702
4. Wetter, M. C., and Welsh, M. J. (1997) *Nature* 389, 294–296
5. Sheppard, D. R., Nishikawa, Y., and Welsh, M. J. (1992) *Science* 255, 160–164
6. Welsh M. J., and Smith A. E. (1993) *Cell* 73, 1251–1254
7. Cutting, G. R., Kasch, L. M., Rosenstein, B. J., Ziaelsken, J., Tsui, L. C., Antonarakis, S. E., and Kazazian, H. H. (1990) *Nature* 346, 366–369
8. Gaddby, D. C., and Nairn, A. C. (1999) *Physiol. Rev.* 79, S7–S107
9. Callebaert, I., Eudes, R., Moron, J. P., and Leth, P. (2004) *CHL Cell Mol. Life Sci.* 61, 230–242
10. Aleksandrov L., Aleksandrov, A. A., Chang, X., and Riordan, J. R. (2002) *J. Biol. Chem.* 277, 15419–15425
11. Berger, A. L., Uka, M., and Welsh, M. J. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102, 345–346
12. Howell, L. O., Borchardt, R., Cole, J., Kaz, A. M., Randak, C., and Cohn J. A., (2004) *Biochem. J.* 378, 151–159
13. Anneran, J. P., Ko, Y. H., and Pedersen, P. L. (2003) *Biochem. J.* 371, 451–462
14. Randak, C., Neth, P., Auerwald, E. A., Eckerskorn, C., Assfalg-Machleidt, L., and Machleidt, W. (1997) *FEBS Lett.* 410, 180–186
15. Randak, C., Auerwald, E. A., Assfalg-Machleidt, I., Reenstra, W. W., and Machleidt, W. (1999) *Biochem. J.* 340, 227–235
16. Randak, C., and Welsh, M. J. (2003) *Cell* 115, 837–850
17. Hyde, S. C., Emley, P., Hartshorn, M. J., Mimnack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., and Higgins C. F. (1990) *Nature* 346, 362–368
18. Lewis, H. A., Buchanan, S. G., Burley, S. K., Conners, K., Dickey, M., Dorwart, M., Fowler, G., Gao, X., Guggino, W. B., Henderikson, W. A., Hunt, J. F., Kearins, M. C., Lorimer, D., Maloney, P. C., Post, K. A., Rajashankar, K. R., Rutter, M. E., Sauder, J. M., Thibeudeau, P. H., Thomas, P. J., Zhang, M., Zhao, X., and Ettorre, S. (2004) *EMBO J.* 23, 292–293
19. Mozersova, E., and Lima, C. D. (2010) *Mol. Cell 5*, 865–875
20. Gill, S. C., and Von Hippel, H. P. (1989) *Annu. Chem. Biol.* 182, 319–326
21. Willis Swope, M., Hogan J. K., Prabhakar, P., Liu X., Tsai, K., Wei, Y., and Fox, T. (2005) *Protein Sci.* 14, 1818–1826
22. Shultz-Gleswender, A., Zhang, Y., Holt, T., McDermott, D., and Olive, M. D. (2004) *LI-COR Biosciences, Lincoln, NE*
23. Wang, W., He, Z., O'Shaughnessy, T. J., Rux, J., and Reenstra, W. W. (2002) *Am. J. Physiol. Cell Physiol.* 282, C177–C1180
24. Lu, N. T., and Pedersen, P. L. (2000) *Arch. Biochem. Biophys.* 372, 7–20
25. Lienhard, G. E., and Secemski, I. I. (1973) *J. Biol. Chem.* 248, 1121–1123
26. Berry, M. B., and Phillips, G. N. (1998) *Proteins* 32, 276
27. Krishnamurthy, H., Lou, H., Kimpilussa, C., and Cukier, R. I. (2005) *Proteins* 58, 88–100
28. Vonrhein, C., Bonisch, H., Schafer, G., and Schulz, G. E. (1998) *J. Mol. Biol.* 282, 167–179
29. Verdon, G., Albers, S. V., van Oosterwijk, N., Dijkstra, B. W., Driessen, A. J. M., and Thunmissen, A. M. W. H. (2003) *J. Mol. Biol.* 334, 255–267
CFTR NBDs Possess Adenylate Kinase Activity

30. Ramaen, O., Sizun, C., Parnard, O., Jacquet, E., and Lallemand, J. Y. (2005) Biochem. J. 391, 481–490
31. Chen, J. H., Chang, X. B., Aleksandrov, A. A., and Riordan, J. R. (2002) J. Membr. Biol. 188, 55–71
32. Ramjeesingh, M., Li, C., Kogan, I., Wang, Y., Huan, L. J., and Bear, C. E. (2001) Biochemistry 40, 10700–10706
33. Chappe, V., Irvine, T., Liao, J., Evangelidis, A., and Hanrahan, J. W. (2005) EMBO J. 24, 2730–2740
34. Rich, D. P., Gregory, R. J., Anderson, M. P., Manavalan, P., Smith, A. E., Welsh, M. J. (1991) Science 253, 205–207
35. Randak, C., and Welsh, M. J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2216–2220
36. Randak, C., and Welsh, M. J. (2005) J. Biol. Chem. 280, 34385–34388
37. Ramjeesingh, M., Ugwu, F., Li, C., Dhani, S., Huan, L. J., Wang, Y., and Bear, C. E. (2003) Biochem. J. 375, 633–641
38. Alberty, R.A., and Goldberg, R. N. (1992) Biochem. 31, 10610–10615
39. Ramjeesingh, M., Li, C., Garami, E., Huan, L. J., Hewaryk, M., Wang, Y., Galley, K., and Bear, C. E. (1997) Biochem. J. 327, 17–21
40. Kold, J. F., Ramjeesingh, M., Stratford, F., Huan, L. J., and Bear, C. E. (2004) J. Biol. Chem. 279, 41664–41669
41. Zimmermann, H. (1996) Prog. Neurobiol. 49, 589–618
42. Elvir-Mairena, J. R., Jovanovic, A., Gomez, L. A., Alekseev, A. E., and Terzic, A. (1996) J. Biol. Chem. 271, 31903–31908
43. Balakrishnan, L., Venter, H., Shilling, R. A., and van Veen, H. W. (2004) J. Biol. Chem. 279, 11273–11280