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Purification, Characterization, and Expression of CFTR Nucleotide-Binding Domains

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The nucleotide binding domains (NBDs) within CFTR were initially predicted to lie in the cell cytoplasm, and to gate anion permeability through a pore that was present in membrane spanning α helices of the overall polypeptide. Our studies designed to characterize CFTR suggest several important features of the isolated nucleotide binding domain. NBD-1 appears to bind nucleotides with similar affinity to the full-length CFTR protein. In solution, the domain contains a high β sheet content and self-associates into ordered polymers with molecular mass greater than 300,000 Daltons. The domain is very lipophilic, disrupts liposomes, and readily enters the planar lipid bilayer. Clinically important mutations in the domain may disrupt the nucleotide binding capabilities of the protein, either through a direct effect on the nucleotide binding site, or through effects that influence the overall folding of the domain *in vitro*. Finally, after expression in human epithelial cells (including epithelial cells from a CF patient), the first nucleotide binding domain targets the plasma membrane even in the absence of other constituents of full-length CFTR and mediates anion permeability in these cells.

KEY WORDS: Cystic fibrosis; ion transport; topology.

INTRODUCTION

The ATP binding cassette (ABC) gene family includes over 30 polypeptides characterized by highly conserved motifs comprised of two membrane spanning regions (each containing six predicted membranes spanning α helices) and two separate domains each containing Walker A and Walker B consensus nucleotide binding sites (NBD-1 and NBD-2).[1–3] The gene family encompasses a broad spectrum of prokaryotic and eukaryotic proteins, several of which are important in health and disease. These include the adrenoleukodystrophy gene, genes mediating resistance to chemotherapeutic agents in human tumors and chloroquine resistance in malaria, and the cystic fibrosis transmembrane conductance regulator (CFTR). Because the nucleotide binding domains throughout the ATP binding cassette gene family are highly conserved (in general, approximately 30% sequence homology), it is reasonable to imagine that these domains subserve similar functions throughout the superfamily. Because the majority of mutations that occur within the CFTR are found within the two nucleotide binding domains of the protein, NBDs have particular relevance to the clinical disease.

Our studies have focused on recombinant overexpression and isolation of CFTR nucleotide binding domain 1 and 2.[4–11] We have analyzed the folded form of the protein, and its nucleotide binding characteristics. In addition, we have analyzed a motif within the domain, known to be highly conserved throughout the ATP binding cassette family (LSGGQXQR sequence), and propose that it may be important in the nucleotide binding site. Our studies have also indicated that the CFTR nucleotide binding domain is capable of inserting in a lipid bilayer and forms anion channel structures in this setting. Finally, when expressed in mammalian (including human) epithelial cells, NBD-1 leads to increased anion permeability and localizes to the plasma membrane. As with other members of

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the ATP binding cassette gene family, therefore, information necessary for targeting of the isolated nucleotide binding region to the plasma membrane is contained within this discrete domain itself.

RECOMBINANT SYNTHESIS OF THE CFTR NUCLEOTIDE-BINDING DOMAIN

We applied two different approaches to recombinant synthesis and purification of CFTR NBD-1. Figure 1 shows an example of the first approach. In this method, the CFTR NBD-1 cDNA (amino acids 426–588) was cloned into the EcoRI site of the pGEX-2T vector (Promega). Protein expression with this vector is under the regulatory control of the Taq promoter. Transfection of the plasmid into DH5α *E. coli,* followed by induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), led to high-level overexpression of a fusion of the glutathione-S-transferase (GST) protein in frame with NBD-1. The NBD-1 could be purified in small amounts over glutathione affinity agarose by this method. However, because the majority of the domain was packaged by the *E. coli* as insoluble inclusion bodies, yield was greatly increased by a protocol involving preparative SDS polyacrylamide gel electrophoresis. In this method, inclusion bodies were isolated from *E. coli* by sonication and slow-speed centrifugation (3000 $g \times 5$ min) at 4 hours after induction. The pellet was solubilized in 8 M urea, and then dialyzed into thrombin cleavage solution. A cleavage site engineered between GST and NBD-1 was then cut overnight at 4°C, and the recombinant NBD-1 from 4 liters of *E. coli* culture separated through a preparative 12% polyacrylamide gel containing 0.8% SDS. NBD-1 obtained by this approach is approximately 4–10 mg of protein from 4 liters of *E. coli* broth (Fig. 1, Lane 4). The NBD-1 can then be refolded over 2–3 days by serial dialysis. The final material is studied in 10 mM Tris, pH 7.4.

In another strategy for isolation of of the NBD-1, plasmids encoding GST cDNA and a thrombin

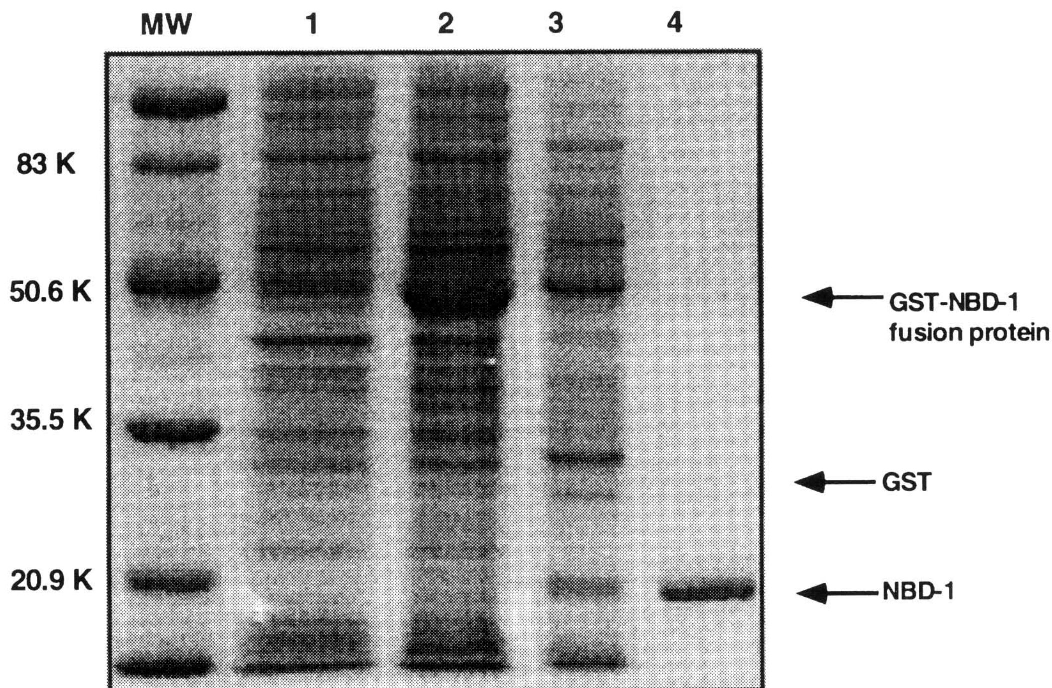

Fig. 1. Purification of CFTR NBD-1 from *E. coli.* Prokaryotic cells (DH5α) were transfected with a plasmid directing the synthesis of the first NBD of CFTR (amino acids 426–588) fused by a thrombin cleavage site to glutathione-S-transferase. Lane 1 shows total cell lysate from approximately 10 μg *E. coli.* After lysis of the bacteria and a slow-speed spin to purify inclusion bodies, the fusion protein (top arrow) is significantly enriched (Lane 2). Lane 3 shows the result of the cleavage of this fusion product into its two constituents (a 31-kDa glutathione-S-transferase fragment and a 21-kDa NBD). Following preparative gel electrophoresis, the NBD is purified to homogeneity (Lane 4).

cleavage site were adapted in order to generate a recombinant baculovirus. The viral constructs generated in this way led to NBD-1 fusion proteins that could be purified by glutathione affinity agarose. The advantage of baculoviral expression was that much larger CFTR polypeptides (including the full-length CFTR) could be synthesized and purified from insect cells in yields suitable for *in vitro* biochemical studies. From the standpoint of NBD purification, however, insect cell cultures were more cumbersome than recombinant protein synthesis in *E. coli.* The requirement for repeated infection and baculoviral purification from eukaryotic cells made this a less useful technique for studying CFTR nucleotide binding domains. As a result, the studies shown here predominantly utilize NBD refolded from *E. coli.*

PHYSICAL CHARACTERISTICS OF THE NBD-1

The CFTR nucleotide binding domains have a pronounced tendency to self-associate into long, ordered polymers in solution. By electron microscopy, these polymers appear as strands, with a molecular mass by sedimentation coefficient analysis of greater than 300,000 Daltons in 10 mm Tris, pH 7.4. Heterogeneity in the size of these strands is a feature that has limited the ability to obtain NBD-1 protein crystals suitable for x-ray diffraction. In our own laboratory, we have found that solubility of NBD-1 is not limiting (solutions up to 25 mg/ml have been studied). However, even modest increases in salt concentration (for example, 30–40 mm Tris, pH 7.4) or decreases in pH below 6.5, lead to rapid precipitation of the NBD-1. We have studied over 2000 hanging drop type conditions in order to obtain protein crystals suitable for x-ray diffraction. The majority of these experiments have been limited by insolubility after small changes in solvent salt concentrations or pH.

By circular dichroism, the nucleotide binding domain is predominantly β sheet (greater than 60–70%) with approximately 10% α helix. This overall CD spectrum may be influenced by the intermolecular interactions of the polypeptide in solution. This structure by CD is not changed by the presence or absence of phenylalanine 508. (The omission of F508 accounts for approximately 70% of defective alleles seen in CF patients, and at least by spectral analysis does not appear to grossly disrupt the final folded form of the domain). The tendency of NBD-1 toward self-association does not appear to be altered by the ΔF508 mutation. This has been investigated by nuclear magnetic resonance, circular dichroism, electron microscopy, UV spectroscopy, and light scattering techniques.[4,6,12] It therefore appears that the F508 mutation does not abrogate the final folded structure of the NBD, and other studies indicate that the ΔF508 NBD-1 remains functional within the full-length CFTR protein. For example, ΔF508 CFTR retains substantial activity in the plasma membrane, the endoplasmic reticulum, and in the planar lipid bilayer.[13–15] Other laboratories have proposed that the folding pathway (as opposed to the final folded form) of the NBD might be disrupted by the F508 mutation, so as to lead to cellular recognition of the ΔF508 CFTR, and rapid degradation by cellular mechanisms such as the ubiquitin/proteasome proteolytic pathway.[16,17]

NUCLEOTIDE BINDING BY CFTR NBD-1 *In Vitro*

The second most common CFTR mutation is the replacement of a glycine by aspartic acid at CFTR position 551 (G551D). This replacement occurs in a highly conserved motif present in all members of the ATP binding cassette gene family (LSG*G*QXQR (AA 548-555 of CFTR); G corresponding to position 551 in italics). We studied the nucleotide binding characteristics of the wild type CFTR NBD-1, and also NBD-1 containing ΔF508 or G551D. As shown in Fig. 2, while the ΔF508 mutation had no effect on binding of the nucleotide analog trinitrophenol ATP, the G551D mutation significantly decreased nucleotide binding by this recombinant CFTR domain. To confirm that glycine within this motif was important in nucleotide interactions, the corresponding glycine-aspartic acid replacement was made in CFTR NBD-2 (G1349D), and nucleotide binding was compared with the purified wild type region. The clinically important NBD-2 mutation G1349D led to decreased nucleotide binding by NBD-2. These results were confirmed in an independent assay (binding by 32p-2azido ATP). In addition, the binding of ATP by either domain could be displaced by Mg_2ATP (half maximal inhibition of binding at approximately 5 mM). These results suggest the possibility that the LSGGQXQR motif might be at or near an ATP binding site within the CFTR. We therefore modeled the domain based on an analysis of its predicted secondary structure and similarities to the known secondary structure of adenylate kinase. In this

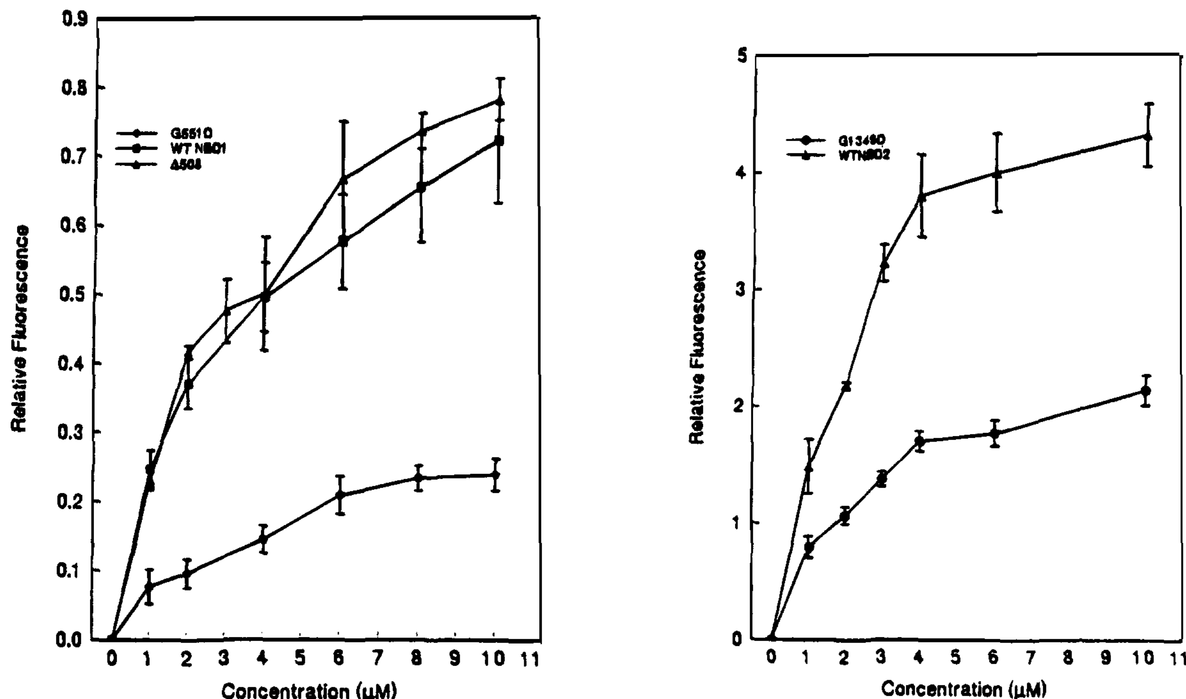

Fig. 2. Nucleotide binding by recombinant domains of CFTR. CFTR NBD-1, as well as NBD-1 containing the ΔF508 and the G551D mutations, were dissolved in 10 mM Tris, pH 7.4. The binding of trinitrophenol ATP (TNP-ATP) was studied as described previously.[6] Although the ΔF508 mutation had little effect on TNP-ATP binding, the G551D mutation significantly decreased the affinity of the recombinant domain for the nucleotide analog. NBD-2 polypeptides with and without the G1349D mutation were compared by trinitrophenol ATP binding. The glycine-to-aspartic acid mutation at position 1349 substantially decreased the affinity of the second nucleotide binding domain for TNP-ATP. Reproduced from the *Journal of Clinical Investigation*, 1994, **94**, 228–236.

model, the LSGGQXQR motif was predicted to fall very near to a nucleotide binding fold within the NBDs.[6] Peptide mapping of covalent azido ATP labeling of NBD-1 indicated that at least one binding site for azido ATP may lie very near this motif.[18] An alternative explanation, that *in vitro* folding of the domain is grossly disrupted by the G551D mutation, was also considered. However, the CD spectrum of the folded G551D NBD-1 did not differ from either ΔF508 or wild type NBD-1, and the G551D CFTR protein is not recognized as misfolded, at least insofar as the cellular quality control pathways responsible for CFTR processing are concerned. Nevertheless, high-resolution structural studies will be necessary in order to determine whether decreased binding by G551D NBD-1 is due to a replacement of a neutral residue with an exposed negatively charged aspartic acid, possibly blocking entry of negatively charged ATP molecules into a nucleotide binding pocket within the NBD-1.

Together with Dr. Harvey Pollard's laboratory, we have previously reported that CFTR NBD-1 is highly lipophilic.[5] The NBD-1 polypeptide enters the planar lipid bilayer and leads to the creation of an anion pore with an unusual capacity to pass current carried by large anions (HEPES, gluconate) in a gated fashion. The channel failed to conduct fluoride or bromide, and was blocked by the phenylanthranylate class anion channel blocker NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid). The ΔF508 and wild type proteins had slightly (≈5 mV) different reversal potentials. The purified NBD-1 also disrupts liposomes, although the interaction is stronger for liposomes comprised of phospoholipids than for sphingolipids.[19,20] Taken together, this data suggests that CFTR NBD-1 is highly lipophilic, and might be capable of entering the eukaryotic plasma membrane. Nucleotide binding domains within prokaryotic members of the ATP binding cassette family (for example, HIS-P, OPP-D, Mal-K, P69) are known to target the plasma membranes of bacteria,[21–25] and in the case of HIS-P and Mal-K assume a transmembrane configuration.

Whether or not the isolated CFTR NBD-1 contains the information necessary for plasma membrane targeting in mammalian cells and a transmembrane

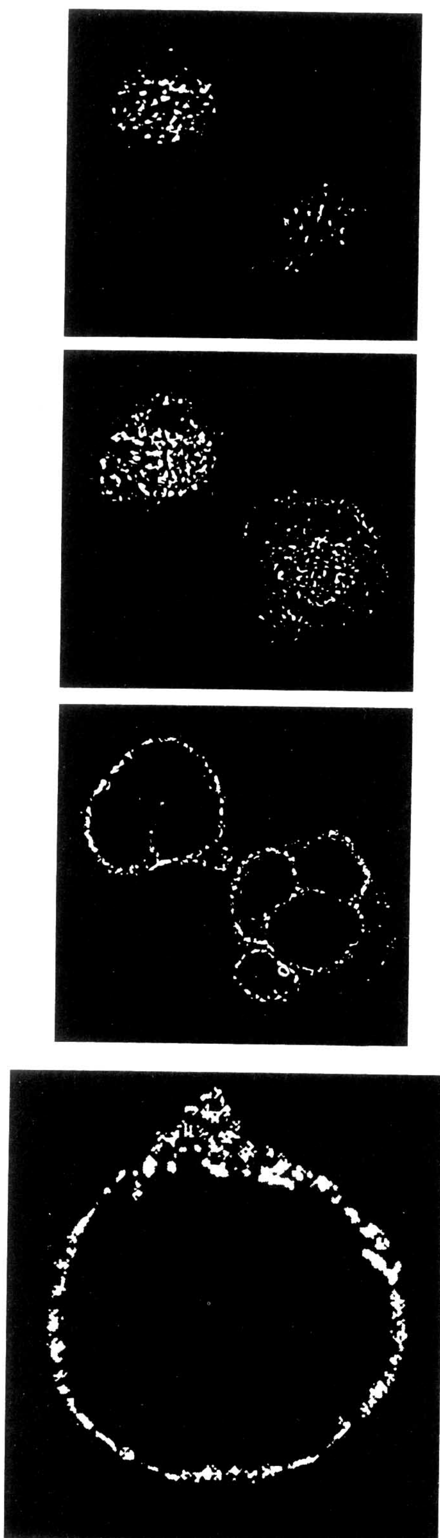

Fig. 3. Localization of the CFTR nucleotide binding domain 1 to the plasma membrane in COS-7 cells. CFTR NBD-1 was cloned into the pTM-1 vector (gift of Dr. J. Engler, University of Alabama at Birmingham) and expressed into Hela cells. A vaccinia virus expressing the domain was obtained by standard techniques.[35] COS-7 cells were infected with the domain at a multiplicity of infection (MOI) of 10. Twelve hours following infection, cells were formaldehyde fixed without detergent permeabilization. The NBD-1 was detected using a polyclonal antibody raised against the domain[4] and recognition was with a fluorescein tagged swine anti-rabbit antibody (DAKL, Inc.). A Photometrics SenSys digital camera and IPlab spectrum software with power microfilm (Signal Analysis Corporation) were used for fluorescent imaging. Excitation was at 623 nm using UPlanApo 100X or UApo/340 40X objectives. The panels indicate descending cross sections using the 40 × objective. The bottom panel is a 100 × magnification of a single cell. Approximately 50% of cells reveal the staining pattern indicative of surface staining.

configuration is not yet known, since CFTR is synthesized as a single polypeptide chain in mammalian cells. In order to investigate the possibility of a transmembrane configuration for the NBD-1, and to determine the relevance of previous findings in the planar lipid bilayer in human cells, we expressed the NBD-1 in living epithelial cells. As shown in Fig. 3, the NBD expressed this way is predominantly localized to the plasma membrane, as detected by confocal immunomicroscopy. In addition, the cellular permeability to anions such as iodide is greatly increased following NBD-1 overexpression (Fig. 4). As with other members of the ATP binding cassette gene family, therefore, the CFTR NBD-1 targets the plasma membrane of mammalian cells, appears capable of assuming a transmembrane configuration, and mediates the appearance of a new anion permeability pathway after expression. Because there is no reason to assume that the remainder of CFTR constrains NBD-1 in such a way as to prevent a strong tendency to enter the plasma membrane, this data supports the notion that structures contributing to the anion pore within CFTR exist in NBD-1 of the full-length protein.

DISCUSSION

The isolation and purification of CFTR NBD-1 has led to useful information concerning the function of the full-length protein. The domain faithfully reflects the nucleotide binding that has been well characterized for full-length CFTR.[6] The observation that the final folded structure is largely maintained in the absence of phenylalanine at position 508 supports other studies indicating that the ΔF508 mutation leads to a

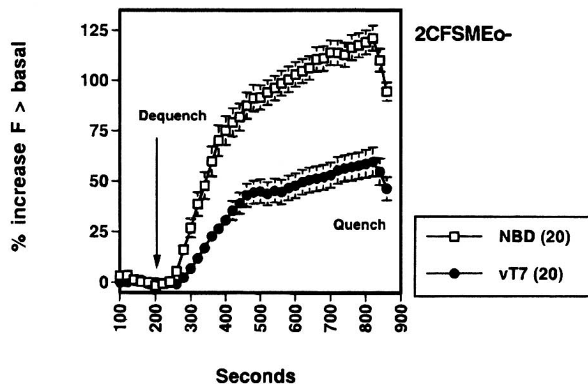

Fig. 4. Permeability effects of the wild type CFTR NBD-1 expression in mammalian cells. 2CFSMEo$^-$ cells (airway epithelial cells derived from a CF patient) were treated with recombinant vaccinia virus (as described above) and studied with the halide sensitive dye SPQ (6-methoxy-N(3-sulfopropyl) quinolinum).. SPQ is quenched in the presence of halides, and is a standard reagent for measuring CFTR expression in epithelial cells. At the arrow, cells were switched to a halide-free medium, and the rate of the SPQ dequench used as a measure of cellular permeability to halides. Wild type NBD-1 expression led to a marked increase in permeability to anions in these cells, strongly suggesting the presence of NBD-1 associated with the plasma membrane. Experiments in which endogenous vaccinia proteins or a control protein (Lac Z) were transfected did not lead to increases in anion permeability in these cells. The results indicate the presence of anion-conductive pathways elicited by expression of NBD-1 in mammalian cells.

functional CFTR protein that is retained in the endoplasmic reticulum due to differences in the folding program or in the time required to reach the final folded state.[13,16] The ΔF508 NBD-1 protein is also capable of a final folded structure that appears to be similar to the wild type domain in solution. On the other hand, a glycine-aspartic acid substitution at position 551, the second most common mutation among cystic fibrosis patients, leads to disruption of nucleotide binding. This finding supports other observations that G551D is normally processed to the plasma membrane, but maintains little or no residual function. The role of the highly conserved LSGGQXQR motif, present in all members of the ATP binding cassette gene family, has not been well defined in the past. It has been suggested that in HIS-P, this domain may transduce a structural change induced by ATP binding or hydrolysis.[25] Our modeling of the domain suggests the possibility that this region of CFTR may be important in the nucleotide binding pocket itself, although high-definition structural studies will be necessary in order to resolve this issue.

CFTR NBD-1 now been shown by several laboratories to be a highly lipophilic molecule.[5,26,27] It readily inserts in the planar lipid bilayer, disrupts liposomes, can be found in the prokaryotic cell membrane, and assumes a transmembrane configuration in SF-9 (insect) cells after baculoviral expression. In addition, the corresponding domain is found at the plasma membrane in many members of the ABC gene family, and the information necessary to target NBDs to the cell surface is contained within the domains themselves. In prokaryotic members of the ATP binding cassette family, the domain has already been shown to assume a transmembrane orientation.[21,22] Taken together with our findings that the CFTR NBD-1 localizes to the mammalian plasma membrane and alters anion permeability, the simplest explanation for the findings in Fig. 3 and 4 is that eukaryotic NBDs are capable of plasma membrane targeting and transmembrane localization.

There is controversy concerning the localization of pore-forming elements within the CFTR. The proposed topology of CFTR indicated that the pore was likely to exist within the α helices of predicted transmembrane segments. Anderson *et al.* showed that point mutations in the first or sixth predicted α helix of CFTR caused alterations in the anion selectivity of the channel, and covalent modifications of these segments decrease conductance through CFTR.[28] These results provided strong evidence that α helices contributed to the lining of the CFTR pore. On the other hand, it has also been shown that omission of the first four α helices of TM-1 results in a CFTR Cl^- channel that appears to be fully functional.[29] Other studies have indicated that deletion of the second half of CFTR (i.e., beyond residue 836) and deletion of a portion of the regulatory domain (residues 708 to 835) still result in an intact anion channel within the overall protein.[30] This analysis places the anion pore within CFTR between residues 265 and 708, a segment of CFTR that contains the fifth and sixth predicted α helices of TM-1, and the CFTR NBD-1. While it has been shown that the TM-1 sixth α helix of CFTR contains residues that are exposed to the extracellular environment,[31] this segment could not be the sole constituent of the CFTR anion pore, unless several CFTR molecules self-associated in such a way as to promote alignment of several helix six segments into a channel structure. Because this sort of self-association is not believed to occur during CFTR assembly,[32] other residues of CFTR between 265 and 708 must contribute to pore formation. The major remaining structural motif within the portion of CFTR that must contain a chloride channel is the first NBD. While further studies will be necessary in order to verify that portions of the NBD-1

contribute to pore formation within the overall protein, it is clear from the studies presented here that CFTR NBD-1 contains sequences necessary and sufficient to form ion channels, both in artificial cell membranes and in living cells.

In conclusion, the ability to synthesize and purify CFTR nucleotide binding domains offers the opportunity to understand the mechanisms underlying mutations that cause cystic fibrosis. The recent suggestion that the ΔF508 mutation disrupts at least one *in vitro* folding paradigm and that the normal folding program can be re-established by inclusion of glycerol (an intervention that also corrects ΔF508 processing in living cells) indicates the potential importance of this strategy toward a better understanding of disease mechanisms.[33–35] On the other hand, a major challenge to the field includes definition of a high-resolution structure of the NBD-1. Such information would add to our understanding of CFTR, and also many other gene products which contain highly homologous domains in the ABC gene family. Finally, efforts toward correlating structure and folding of purified domains *in vitro* need to be compared whenever possible with living cells, so that conclusions drawn after refolding can be most clearly connected to the *in vivo* situation.

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