# The Nucleotide Binding Folds of the Cystic Fibrosis Transmembrane Conductance Regulator Are Extracellularly Accessible†

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ABSTRACT: Analysis of the primary sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) has suggested the presence of two predicted cytoplasmic regions of the protein which are thought to be nucleotide binding folds (NBF1 and NBF2). Previous studies have shown that purified recombinant NBF1 can form anion conducting channels in planar phospholipid bilayers [Arispe et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1539−1543] and that the bacterial His P protein (analogous to a NBF) can be extracellularly labeled with a membrane-impermeant reagent [Baichwal et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 620−624]. Based on these observations, it is reasonable to hypothesize that the NBFs from the CFTR are associated with the plasma membrane and have extracellularly-accessible regions. Direct biochemical evidence for this was obtained by determining the ability of the individual NBFs, expressed in intact Hi5 cells, to be chemically modified with the membrane-impermeant reagent NHS-biotin. The results indicate that both NBF1 and NBF2, in intact cells, can be chemically modified by extracellular NHS-biotin. The negative control, the cytosolic enzyme $\beta$-galactosidase, was not significantly labeled under these conditions, verifying the extracellular nature of the labeling reaction. When the surface-accessibility of a NBF1 construct containing the CF-causing mutation $\Delta$F508 was analyzed, similar labeling was observed, suggesting that the mutation does not affect this aspect of the CFTR's structure. These data support the conclusion that, under certain conditions, the NBFs are capable of spanning the plasma membrane, perhaps constituting a portion of the CFTR's ion conducting channel.

The cystic fibrosis transmembrane conductance regulator (CFTR)[1] is a 1480 amino acid integral membrane protein found in the apical plasma membrane of epithelial cells (*1, 2, 3*). Mutations in the CFTR cause the fatal disease cystic fibrosis, the most common inherited disease in the Caucasian population (*4*). This protein is thought to be a chloride channel which is regulated by phosphorylation and intracellular ATP (*5, 6, 7, 8, 9*). The CFTR has been classified as a member of a family of membrane proteins termed traffic ATPases or ATP Binding Cassette (ABC) transporters (*10, 11*). Members of this family transport a variety of molecules across the cell membrane and share common consensus sequences known to bind ATP.

The CFTR is predicted to contain 12 transmembrane domains, 2 nucleotide binding domains (NBF1 and NBF2), and a highly charged 300 amino acid cytoplasmic loop designated the R domain (*1*). As of yet, little direct information is available regarding the three-dimensional structure of the protein, and the only structural model available is based upon primary sequence homologies and hydophobicity analyses. The current model of the CFTR's structure has led researchers to believe that the two NBFs are intracellular and that they regulate the channel properties of the CFTR by ATP binding and possible ATP hydrolysis (*8, 9*). Mutagenesis experiments (*12*) have led to the supposition that the transmembrane domains may form the pore of the CFTR chloride channel.

Evidence exists which suggests that the traditional CFTR structural model described above may not be correct. Purified recombinant NBF1 alone (amino acids 426−588), reconstituted into planar lipid bilayers, was able to mediate anion conductance across the bilayer (*13*). This suggests that NBF1 alone can interact with a lipid bilayer and that association is in a manner which facilitates transport of small molecules across that membrane. In addition, a subunit of the well-characterized bacterial histidine permease, HisP, exhibits extracellular accessibility as determined by chemical modification with NHS-biotin (*14*). The HisP is structurally similar to the NBFs of the CFTR (*10*). These observations warrant additional research into the location and function of the NBFs of the CFTR in order to generate a more accurate model of the structure of this medically relevant membrane protein. This is important for the understanding of both the structure/function relationships within the polypeptide and the mechanisms by which mutations cause disease. It is the objective of this study to address the structure of the NBFs, in relation to the plasma membrane, and determine whether they are exposed to the extracellular surface of the cell.

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[1] Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; NBF, nucleotide binding fold; NHS-biotin, *N*-hydroxysuccinimidobiotin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TCA, trichloroacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

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## EXPERIMENTAL PROCEDURES

*Constructs and Virus Production*. Using the pBluescript (Stratagene) vector, site-directed mutagenesis (*15*) was used to introduce unique *Not*I restriction endonuclease sites into the CFTR cDNA at positions 1410 and 1913 (to generate the NBF1 construct, amino acids 429−591) or into positions 1890 and 2630 (to generate the NBF2 construct, amino acids 1210−1414). The 3′ mutagenic oligonucleotides also introduced a triplet of stop codons just prior to the engineered *Not*I site and the 5′ mutagenic oligonucleotides introduced a methionine residue just after the *Not*I site in the NBF1 construct (corresponding to amino acid 429 of CFTR). The NBF2 construct utilized a preexisting methionine. Mutagenesis was also used to generate a derivative of NBF1 containing the single amino acid deletion corresponding to ΔF508. The flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was inserted into the carboxy terminus of the NBF constructs using site-directed mutagenesis as before. This epitope provides a means of detecting the presence of a particular NBF via protein immunoblots using an anti-flag monoclonal antibody ("M2", IBI/Kodak). Mutagenized DNA was sequenced in its entirety to verify the presence of the correct mutations and to ensure no other mutations were produced. The constructs were then excised from pBluescript using *Not*I and subcloned into the baculovirus transfer vector pVL1392 (InVitrogen). Recombinant baculovirus was made according to established procedures (*16*). The $\beta$-galactosidase baculovirus construct was obtained from InVitrogen.

The baculovirus construct expressing both α and $\beta$ subunits of the sheep Na,K-ATPase was made by subcloning the $\beta$ subunit cDNA downstream of the polyhedrin promoter and the α subunit downstream of the P10 promoter in the transfer vector p2Bac (InVitrogen). The recombinant virus was made according to established procedures, and immunoblot analysis verified the expression of both subunits in infected insect cells (unpublished experiments).

*Na,K-ATPase Assays*. Membranes were made from Hi5 cells, infected with the sheep α+$\beta$ Na,K-ATPase baculovirus for 3−4 days, according to previously described procedures (*17*). In brief, cells were harvested, washed, and resuspended in hypotonic lysis buffer. The cells were disrupted via Dounce homogenization, and the solution was centrifuged for 5 min at 500*g* to remove intact cells and aggregates of debris. The supernatant was centrifuged at 100000*g* for 30 min at 4 °C. The membranes (the pellet from the previous step) were washed with NaI for 10 min and centrifuged again at 100000*g*. The pellet was washed 2−3 times with 1 mM Tris, pH 7.4, 1 mM EDTA and stored in 25 mM imidazole, 0.4 M NaCl, 0.4 M KCl, 1 mM Tris, pH 6.9, and 1 mM EDTA at −70 °C until use. Na,K-ATPase activity was assayed spectrophotometrically as described (*17*).

*Cell Growth and Surface Labeling*. Hi5 insect cells were grown in TNM-FH media (Sigma) supplemented with 10% fetal bovine serum, 5 mM glutamine, and 40 $\mu$g/mL gentamicin in T150 tissue culture flasks at 27 °C. To initiate an experiment, cells in a single T150 flask were infected with the appropriate baculovirus construct at a multiplicity of infection of approximately 1. Two days following infection, cells were chilled on ice and the media aspirated. The cells were washed twice with 20 mL of insect buffer (6.8 mM $CaCl_2$, 55 mM KCl, 7.3 mM $NaH_2PO_4$, 11.2 mM $MgSO_4$, 11.2 mM $MgCl_2$, and 78 mM sucrose, pH 6.2) at 4 °C and then incubated with 12 mL of 0.5 mg/mL NHS-biotin (*N*-hydroxysuccinimidobiotin, Pierce) in insect buffer at 4 °C for 15 min. Following incubation, the reaction was quenched by the addition of 20 mL of serum-free TNM-FH media at 4 °C for 10 min and then washed twice more with 20 mL of insect buffer alone. The labeled cells were then dissolved in 9 mL of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton, 1% CHAPS, 1 mM Pefabloc, and 10 $\mu$g/mL aprotinin). Insoluble material was pelleted via 14000*g* centrifugation, and the biotinylated proteins in the supernatant (designated "labeled cell extract") were isolated via chromatography on immobilized monomeric avidin.

*Isolation of Hi5 Membranes Containing NBF1*. Hi5 insect cells expressing recombinant protein were harvested and resuspended with douncing in 10 mL of 0.25 M sucrose, 1 mM Tris, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$, 10 $\mu$g/mL aprotinin, and 1 mM Pefabloc. The resulting homogenate was then centrifuged 5 min at 500*g*. The supernatant fraction was then subjected to a 30 min 100000*g* centrifugation. The supernatant from this centrifugation was discarded, and the membrane pellet was then resuspended in 5 mL of the aforementioned buffer by douncing. One milliliter of 6 M NaI, 10 mM $MgCl_2$, and 10 mM Tris, pH 7.4, was added dropwise while gently vortexing the sample. After incubating on ice for 10 min, the solution was again subjected to a 30 min 100000*g* centrifugation. The supernatant was discarded and the membrane pellet washed twice by resuspension with gentle douncing in 10 mL of 1 mM Tris, pH 7.4, 1 mM EDTA. After the final 100000*g* centrifugation, the membrane pellet was resuspended in 5 mL of buffer containing 25 mM imidazole, 0.4 M NaCl, 0.4 M KCl, 1 mM Tris, pH 6.9, and 1 mM EDTA and stored at −70 °C until further analysis.

*Isolation of Biotinylated Proteins*. A 2 mL immobilized monomeric avidin (Pierce) column was prepared according to the manufacturer's instructions and equilibrated with lysis buffer. Labeled cell extracts were loaded onto the column which was then washed sequentially with 10 mL of PBS, 0.1% Triton; 20 mL of PBS, 0.1% Triton, 0.5 M NaCl; and 10 mL of PBS, 0.1% Triton to remove nonbiotinylated proteins. The flow-through was saved and designated "unlabeled material". Biotinylated proteins were then eluted from the column with 2 mM *d*-biotin, 0.1% Triton in 1 mL fractions (designated "labeled material").

*Detection of Labeled (Biotin-Eluted) Proteins*. The protein-containing, biotin-eluted fractions ("labeled material") from the monomeric avidin column were pooled and TCA-precipitated, as were equivalent amounts of the precolumn cell extract and the postcolumn extract (unlabeled material which flowed through the column). Following washing in acetone, the samples were resuspended in SDS−PAGE sample buffer (62.5 mM Tris, pH 6.8, 6 M urea, 2% SDS, and 0.001% bromophenol blue). After incubation at ambient temperature for at least 30 min, the samples were subjected to SDS−PAGE (4−20% polyacrylamide, precast gels from BioRad). Electrophoretic transfer of the proteins onto PVDF membrane (Amersham) was performed (*18*). The membrane was blocked overnight in TBS, 0.1% Tween, and 5% nonfat milk and then incubated either with the monoclonal anti-flag antibody (M2, IBI/Kodak, 0.5 $\mu$g/mL) to detect the NBF or with the monoclonal anti-$\beta$-galactosidase antibody (Boeh-

ringer Mannheim, 1.25 $\mu$g/mL) for 1 h. After this incubation, the membranes were washed (TBS, 0.1% Tween) and incubated with anti-mouse antibody conjugated to horseradish peroxidase (Sigma, 1:5000) for 1 h. The membranes were washed again, and the signal was developed using enhanced chemiluminescence (ECL, Amersham).

*ATP Binding Studies*. After a 2 day infection period with baculovirus expressing recombinant NBF1, a T-150 flask of Hi5 insect cells was harvested, and the cells were homogenized by douncing in 3 mL of 50 mM Tris, pH 7.4, containing 10 $\mu$g/mL aprotinin and 10 $\mu$M Pefabloc. The cell homogenate was then subjected to a 40000*g* centrifugation at 4 °C and dialyzed overnight against 10 mM Tris, pH 7.4. Following dialysis, the homogenate was again centrifuged at 40000*g* for 30 min. One milliliter of the supernatant was then tumbled for 1 h at 4 °C with 40 $\mu$l of washed (10 mM Tris, pH 7.4) agarose-coupled ATP (C8, Sigma) and/or free ATP (sodium salt). Following the 1 h incubation, the beads were washed 3 times with 1.5 mL of 10 mM Tris, pH 7.4, and the bound proteins eluted with SDS−PAGE sample buffer (62.5 mM Tris, pH 6.8, 6 M urea, 2% SDS, and 0.001% bromophenol blue) and analyzed via SDS−PAGE and immunoblot analysis using the M2 monoclonal antibody.

## RESULTS

To determine if presumed cytosolic portions of the CFTR have accessibility to the cell surface, various constructs containing particular domains of the CFTR were expressed and characterized in insect cells via the baculovirus system. This particular system has been extensively used to generate a wealth of biochemical and physiological data regarding the characterization of functional CFTR (*18*, *19*, *20*, *21*, *22*, *23*). Labeling of extracellularly accessible proteins was accomplished using a biotinylation reagent, NHS-biotin, which has previously been shown to be a membrane-impermeant reagent (*24*). After labeling and solubilization, the covalent incorporation of biotin was ascertained by avidin chromatography (experiment diagrammed in Figure 1). This approach takes advantage of a monomeric avidin column developed to facilitate separation and recovery of biotinylated proteins through a reversible avidin−biotin interaction (*25*). If a protein was labeled by membrane-impermeant NHS-biotin, then it should interact with and be retained on the avidin column. The bound material can then be specifically eluted with excess free biotin. Unlabeled (not surface-accessible) proteins will be found in the avidin column flow-through. The presence (or absence) of the protein of interest in the biotinylated fraction was determined via protein immunoblots probed with antibodies specific for the protein under study. This method of detecting biotinylated proteins was chosen after more direct means proved problematic. For example, experiments were performed where biotinylated samples corresponding to total cell homogenates prepared from labeled cells were analyzed via protein blots probed with horseradish peroxidase (HRP)conjugated avidin. Also, experiments were performed where the epitope-tagged (flagged) NBF was immunoprecipitated after labeling with NHS-biotin using the anti-flag antibody, followed by protein blots probed with HRP-conjugated avidin. In both cases, a very high background of signal was seen (data not shown) due to both the large number of different membrane proteins that were labeled in intact cells with NHS-biotin and the high sensitivity of using HRP coupled to chemiluminescence

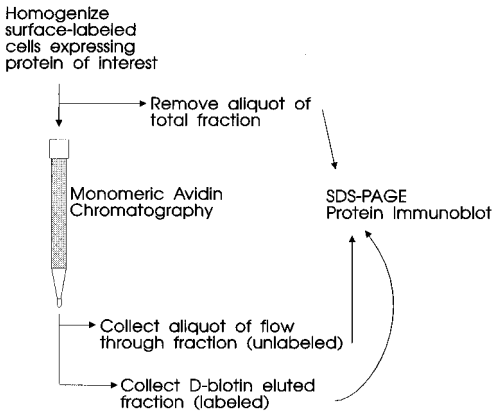


FIGURE 1: Use of monomeric avidin chromatography in determination of extracellular labeling. The diagram illustrates separation of proteins based on the extent of labeling by an extracellular probe. As described under Experimental Procedures, intact cells are labeled with NHS-biotin. Extracts are prepared and subjected to avidin affinity chromatography to separate the sample into biotinylated and nonbiotinylated fractions. Unlabeled proteins will pass through the column, and biotinylated (hence surface-accessible) proteins will be retained by the affinity matrix. The retained proteins can be subsequently eluted with free biotin. Three fractions are generated from this procedure: total extract (sample of extract prior to chromatography); flow-through (nonbiotinylated proteins); and eluted proteins (labeled with biotin).

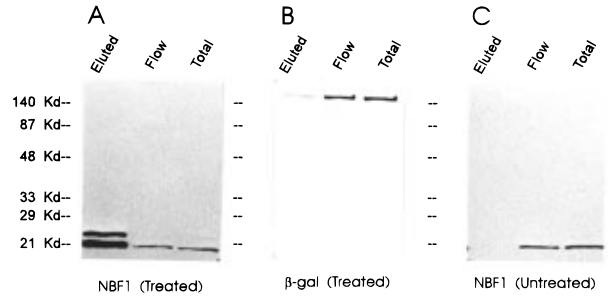


FIGURE 2: Immunoblot analysis of avidin-fractionated NBF1 and $\beta$-galactosidase. Samples were prepared as described under Experimental Procedures. "Total" represents the cell homogenate prior to avidin chromatography. "Flow" represents the material which flowed through the column. "Eluted" represents the material specifically eluted from the column with excess free biotin. The same relative amounts of protein were loaded in each representative set of samples (i.e., all "Eluted" samples contained equal protein; all "Flow" contained equal protein), facilitating the direct comparison between experiments. (Panel A) Luminogram of immunoblot probed with M2 anti-flag antibody. Hi5 cells were infected with the NBF1 baculovirus and surfaced-labeled ("treated") with NHS-biotin. (Panel B) Luminogram of immunoblot probed with anti-$\beta$-galactosidase antibody. Hi5 cells were infected with the $\beta$-galactosidase baculovirus and surfaced-labeled with NHS-biotin. (Panel C) Same as in panel A, but the cells were not exposed ("Untreated") to the NHS-biotin reagent.

detection. This necessitated using the approach described here where the biotinylated proteins were first isolated using immobilized avidin, followed by the detection of the labeled protein via a standard protein immunoblot probed with primary antibodies directed against the protein of interest.

Figure 2A shows the results of an analysis of NBF1 expressed in Hi5 cells. Cells were labeled with NHS-biotin after 2 days of infection, and fractions corresponding to the total extract ("Total"), the avidin column flow-through ("Flow") and the biotin-eluted material ("Eluted") were analyzed via an immunoblot probed with an antibody specific for the NBF construct (the anti-flag monoclonal antibody). As shown, significant amounts of labeled NBF1 are found

in the fraction eluted with free biotin, indicating that this recombinant protein is biotinylated in intact cells. Note that, in the hands of the authors, recombinant NBF1 always migrates as a doublet on SDS−PAGE. This may be due to covalent posttranslational modification of the recombinant domain (data not shown). A longer development of the immunoblot used in Figure 2A reveals the doublet is present in all lanes (data not shown), indicating that this doublet is a feature of the NBF1 that is not affected by NHS-biotin labeling. Unlabeled NBF1 (Figure 2C) does not nonspecifically adsorb to the avidin column, witnessed by the absence of NBF1 in the eluted lane in Figure 2C (again, longer development reveals the NBF1 doublet discussed above; data not shown). This indicates that the interaction of NBF1 with the avidin matrix, after surface labeling with NHS-biotin, was specific and dependent upon the recombinant protein actually being biotinylated.

The negative control for this experiment was $\beta$-galactosidase, a cytoplasmic enzyme. Here, a separate flask of cells was infected for 2 days with a $\beta$-galactosidase baculovirus and surfaced-labeled with NHS-biotin. Figure 2B shows the luminogram from an immunoblot probed with an anti-$\beta$-galactosidase antibody. As shown, insignificant amounts of this protein are recovered in the eluted fraction, suggesting that $\beta$-galactosidase is not labeled and that NHS-biotin is, under the labeling conditions described here, a truly membrane-impermeant reagent. This confirms the conclusion that the NBF1 labeling observed in the eluted fraction in Figure 2A is due to extracellular labeling of the NBF. The slight degree of labeled $\beta$-galactosidase observed in the eluted fraction (Figure 2B) is most likely due to a small percentage of the infected cells being nonviable and permeable to NHS-biotin. Cell viability was, in fact, determined to be slightly less than 100% (data not shown) by trypan blue exclusion. No differences were observed in the viability between NBF- or $\beta$-galactosidase-infected cells.

The approach of SDS−PAGE and immunoblot analysis facilitates a relatively quantitative determination of extracellularly-labeled protein in respect to total protein. The total protein extract (precolumn) and the flow-through sample were loaded in lanes adjacent to the biotinylated proteins (biotin-eluted material) isolated using the avidin column. The resulting protein immunoblot was then probed using the appropriate antibodies. Surface-accessible proteins exhibit a significantly stronger signal in the eluted lane relative to the total or flow-through lane. The use of this approach yielded consistently reproducible results which indicated that NBF1 had extracellular accessibility and that the similarly expressed cytosolic control $\beta$-galactosidase did not (Figure 2A versus Figure 2B).

If the NBF is accessible to the outside of the cell, which seems apparent based on the above findings, then the recombinant protein should be associated with the cell's plasma membrane. This was verified by preparing a membrane fraction enriched in the Na,K-ATPase and demonstrating that the NBF was also present in this preparation. Insect cells have little or no endogenous Na,K-ATPase activity (*26, 27*). In order to use this traditional plasma membrane protein as a marker enzyme, a recombinant baculovirus encoding the sheep Na,K-ATPase was made, and this was expressed in the Hi5 cells. A membrane fraction was prepared involving low- and high-speed centrifugation and a membrane wash using NaI (*28, 17*). This procedure

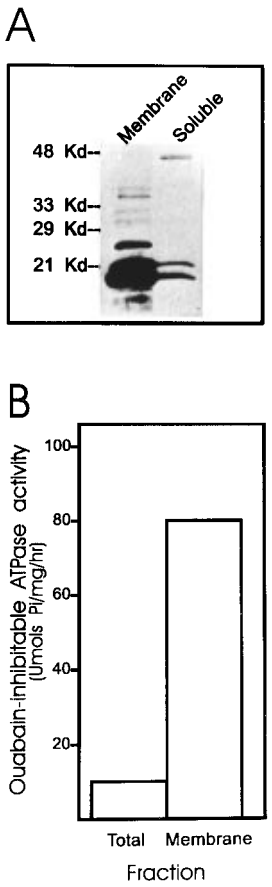


FIGURE 3: Colocalization of the NBF1 protein with an enriched membrane fraction. Hi-5 insect cells expressing recombinant Na,K-ATPase (a plasma membrane marker) were fractionated, and ouabain-inhibitable ATPase activity was measured as described under Experimental Procedures (panel B). Hi-5 insect cells expressing recombinant NBF1 were fractionated using the same procedure and analyzed for the presence of NBF1 via a protein immunoblot probed with the M2 anti-flag antibody (panel A). 20 $\mu$g of total protein was analyzed in each lane, and this represents 1/75 and 1/300 of the total membrane protein and total soluble (cytosolic) protein, respectively.

enriches membranes by approximately 8−10-fold (Figure 3B), determined by an enrichment in the activity of the Na,K-ATPase. The contention that this is indeed plasma membrane enrichment is supported by previous confocal microscopy studies which showed that the recombinant Na,K-ATPase, expressed in insect cells (Sf9), is indeed on the plasma membrane (*26, 27*). This membrane preparation procedure was then performed on cells expressing the recombinant NBF1 construct. Figure 3A shows results of these experiments demonstrating the presence of significant amounts of recombinant NBF1 determined by protein immunoblot analysis of the membrane fraction. Quantitation of the immunoblot signal in Figure 3A showed that approximately 60% of the total expressed NBF1 was in the membrane fraction. These data suggest that the expressed NBF1 is associated with the plasma membrane of the host cell.

If the NBF1 polypeptide was misfolded, resulting in the inappropriate exposure of hydrophobic regions, then the above-described membrane interaction may be a nonspecific event, rather than a real phenomenon based on the structure of the native NBF. Evidence that the recombinant NBF1 was indeed correctly folded and functionally active was provided by determining that the expressed protein was

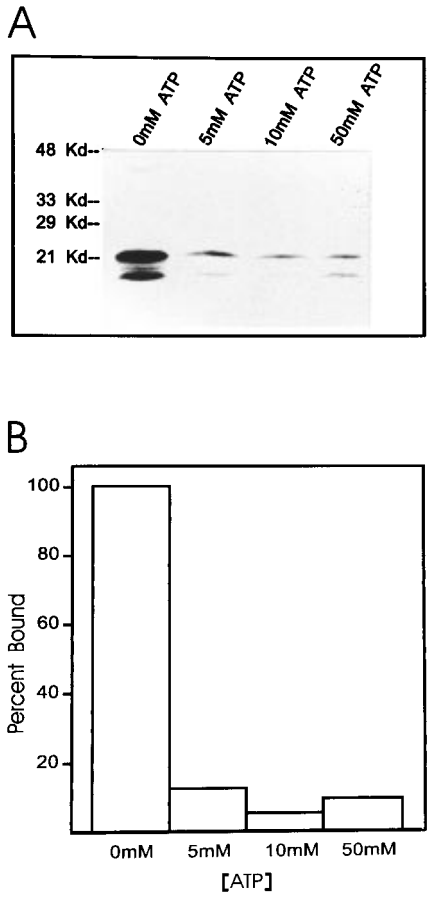


FIGURE 4: Recombinant NBF1 is capable of specifically interacting with an ATP−agarose column. Whole cell extracts of cells expressing recombinant NBF1 were dialyzed and then incubated with ATP−agarose in the presence of increasing concentrations of free ATP. The proteins binding with the ATP−agarose were then eluted and analyzed via a protein immunoblot probed with the M2 anti-flag antibody (panel A). The results of the luminogram generated from this protein immunoblot were then analyzed densitometrically to determine the relative quantity of NBF1 bound to the ATP−agarose column. The results of this analysis are presented in the bar-graph form (panel B) arbitrarily setting the value of NBF1 binding to the ATP−agarose as 100%.

competent to bind ATP (Figure 4). Infected Hi5 cells were lysed, and the lysate was dialyzed overnight to ensure that endogenous ATP was removed. The extract was incubated with ATP−agarose, and the bound material was isolated and analyzed via immunoblots probed with the M2 antibody (to detect NBF1). Experiments were also performed where the incubation with the ATP beads was accompanied by the presence of increasing amounts of free ATP. As shown (Figure 4), the NBF1 was able to bind to the ATP−agarose beads, and this binding was specific based on the ability for free ATP to compete for the binding. The concentration of free ATP required for competition was in the same range as that previously reported for inhibition of NBF1 binding to an ATP affinity matrix (*29*).

Figure 5 shows the results of surface-labeling experiments performed on cells expressing either NBF1ΔF508 or NBF2. Both of these proteins were detected on the immunoblot using the same antibody (the anti-flag monoclonal) as described above for studies with NBF1. Figure 5A indicates that, similar to data seen for the wild-type NBF1, the mutant NBF1ΔF508 is also labeled with NHS-biotin ("eluted" lane). Since the same antibody was used for detection, it was

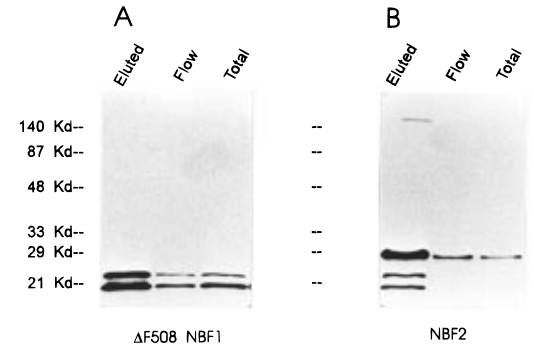


FIGURE 5: Immunoblot analysis of avidin-fractionated NBF1ΔF508 and NBF2. See the legend to Figure 2 for a description of each lane. The same relative amounts of protein were loaded on each lane so that the data from each panel can be directly compared. Both panels represent luminograms obtained from immunoblots probed with the anti-flag antibody. (Panel A) Hi5 cells were infected with the NBF1ΔF508 baculovirus and surfaced-labeled with NHS-biotin. (Panel B) Hi5 cells were infected with the NBF2 baculovirus and surfaced-labeled with NHS-biotin. The faint bands visualized below NBF2 are probably degradation products.

possible to quantitate the relative amounts of wild-type NBF1 found in the labeled fraction versus the amount observed for NBF1ΔF508. No significant differences in signal intensity were observed, suggesting that the same amount of either version of NBF1 was accessible to the surface of the cell. Experiments with NBF2 (Figure 5B) also revealed that, similar to NBF1, this domain is labeled with NHS-biotin in intact cells ("eluted" lane). Therefore, it is reasonable to conclude that both NBF1 and NBF2 are capable of spanning the plasma membrane of the cell.

## DISCUSSION

Predictions of function relative to a domain of a particular protein are dependent on the cellular location of that domain as well as the orientation of that domain within the polypeptide. The analysis of the structure of many proteins is based on comparisons to similar, well-characterized proteins whose structure has been elucidated. This is a difficult exercise for the CFTR, as the X-ray diffraction-derived structure for this, or any related protein (members of the superfamily of traffic ATPases), is unavailable. It is therefore difficult to assign, with confidence, particular functions to specific structures of the CFTR in the absence of new biochemical or biophysical data.

The NBFs of the CFTR have been postulated by sequence analysis to be intracellular, and therefore many discussions of their function are based upon the premise that the NBFs are indeed cytosolic. The present work has directly demonstrated that the NBFs are capable of spanning the eukaryotic cell plasma membrane and are accessible to the outside of the cell. This is not predicted by the current model and underscores the value of biochemical and biophysical assessments of protein structure and function. The findings presented here are in accord with two separate observations. First, purified NBF1 alone, in the absence of any CFTR transmembrane domains, was able to conduct anions across a planar lipid bilayer (*13*). For this to occur, the NBF1 must be capable of forming a pore across the bilayer. Second, a bacterial counterpart to a NBF, HisP, was shown to be surface-accessible using the same reagent (NHS-biotin) employed in the present study (*14*). Therefore, the present conclusions that NBF1 and NBF2 are capable of spanning

the plasma membrane are supportive, not contradictory, of previously published data. From these observations (current study, *13*), it is reasonable to hypothesize that the NBFs are indeed a component of the CFTR-conducting pore. Previous mutagenesis studies showed altered halide selectivity upon the mutagenesis of certain transmembrane residues (*12*). It is possible that these amino acid substitutions in transmembrane domains affected the mechanism by which the NBF participates in the channel properties of the CFTR. This leads to one caveat, which is the fact that the experiments described here were performed on individual domains rather than with the domains expressed in context with the intact CFTR protein. While the data presented here clearly demonstrate that the NBFs are extracellularly accessible, future studies will be required to determine the role of the rest of the CFTR polypeptide on this feature of the NBFs. Indeed, as part of the regulation of the CFTR's channel activity, the interaction of the NBFs with the membrane may be modulated. Thus, other regions of the CFTR may influence the NBFs' surface accessibility.

It has also been hypothesized that the ΔF508 mutation may have a deleterious effect upon the structural stability of the protein (*30*, *31*). Since the present study demonstrates that NBF1 is partly membrane-associated and surface-accessible, one might conclude that the mutation may result in an alteration of this accessibility to the cell surface and contribute to the mechanism of CF disease. However, the current data indicate little or no difference in the surface accessibility of NBF1 with or without ΔF508 when expressed in cells grown at 27 °C. If the NBFs are contributing to the channel properties of the CFTR, it is understandable that the ΔF508 does not affect the surface accessibility of the NBF1, since the ΔF508 CFTR protein appears to be a functional chloride channel (*32*, *33*, *34*, *35*) when processed to the plasma membrane.

One caveat to these studies is the possibility that the membrane-bound form of the NBF is nonspecifically associated with the membrane due to misfolding. It seems unlikely that the NBF polypeptide is simply nonspecifically adsorbed onto the membrane. If this were true, then the 1.0 M NaI wash would have removed the material from the membrane. Also, while it has been demonstrated that NBF can, under certain circumstances, bind ATP, it is not clear that the membrane-bound form of the NBF binds ATP. This would exclude the use of ATP binding to access the functionality of the membrane-bound form of the NBF. ATP binding experiments were indeed performed using the membrane-associated fraction of NBF1. In one experiment, detergent extracts were made from the membrane fraction and subject to ATP−agarose affinity chromatography. It was found that the NBF1 did not interact with the ATP resin (data not shown). This is in accord with earlier findings, which demonstrated that detergents inhibited the ATPase activity of the MDR p-glycoprotein (*36*), a homolog of the CFTR. Hence, it was technically difficult to demonstrate ATP binding to a detergent extract of NBF, since it was likely that the detergent itself may interfere with the interaction. Furthermore, 8-azido-ATP labeling experiments were performed on the membrane fraction in the absence of detergents. Here, as above, it was not possible to demonstrate ATP binding to the membrane-bound form of NBF1. However, it is possible, based on the emerging model of the CFTR, that the membrane-bound form of the NBF1 is intimately involved in channel activity (*13*) and no longer binds to ATP once the nucleotide is hydrolyzed and the channel is opened.

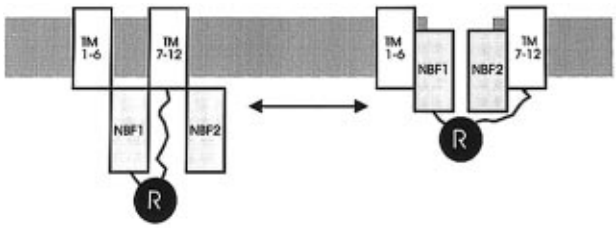


FIGURE 6: Hypothetical model of the CFTR. This model takes into account recent data that suggest that the NBFs are accessible to the outside of the cell. The model describes a conformational change leading from the closed state to the open state. This conformational change is concomitant with the membrane insertion of the NBFs. See the text for a discussion of this model.

Figure 6 depicts an alternative model for the CFTR which is based on the current data. The novel feature of this model is the surface-accessibility of the NBFs, as described in the current work. Instead of showing the NBFs as being strictly cytoplasmic domains, the model suggests that a portion of the NBF is actually afforded access, if only temporarily, to the outside of the cell. This is not to mean that the nucleotide binding site of the NBF is extracellular; rather, the domain is configured in such a manner so as to be accessible to both the intracellular and extracellular compartments of the cell, possibly in a temporal fashion. Furthermore, it is possible that this membrane orientation may be a regulated event, which is supported by data showing that the halide conductance mediated by pure NBF1 can be modulated by ATP (*13*). Perhaps one conformation of the CFTR involves the interaction of the NBF(s) with the membrane and a different conformation involves a more cytosolic orientation.

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