Cystic Fibrosis Transmembrane Conductance Regulator: The First Nucleotide Binding Fold Targets the Membrane with Retention of Its ATP Binding Function†

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ABSTRACT: Most cases of cystic fibrosis are caused by a single deletion mutation (ΔF508) within the first nucleotide binding fold (NBF1) of the CFTR protein (cystic fibrosis transmembrane conductance regulator). NBF1 is classically defined as amino acid residues phenylalanine 433 through serine 589, encoded by exons 10−12, and only part of exon 9, of the CFTR gene. This assignment is based on sequence homology of this region of the CFTR protein with that of other nucleotide binding proteins. Here, we report that when the complete modular unit encoded precisely by exons 9−12 is expressed in *Escherichia coli* as glycine 404 through serine 589, i.e., as [G404−N432]NBF1 or as ΔF508[G404−N432]NBF1, the resultant proteins target the cytoplasmic membrane. Significantly, [G404−N432]NBF1 is readily labeled from the outside of intact *E. coli* spheroplasts with the water soluble, membrane impermeable probe Biotin-X-NHS, sulfosuccinimidyl-6-(biotinamido)-hexanoate. Similar findings were observed with the disease causing mutant ΔF508[G404−N432]NBF1. Three different control experiments which involved (1) assays for known cytosolic *E. coli* enzymes, (2) immuno-gold electron microscopy with antibody having an epitope for the biotin moiety, and (3) tests for biotinylation of the cytosolic component, Enzyme 1 of the glucose phosphotransferase system, demonstrated that the spheroplasts used in this study are neither leaky nor permeable to Biotin-X-NHS. In addition, membrane-associated [G404−N432]NBF1, upon solubilization with Triton X-100, was found to bind to an ATP−agarose column and be released therefrom by elution with ATP, emphasizing retention of a native-like structure. In sharp contrast, NBF1 localizes to the cytosol when the [G404−N432]-N-terminal region is replaced with the maltose binding protein. The novel findings reported here implicate a role of the N-terminal region of NBF1 in its subcellular localization and are directly relevant to our understanding of the membrane structure, function, and trafficking of CFTR.

Cystic fibrosis is caused by mutations within the CFTR[1] protein, an integral membrane protein comprised within a single polypeptide chain of 1480 amino acids (Riordan et al., 1989). CFTR contains five major domains, including two nucleotide binding folds (NBF1 and NBF2), a regulatory domain (R), and two transmembrane regions (TMSs) (Riordan et al., 1989; Carol et al., 1993). The latter, at least in part, form an anion channel that is believed to require for optimal function both ATP binding and ATP hydrolysis mediated by one or both nucleotide binding folds (Thomas et al., 1991; Anderson et al., 1991a,b; Hartman et al., 1992; Smith et al., 1993; Ko et al., 1995; Carson et al., 1995) and mediated also by phosphorylation of the R domain by protein kinase A and/or other cellular kinases (Tabcharani et al., 1991; Cheng et al., 1991). The predominant anion translocated by CFTR is believed to be Cl^- (Riordan et al., 1989; Carol et al., 1993), although evidence for ATP translocation has been reported also (Resin et al., 1994; Schwiebert et al., 1995) but is currently subject to debate (Reddy et al., 1996; Li et al., 1996).

Over 400 putative disease-causing mutations have been reported by the CF Genetic Analysis Consortium (Tsui, 1992). Of these, the deletion mutation, ΔF508, residing near the center of NBF1 accounts for most cases of the disease (Kerem et al., 1990). Unlike wild type CFTR which at 37 °C normally moves from the endoplasmic reticulum to the Golgi apparatus, and finally to the plasma membrane, ΔF508 CFTR is unable to leave the endoplasmic reticulum (Cheng et al., 1990; Rich et al., 1991; Anderson et al., 1991a,b; Denning et al., 1992a,b). It is believed that, because of a misfolding problem (Thomas et al., 1992), ΔF508 CFTR is removed from the normal folding pathway and targeted for degradation. However, at 25 °C, ΔF508 CFTR evidently folds sufficiently to escape the degradation pathway and moves to the plasma membrane where it becomes partially functional as a Cl^- channel (Denning et al., 1992a,b; Li et al., 1993).

The cellular location of NBF1 has not been clearly resolved. The original model (Riordan et al., 1989) based

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[1] Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; NBF1, first nucleotide binding fold; NBF2, second nucleotide binding fold; TMSs, transmembrane spanning regions; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Biotin-X-NHS, sulfosuccinimidyl-6-(biotinamido)-hexanoate; EM, electron microscopy; ECPCR, expression cassette polymerase chain reaction; G6PDH, glucose-6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase.

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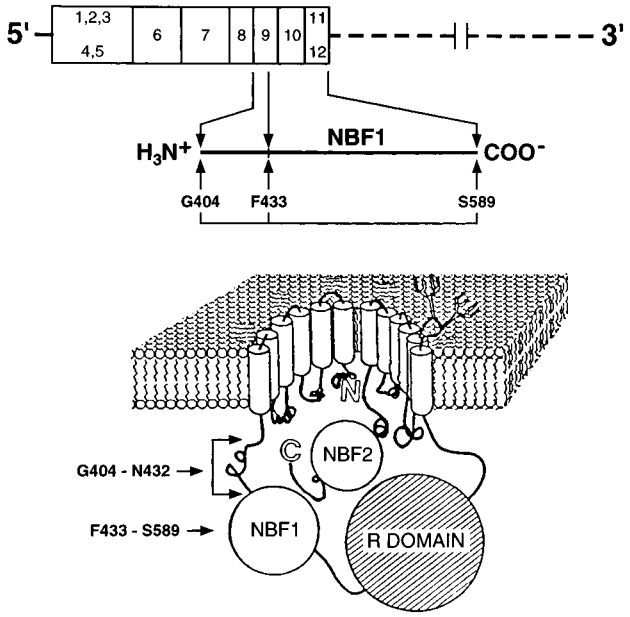

FIGURE 1: Diagram of the CFTR gene illustrating the region encoding NBF1. The nucleotide binding fold NBF1 was originally defined as comprising F433−S589 (Riordan et al., 1989) on the basis of its homology with other nucleotide-binding proteins. However, this definition excludes that part of exon 9 encoding [G404−N432] (upper diagram). The purpose of this study was to express the complete modular unit for [G404−N432]NBF1 encoded by exons 9−12, i.e., G404−S589, to establish whether NBF1 would target the membrane or remain in the cytosol as depicted in the original CFTR model (lower diagram) (Riordan et al., 1989).

only on primary sequence homologies and hydrophobicity analysis led to the view that NBF1 localizes exclusively in the cytosol. To date, no experimental data have been reported in support of a cytosolic location of NBF1. Rather, experimentally based models for CFTR or related transporters depict NBF1 as being membrane-assoicated (Arispe et al., 1992; Biachwal et al., 1993; Loo & Clarke, 1995). Thus, *in vitro* studies with a recombinant NBF1 protein preparation demonstrated its capacity to reconstitute with planar lipid bilayers and to mediate anion conductance (Arispe et al., 1992). Moreover, studies of an NBF1-like subunit of the bacterial histidine transporter (His-P) resulted in its labeling with a membrane impermeant probe from the extracellular surface (Biachwal et al., 1993). Finally, recent studies with P-glycoprotein (MDR-1), which exhibits considerable sequence homology with CFTR, implicate both nucleotide domains, NBF1 and NBF2, as being membrane-associated (Loo & Clarke, 1995).

To establish as directly as possible whether NBF1 of CFTR has a propensity to interact with biological membranes *in vivo*, we have examined this question using an *Eesherichia coli* expression system. As *E. coli* contain neither an endoplasmic reticulum nor a Golgi apparatus, NBF1 upon its expression is expected either to remain in the cytosol or to target the membrane. The cDNA for NBF1 ligated into the expression vector used in these studies includes precisely all of exons 9−12 of the CFTR gene (Figure 1). This modular unit encodes amino acid residues F433−S589, originally defined as NBF1 on the basis of homology arguments (Riordan et al., 1989), together with the prior 29 amino acids [G404−N432]. Experiments described below show that [G404−N432]NBF1 targets and incorporates into the *E. coli* cytoplasmic membrane with retention of its ATP binding function and implicate a role for the N-terminal G404−N432 region in this process.

EXPERIMENTAL PROCEDURES

Materials

Templates employed for the generation of NBFs were clone T16-1 and the plasmid pTM1 CFTR3ΔF508, which were obtained from the American Type Culture Collection and as a gift from M. Welsh (The University of Iowa), respectively. Primers used in the PCR procedures were synthesized in The Johns Hopkins University Protein/Peptide/DNA Synthesis Facility using an Applied Biosysems synthesizer, model 380B. The pET28b expression vector and *E. coli* strain BLR(DE3) were obtained from Novagen. The restriction enzymes and T4 DNA ligase were from New England BioLabs (NEB). The Gene Amp PCR reagent kit and AmpliTaq DNA Polymerase were purchased from Perkin-Elmer. The Plasmid Maxi Kit and Qiaquik gel extraction kit were from Qiagen, and the following were from Sigma: glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH), and ATP−agarose, where the agarose is attached to ribose hydroxyls of ATP. Water soluble Biotin-X-NHS was from Calbiochem/Novabiochem. Affinity-purified mouse IgG (against the biotin moiety) and goat secondary antibody (against mouse IgG complexed to EM grade colloidal gold) were obtained from Jackson Immuno Research Laboratories. The company Ted Pella supplied LR White, uranyl acetate, and other research chemicals used in the EM studies. Pierce provided the Micro Coomassie Dye Binding Assay Kit. Polyclonal rabbit IgG having an epitope for MBP was from NEB. All other reagents were of the highest quality available from commercial sources. Purified polyclonal goat IgG against Enzyme I, a cytoplasmic *E. coli* protein associated with the glucose phosphotransferase system, was a gift from S. Roseman (Department of Biology, The Johns Hopkins University).

Methods

Construction of the Prokaryotic Expression Vector Containing the cDNA Encoding [G404−N432]NBF1 and the ΔF508 Mutant Protein. The expression cassette polymerase chain reaction, ECPCR (Saiki et al., 1985; MacFerrin et al., 1990), was employed to synthesize the cDNA encoding [G404−N432]NBF1 and its ΔF508 mutant form as summarized in Figure 2A. Restriction enzyme sites (*Nco*I and *Xho*I), GC clamps, and a stop codon were incorporated in the primers with the *Nco*I site in the forward primer and the *Xho*I site in the reverse primer. The sequences of the primers used to define and amplify the DNA corresponding to [G404−N432]NBF1 from base 1342 (G404) to base 1899 (S589) are as follows: forward primer (27 bases), 5′-GCGCCCATGGGATTTGGGGAATTATTT-3′; and reverse primer (34 bases), 5′-CGCGCTCGAGTTAGCTTTCAAATATTTCTTTTTC-3′.

The ECPCR was conducted in a 100 μL reaction volume composed of 20 mM Tris-HCl (pH 8.8), 2.5 units of TaqStart/AmpliTaq DNA polymerase, 0.15 μM primers, 22.5 ng of template, 100 μM dNTPs, 1.5 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, and 0.1% Triton X-100. The conditions for each PCR cycle were as follows: denaturation, 94 °C for 1 min; annealing, 60 °C for 1 min; and elongation, 72

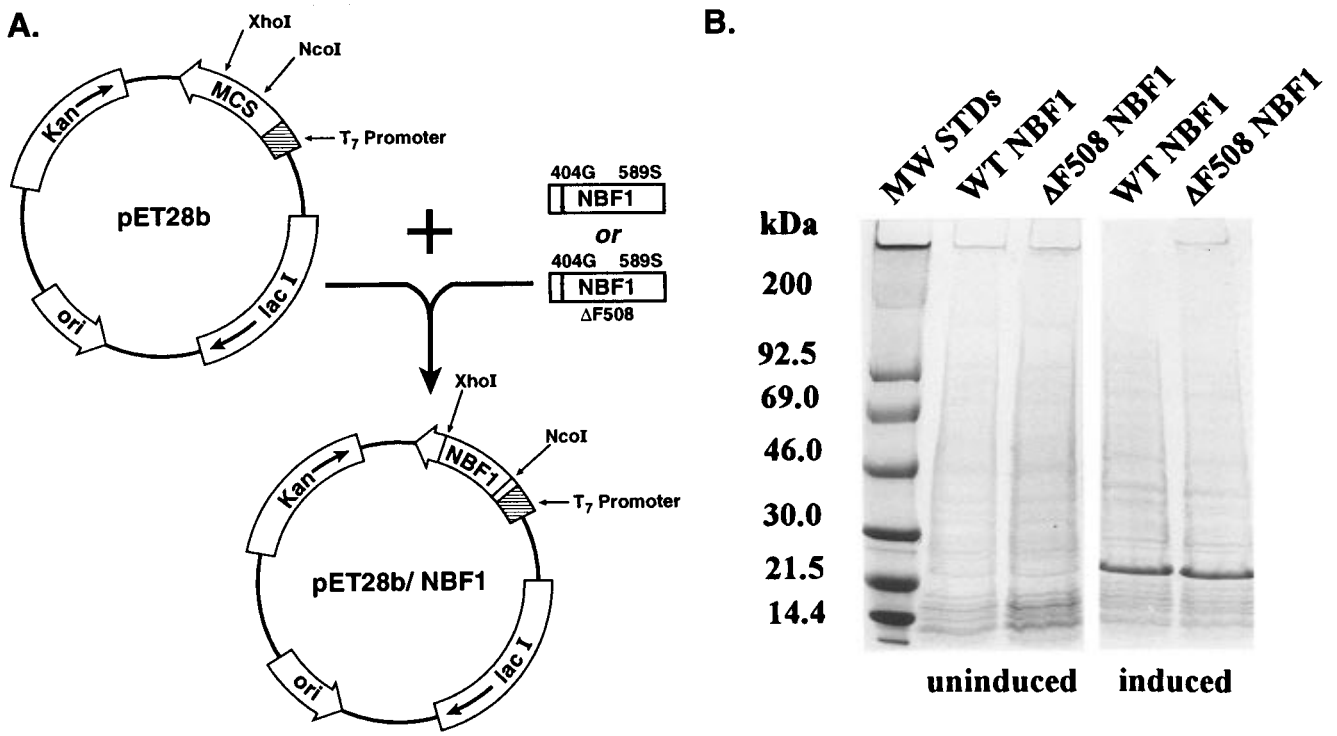

FIGURE 2: (A) Construction of the prokaryotic expression vector for [G404−N432]NBF1 and ΔF508[G404−N432]NBF1. PCR-generated cDNA fragments of CFTR encoding both the wild type and ΔF508[G404−N432]NBF1 proteins were incorporated into the pET28b vector at the *Nco*I and *Xho*I sites under the control of the T7 promoter (see Methods). (B) Analysis by SDS−PAGE of the wild type and the ΔF508[G404−N432]NBF1 protein after expression in *E. coli*. Cells were induced with 0.3 mM IPTG at 37 °C for 3 h. SDS−PAGE was then performed by the method of Laemmli (1970) on 100 μL of the whole cell culture (see Methods) and stained with Coomassie blue dye. The arrow indicates the expected molecular mass (186 amino acid residues, ∼21 kDa) of the [G404−N432]NBF1 protein.

°C for 1 min. The cycle was repeated 30 times. Clone T16-1 and plasmid pTM1 CFTR3ΔF508 were used as PCR templates to amplify the wild type and ΔF508 forms of [G404−N432]NBF1, respectively. These templates were purified by use of Qiagen's Plasmid Maxi-Kit. The PCR-amplified products (580 and 577 base pairs, respectively) were digested with *Nco*I and *Xho*I restriction enzymes and the digested PCR products were subsequently subjected to agarose gel electrophoresis and further purified by use of Qiagen's Qiaquik Gel Extraction Kit followed by ligation into a *Nco*I/*Xho*I-cut pET28b prokaryotic expression vector.

Competent INVαF′ *E. coli* cells were transformed with the above ligation mixture and grown on LB/agar/kanamycin plates. Plasmids purified from kanamycin resistant colonies were subjected to *Hinc*II restriction enzyme digestion analysis. In addition, the plasmid was sequenced via an automated sequencing method (Ansorg, 1987) by the Genetic Resources Core Facility at The Johns Hopkins University to confirm the fidelity of the PCR step and formation of the proper construct. Following this, the correct plasmids were used to cause BLR(DE3) *E. coli* strain to express the [G404−N432]NBF1 protein. All other molecular biological methods employed were conducted by standard procedures (Sambrook et al., 1989).

Expression of [G404−N432]NBF1 and Membrane Preparation from E. coli Cells. BRL(DE3) *E. coli* cells harboring the appropriate recombinant plasmids were grown at 37 °C in LB media containing kanamycin (30 μg/mL) until the absorbance at 600 nm reached approximately 0.5 unit (∼2 × 10^8 cells/mL). Protein production was induced inside the *E. coli* cells with the addition of isopropyl thiogalactoside (IPTG) at 0.3 mM. A combination of lysozyme and sodium deoxycholate was used to lyse the cells (Witholt et al., 1975; Marston, 1987). The cell lysate was centrifuged (Sorvall Centrifuge, model RC 2-B; GSA 4.25 rotor; 5000*g*, 30 min at 4 °C) to remove broken cell walls, inclusion bodies, and other debris, after which the supernatant was further fractionated using a Spinco centrifuge fitted with a Ti 65 rotor at 40000*g* for 1 h at 4 °C to obtain the membrane fraction (Filip et al., 1973). To visualize the [G404−N432]NBF1 proteins, SDS−PAGE was carried out as originally described by Laemmli (1970) and the gels were stained with Coomassie blue dye.

Expression of NBF1 Fused to the Maltose Binding Protein (MBP). Recombinant plasmids containing MBP−NBF1 were prepared exactly as previously described (Ko et al., 1993). MBP−NBF1 was then expressed in TB1 *E. coli* cells after induction with IPTG, also exactly as previously described (Ko et al., 1993).

N-Terminal Sequence Analysis. Gas phase sequencing of the N terminus (Edman, 1950; Hunkapillar & Hood, 1983) of the overexpressed proteins from the polyvinylidenedifluoride (PVDF) membrane was performed to establish that the overexpressed product was the desired [G404−N432]NBF1. To do this, the *E. coli* whole cell lysates containing ∼150 pmol of [G404−N432]NBF1 were electroblotted from the SDS−PAGE gel onto a PVDF membrane. The ∼21 kDa molecular mass protein was excised and subjected to Edman degradation chemistry (Edman, 1950; Hunkapillar, 1983), using an Applied Biosystems 475A Protein Sequencing System that employed both gas- and liquid-pulsed phases.

Determination of Protein. Proteins were estimated by the method of Bradford (1973) or by using the Micro Coomassie Dye Binding Assay Kit supplied by Pierce.

Western Blot Analysis. After conducting SDS−PAGE, the proteins on the gel were transferred electrophoretically onto a PVDF membrane (1 h at 100 V, 0.2 A, at 4 °C in 10 mM CAPS/10% methanol transfer buffer at pH 11). The product was then blocked for 1 h with 2% BSA plus 5% nonfat dry milk in PBS-T (80 mM Na_2HPO_4/20 mM NaH_2PO_4/100 mM NaCl/0.1% Tween-20 at pH 7.5), incubated for 1 h at 23 °C with the monoclonal antibody having an epitope against NBF1, and then further incubated for 1 h at 23 °C with the secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG). The immunoreactive bands were detected by the enhanced chemiluminescence (ECL) system of Amersham Life Sciences.

Preparation of Spheroplasts. Spheroplasts from the *E. coli* expressing [G404−N432]NBF1 proteins as noted above were prepared essentially as described by Witholt et al. (1976) with a slight modification. Briefly, 2 mL of 200 mM HEPES at pH 8.0 was added to resuspend the cell pellet harvested from a 50 mL cell culture medium. To this suspension was added 2 mL of HES buffer [200 mM HEPES (pH 8.0), 1.0 M sucrose, and 1 mM EDTA] to give a concentration of 10−40 mg of cells/mL. Then, the mixture was subjected to a lysozyme treatment (60 μg of lysozyme/ml) for 30 min on ice followed by osmotic shock with an equal volume of water for 30 min at 25 °C. $MgCl_2$ at a final concentration of 20 mM was added to stabilize the spheroplasts. Formation of the spheroplasts was followed by the decrease in absorbance at either 399 or 450 nm, and their presence was visualized by EM after staining with 1% uranyl acetate.

Biotinylation of Spheroplasts. Biotinylation was performed at 23 °C for 5 min either on the intact spheroplasts prepared as above or on lysed spheroplasts made by sonication with Bronwill's Biosonik sonicator (30 s for three times with the probe intensity set at 20% maximum). The biotinylating agent, Biotin-X-NHS, was dissolved in water. The working concentration of the probe was 2 mM for 184 μL of spheroplasts or lysed spheroplasts, both prepared from 1.3 mL of *E. coli* cell culture. After completion of the biotinylation, the lysed spheroplasts were centrifuged at 16000*g* for 5 min to give supernatant and pellet fractions (see Figure 4B). The intact spheroplasts and the supernatant and pellet from the lysed spheroplasts were subjected to SDS−PAGE, after which the separated proteins were electrophoretically transferred onto a nitrocellulose membrane. Then, the biotinylated proteins were detected by colorimetric enzymatic reactions with either alkaline phosphatase (APase) or horseradish peroxidase (HRP) both conjugated to streptavidin.

G6PDH and IDH Assays. The supernatant amounting to 250 μL obtained by centrifuging 250 μL of spheroplasts (equivalent to 1.6 mL of *E. coli* cell culture) at 16000*g* for 5 min at 4 °C was used to determine the presence and activities of cytosolic enzymes, G6PDH, and IDH. The assays were performed according to the protocol (procedure 345-UV) furnished by Sigma. The reaction producing NADPH was monitored spectrophotometrically by measuring the increase in absorbance at 340 nm.

Binding of [G404−N432]NBF1 to an ATP−Agarose Column. The membrane fraction prepared as described above from 100 mL of *E. coli* cell culture was solubilized in 200 μL of ATP column buffer containing 10 mM MOPS (pH 7.2), 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100 (v/v), and 1 mM phenylmethanesulfonyl fluoride (PMSF). This membrane fraction contained approximately 1 mg of the total protein and approximately 30% of the total membrane protein corresponding to [G404−N432]NBF1 as estimated from the extent of staining of the SDS−PAGE gel with Coomassie blue dye. The solubilized membrane fraction was loaded onto a 1 mL ATP−agarose affinity column (contained within a 3 mL syringe) at a flow rate of 0.1 mL/min. Subsequently, the column was washed with 12 mL of column buffer, after which the [G404−N432]NBF1 fraction was eluted with 2 mL of 100 mM ATP. The protein eluted from the colum was subjected to SDS−PAGE and Western blot analysis. The product was visualized on the SDS−PAGE gel by staining with Coomassie blue dye and on the Western blot by use of the ECL detection system, after completing a treatment with a monoclonal antibody raised against NBF1.

EM Preparations. *(A) Fixation of spheroplasts*. The spheroplasts were pelleted, and the medium was replaced with 1.5 mL of 3% paraformaldehyde and 0.05% glutaraldehyde in phosphate-buffered saline solution (PBS) containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ at pH 7.3 in a 1.5 mL Eppendorf tube. The uncapped tubes containing the fixative were placed in an ice bath between two 325 mL water loads in a microwave oven (Pelco, model 3400). Samples were pulsed with microwaves (Giberson & Demaree, 1995; Parton, 1995) with a 10 s pulse/20 s rest/10 s pulse cycle at 100% power with the fixative temperature not exceeding 30 °C. The pellets remained in the fixative for 5 min and were then rinsed briefly in PBS followed by a rinse with 0.1 M sodium cacodylate, both containing 50 mM ammonium chloride. Samples were then pulsed as before with microwaves in $K_3Fe(CN)_6$ solution reduced in 1% osmium tetroxide, rinsed in distilled deionized water, and stained in 1% uranyl acetate aqueous solution for 15 min. The pellets were quickly dehydrated through a graded series of ethanol, infiltrated with Eponate (Pella), and cured overnight at 50 °C. Ultrathin sections were cut on a low-angle Diatome diamond knife and collected on 400 mesh Formvar-coated nickel grids.

(B) Fixation of E. coli cells. The bacteria were pelleted at 13000 rpm in an Eppendorf microcentrifuge for 10 s and then resuspended in 1 × PBS at pH 7.4 for 1 min. Cells were fixed in suspension with 3% formaldehyde plus 2% glutaraldehyde in 30 mM phosphate buffer at pH 7.25 for 1 h at 4 °C on a rotator. Cells were pelleted at 8000 rpm in the Eppendorf microcentrifuge for 1 min and rinsed in phosphate buffer, three changes, for 10 min each. Excess glutaraldehyde was reduced for 1 h in 50 mM NH_4Cl at 4 °C, and all subsequent reaction mixtures were kept cold. Pellets were rinsed in 30 mM phosphate buffer as above. Residual phosphate buffer was substituted with 0.1 M sodium cacodylate at pH 7.4 for 10 min. Cell pellets were fixed under a fume hood in 1% osmium tetroxide in 0.1 M sodium cacodylate at pH 7.4 for 1 h to stabilize the membranes. Samples were rinsed in distilled water, two changes, for 5 min each, and then dehydrated in a graded series of ethanol up to 90%. Pellets were gradually infiltrated with a solution containing 2 parts of LR white and 1 part of 90% ethanol and rotated overnight in the cold room at 4 °C. Infiltration was continued the following day with 100% LR white twice over a 4 h period. Samples were transferred to fresh LR white, in gelatin capsules, and polymerized in a 50 °C oven overnight. For microtomy, the cured gelatin encapsulated

blocks were trimmed with an alcohol-cleaned razor blade, and semithin sections (0.5 μm) were cut with a glass blade to expose the embedded section. Then, thin sections (70 nm) were cut with a Diatome low-angle diamond knife on a Reichert Ultra Cut E Microtome. Selected thin sections were transferred to 400 mesh Formvar-coated nickel grids and immunolabeled within 24 h.

Immunolabeling. (A) Spheroplasts following Biotinylation. Grids were floated on 1% sodium metaperiodate aqueous solution for 15 min and rinsed with, and then incubated on, 0.14% glycine in Tris-buffered saline solution (TBS) for 15 min. Samples were blocked in 5% bovine serum albumin (BSA) in Tris-based saline solution (TBST, 30 mM Tris-HCl/137 mM NaCl/0.1% Tween-20) for 10 min and then incubated in mouse anti-biotin primary antibody (1:50) in TBST containing BSA for 1 h at 25 °C. After several rinses in TBS, the grids were reblocked and then floated on 12 nm gold-conjugated, goat anti-mouse IgG (1:40) in TBS for 1 h at room temperature. Sections were rinsed, dried, and stained with lead citrate, after which they were viewed on a Zeiss 10B transmission electron microscope (TEM) operating at 60 kV. The magnification was calibrated using an appropriate diffraction grating replica. A Photomicrograph Scale Marker was used to draw scale marks on the print of each micrograph.

(B) E. coli Cells following Expression of NBF1 in Fusion with the Maltose Binding Protein (MBP). The experimental conditions for immunolabeling *E. coli* cells were identical to those employed for spheroplasts except that the size of the gold particles was 5 nm instead of 12 nm. MBP−NBF1 was visualized by using either a monoclonal antibody against NBF1 or a polyclonal antibody having the epitope for MBP, followed by reaction with 5 nm gold-conjugated secondary antibodies (1:40).

RESULTS

Rationale and Experimental System. To address the question of whether the first nucleotide binding fold (NBF1) of CFTR preferentially assumes a cytosolic or a membrane location, two factors were initially taken into consideration. The first related to the boundaries of the cDNA segment within the NBF1 region of the CFTR gene that should be selected for expression. Rather than expressing NBF1 as it is classically defined, i.e., as F433−S589 (Figure 1), we chose to express this region with the 29 prior amino acids, G404−N432. In this way, the complete modular unit consisting precisely of exons 9−12 of the CFTR gene would be expressed. The second factor considered was the cell line to be selected for expressing the cDNA encoding [G404−N432]NBF1. *E. coli* was chosen both because it has neither an endoplasmic reticulum nor a Golgi apparatus and because of recent work (Geller et al., 1996) with the N-terminal half of Ste6, a yeast plasma membrane protein related to CFTR. These studies have shown that Ste6 targets and incorporates into the *E. coli* cytoplasmic membrane with the same topology as in its native eukaryotic plasma membrane environment. Thus, obtaining a direct answer to the fundamental question of whether [G404−N432]NBF1 prefers to localize to a cystosolic or a membrane compartment *in vivo* would be both justified and greatly simplified by its expression in *E. coli*. Depending on the results obtained, these studies could be pursued in greater depth in future work in the more complicated eukaryotic system where at least three different membrane compartments are involved in the trafficking pathways of CFTR.

Expression of [G404−N432]NBF1 in E. coli. To express [G404−N432]NBF1 and its ΔF508 mutant form in *E. coli*, the expression cassette polymerase chain reaction was employed to precisely synthesize cDNA fragments of CFTR and ΔF508 CFTR encoding these proteins (see Methods). As shown in Figure 2A, PCR-amplified fragments thereof were then inserted into the *Xho*I and *Nco*I restriction sites of the pET28b prokaryotic expression vector operating under the control of the T7 promoter. These recombinant plasmids were used to transform *E. coli* strain BLR(DE3) followed by induction with IPTG. Figure 2B shows that the expressed wild type and the ΔF508 mutant protein, both exhibiting an apparent molecular mass near 21 kDa, are observed only in SDS−PAGE gels loaded with *E. coli* that have been induced with IPTG.

Membrane Localization of the [G404−N432]NBF1 Protein and Its ΔF508 Mutant Form. To establish the subcellular localization of the expressed NBF1 proteins, IPTG-induced *E. coli* cells transformed with the expression vectors containing [G404−N432]NBF1 and ΔF508[G404−N432]NBF1 cDNAs were lysed with lysozyme and deoxycholate and centrifuged at low speed to remove cell debris and broken cells (see Methods). During this step, any inclusion bodies were sedimented and entirely eliminated. The resulting supernatant was then centrifuged at a higher *g* force to obtain the membrane and cytosolic fractions. Following SDS−PAGE of the aliquots of these fractions, proteins were transferred electrophoretically onto a PVDF membrane and treated with a monoclonal antibody against NBF1. Figure 3A shows that [G404−N432]NBF1 and its ΔF508 mutant forms are detected only in the membrane fraction. The very lightly stained band in the cytosolic fraction is nonspecific, as it is detected in the absence of the primary antibody (Figure 3B). As shown in Figure 3C, SDS−PAGE gels of the same membrane and cytosolic fractions, when subjected to electrophoresis and stained with Coomassie dye, also reveal distinct bands with apparent molecular masses near 21 kDa. As in Figure 3A, it can be seen that these bands are not observed in the cytosolic fraction. Identification of the bands as [G404−N432]NBF1 products was made in separate experiments by N-terminal sequencing analysis after SDS−PAGE and electrophoretic transfer of proteins onto PVDF membranes (Figure 3D). Significantly, the first amino acid residue detected is G404, and all other amino acid residues through K447 correspond exactly to those within CFTR. The electron micrograph presented in Figure 3E, which represents the *E. coli* membrane fraction used in these experiments, confirms its expected double membrane, vesicular properties, with diameter sizes on the average of ∼90 nm. It is important to note that there is no evidence of darker-stained material without a double membrane, characteristic of inclusion bodies.

Biotinylation of [G404−N432]NBF1 and Its ΔF508 Mutant Form in E. coli Spheroplasts. As shown in Figure 4A, interaction of [G404−N432]NBF1 with the *E. coli* cytoplasmic membrane may occur via association with its inner surface, by partial insertion into this surface, or via a transmembrane insertion. To distinguish among these

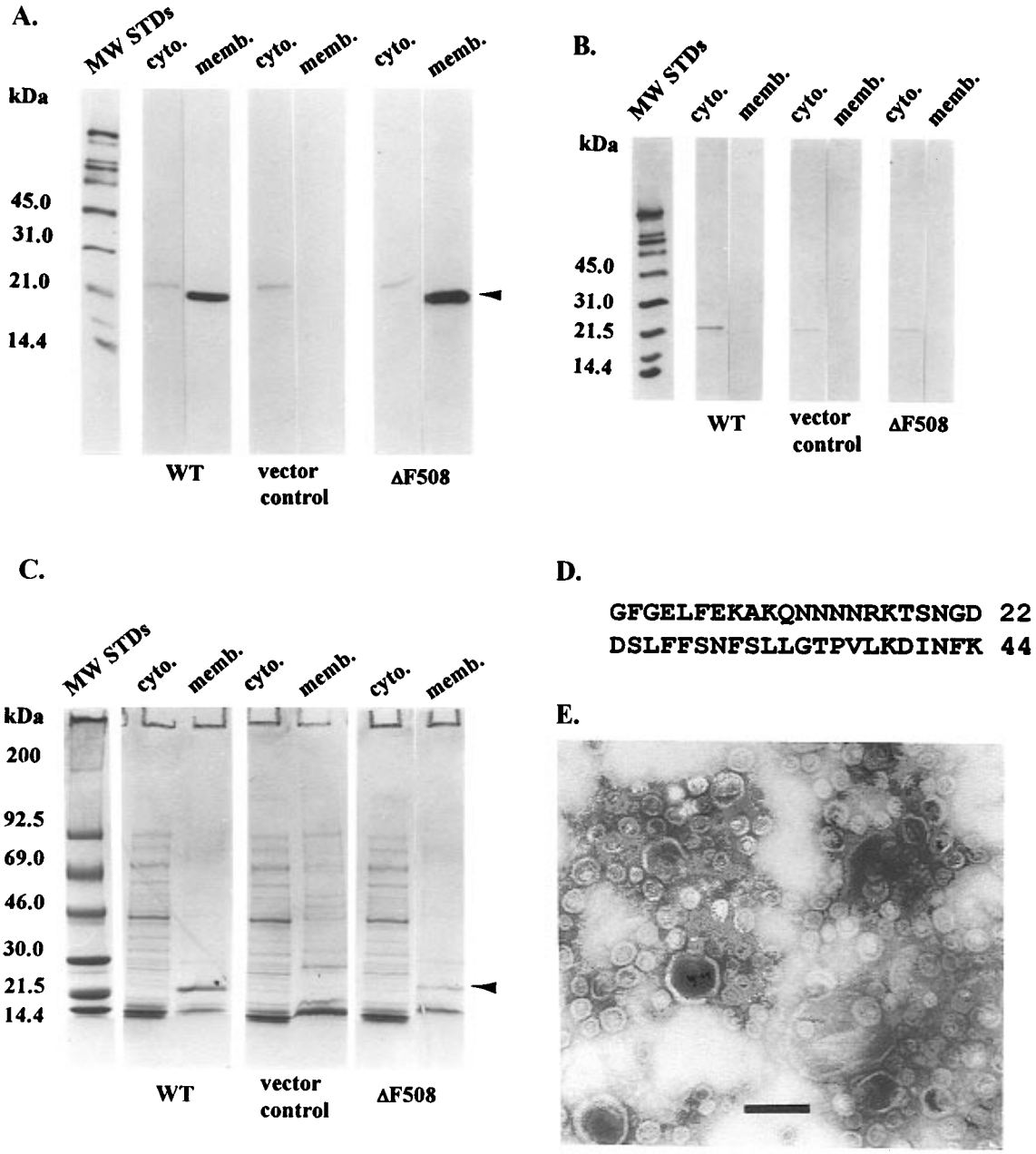

FIGURE 3: Membrane localization of [G404−N432]NBF1 and its ΔF508 mutant form after expression in *E. coli*. (A) Western blot analysis. After expression of [G404−N432]NBF1 and its ΔF508 mutant form in *E. coli*, cells were subfractionated into membrane and cytosolic fractions exactly as described under Methods and subjected to SDS−PAGE using 20 μL corresponding, respectively, to 5 and 0.125 mL of cell culture. Then, the proteins were transferred electrophoretically onto a PVDF membrane and subsequently detected by the ECL system using a monoclonal antibody raised against NBF1, also described under Methods. The arrow designates the NBF1 proteins. (B) Control for experiment A. All conditions are identical to those in part A except the treatment with the monoclonal primary antibody was omitted in order to demonstrate that the bands observed in part A in the cytosolic fraction, but not those representing the membrane fraction, are nonspecific. (C) Detection of membrane association of [G404−N432]NBF1 and its ΔF508 mutant form by Coomassie blue dye staining. Conditions were identical to those in part A except the proteins were not transferred to the PVDF membrane after the SDS−PAGE was performed. Rather, the gel was stained directly with Coomasie blue dye. The band indicated by the arrow is present only in the membrane fraction. (D) Confirmation that the ~21 kDa band is [G404−N432]NBF1 by N-terminal Sequencing. N-Terminal sequencing analysis was performed on the 21 kDa band after transfer onto a PVDF membrane (see Methods). Forty-four amino acid residues which included all 29 corresponding to G404-N432 plus 15 additional residues within NBF1 (F433−K447) were sequenced. (E) Confirmation that the fraction designated as "membrane" in parts A−C contains vesicles. The membrane fraction prepared as described under Methods was negatively stained with uranyl acetate and visualized by electron microscopy. The bar corresponds to 0.1 μm. Only membrane vesicles are observed. There is no sign of darker-stained material without a double membrane, characteristic of inclusion bodies.

possibilities, it seemed simplest to prepare intact *E. coli* spheroplasts and first establish whether [G404−N432]NBF1 is accessible from the outside to a membrane impermeant labeling agent. For this purpose, the water soluble, membrane impermeant agent Biotin-X-NHS which covalently labels accessible lysine residues via its biotin moiety (Von Boxberg et al., 1990) was used.

The design of the labeling experiment is illustrated in Figure 4B. It entails incubating both intact spheroplasts (Figure 4C) and lysed spheroplasts with Biotin-X-NHS, followed by SDS−PAGE, electrophoretic transfer to nitrocellulose membranes, and detection of biotin-labeled proteins with avidin conjugated to either APase (Figure 5A, upper panel) or HRP (Figure 5A, lower panel). The fifth lane from

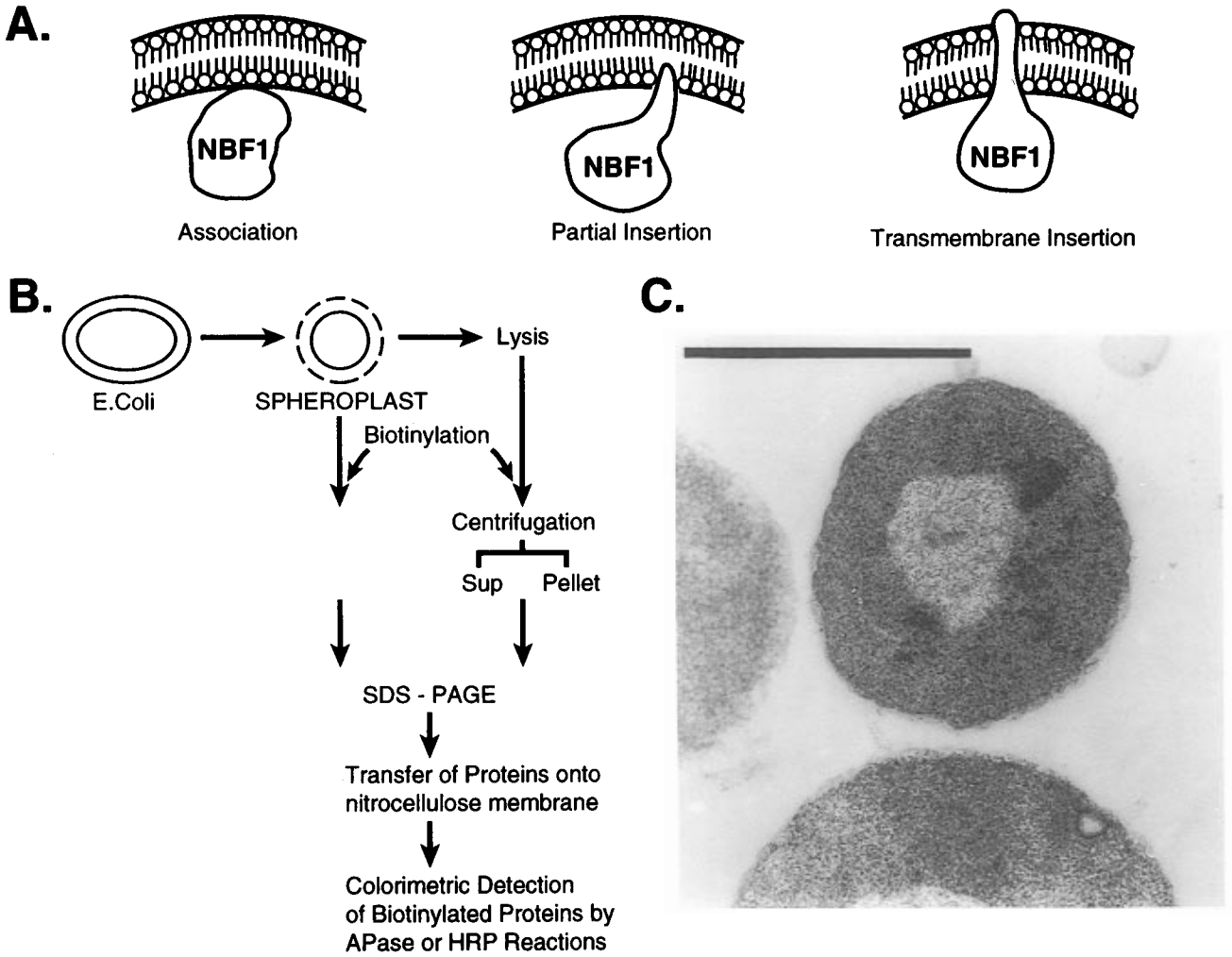

FIGURE 4: (A) Diagram illustrating three possible modes of interaction of wild type and ΔF508[G404−N432]NBF1 with the *E. coli* membrane. NBF1 could be loosely associated (left panel), partially inserted (center panel), or traversing the membrane (right panel). (B) Scheme designed to establish whether NBF1 can be labeled in spheroplasts with a membrane impermeable biotinylating probe (see Methods for details). (C) Electron micrograph of *E. coli* spheroplasts containing membrane-associated NBF1. Thin sections of the spheroplasts were obtained and visualized exactly as described under Methods. The bar corresponds to 1.0 μm.

the left in Figure 5A, which represents insoluble material containing the membrane fraction (pellet) obtained after biotinylating lysed spheroplasts derived from *E. coli* expressing [G404−N432]NBF1, shows a distinct band near 21 kDa after detection with either avidin conjugated to APase or HRP. This band is absent in the cytosolic (supernatant) fraction (lane 4 from the left). It is absent also in both cytosolic (supernatant) and membrane (pellet) fractions obtained after biotinylating lysed spheroplasts derived from *E. coli* transformed with vector alone (lanes 2 and 3 from the left, respectively).

In lanes 6−9 from the left, which summarize results obtained upon incubating intact spheroplasts with the biotinylating agent, it can be seen that the band near 21 kDa corresponding to [G404−N432]NBF1 is present in the spheroplasts derived from *E. coli* expressing this protein (the eighth lane) but not in the same fraction derived from the *E. coli* containing vector alone (the seventh lane). It can also be seen that omission of the biotinylating agent from the incubation medium of intact spheroplasts, as expected, eliminates all protein bands detected by the two avidin-conjugated agents (the sixth and the ninth lanes from the left).

Figure 5B shows that results essentially identical to those described above for wild type [G404−N432]NBF1 were obtained in biotinylation experiments with the ΔF508 mutant form. The third lane from the left in this figure, which represents the membrane fraction (pellet) obtained after biotinylating lysed spheroplasts derived from *E. coli* expressing ΔF508 [G404−N432]NBF1, shows a distinct band near 21 kDa detected with both APase (upper panel) and HRP (lower panel) avidin-conjugated agents. This band is absent in the cytosolic (supernatant) fraction (the second lane). In the fourth lane, which represents results obtained in similar experiments after incubating intact spheroplasts with the biotinylating agent, a band near 21 kDa detected with the avidin- conjugated agents is observed. In a control experiment, deletion of the biotinylating agent eliminates all bands, as expected (the fifth lane).

Finally, two other points should be made. First, in experiments not reported here, bands in the 21 kDa region observed in the above biotinylation experiments (Figure 5A,B) to interact with avidin-conjugated agents were positively identified as [G404−N432]NBF1 products by N-terminal sequence analysis. Second, the amount of [G404−N432]NBF1 biotinylated in intact spheroplasts relative to the total amount of overexpressed [G404−N432]NBF1 protein is estimated to be about 50% (Figure 5A, compare lane 8 and lane 5).

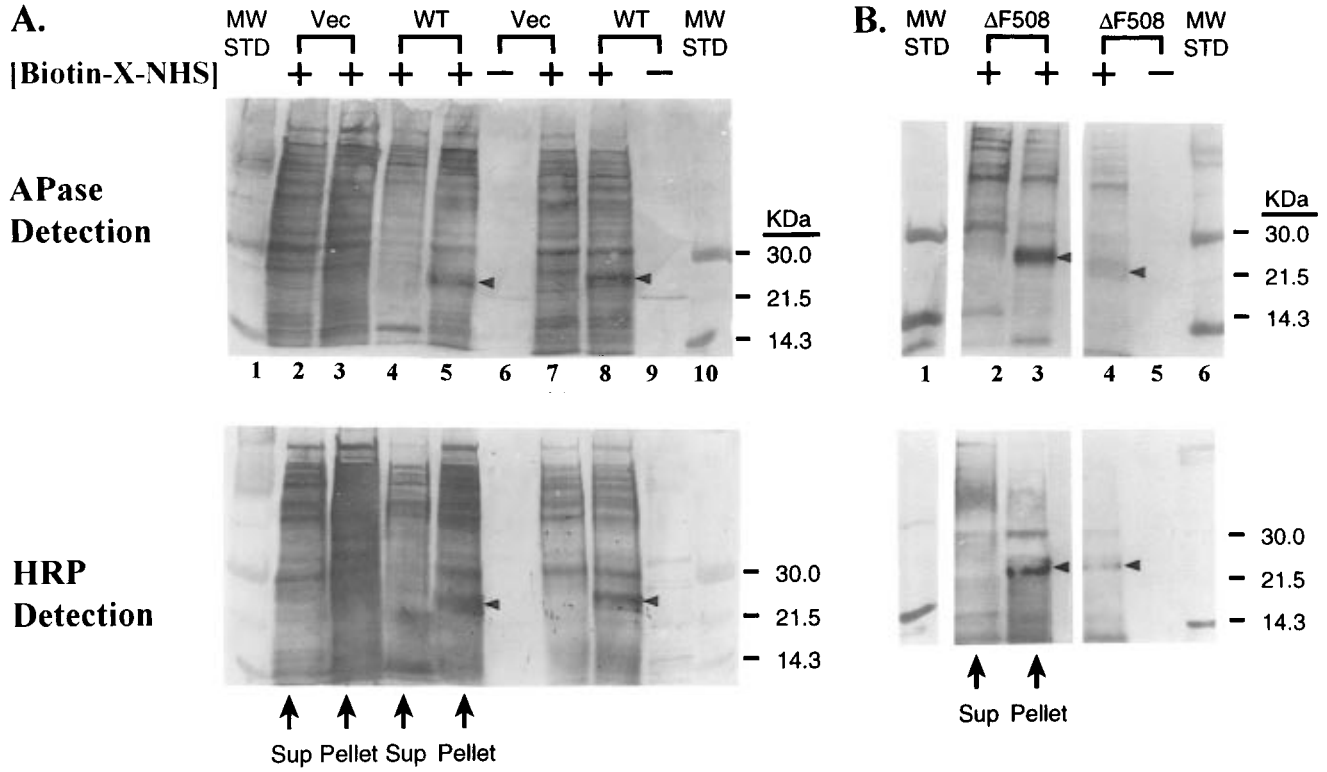

FIGURE 5: (A) Biotinylation of wild type [G404−N432]NBF1 in *E. coli* spheroplasts. After preparation and biotinylation of the spheroplasts with the impermeant probe Biotin-X-NHS exactly as described under Methods, spheroplasts derived from 50 μL of cell culture were subjected to SDS−PAGE. Biotinylated proteins were electrophoretically transferred onto a nitrocellulose membrane and subjected to an interaction with avidin conjugated either to alkaline phosphatase APase (upper panel) or to horseradish peroxidase, HRP (lower panel). The biotinylated proteins were detected colorimetrically by use of chromogenic substrates for each type of enyzme. Lanes 1 (far left) and 10 (far right) represent molecular mass markers. Lanes 2−5 represent proteins derived from biotinylating lysed spheroplasts, whereas lanes 6−9 represent proteins derived from intact spheroplasts. The arrow designates [G404−N432]NBF1, as determined by biotinylation and N-terminal sequence analyses (see Methods). Spheroplasts harboring only the vector alone fail to give rise to most bands as expected (lanes 6 and 9). (B) Biotinylation of ΔF508[G404−N432]NBF1 in *E. coli* spheroplasts. Conditions were exactly as those described in part A. The arrow designates ΔF508[G404−N432]NBF1 as determined by biotinylation and N-terminal sequence analysis (see Methods). Lanes 2 and 3 represent proteins derived from intact spheroplasts that had been lysed prior to biotinylation, whereas lane 4 represents proteins from intact spheroplasts that had been subjected to biotinylation. In lane 5, the biotinylation agent was deleted in the experiment with intact spheroplasts.

Control Experiments Demonstrating That the Spheroplasts Used for Biotinylation Studies Were Intact and That the Probe Used Is Impermeant. Although the above studies clearly implicate a transmembrane orientation of wild type [G404−N432]NBF1 and its ΔF508 mutant form, it could be argued that these proteins are bound to the cytosolic membrane surface and that biotinylation is due to spheroplasts of poor quality that are readily permeable to the agent (Biotin-X-NHS) used. Results of four different types of experiments failed to provide evidence for this possibility. First, freshly prepared spheroplasts fixed with paraformaldehyde/glutaraldehyde and stained with osmium tetroxide appear intact under the electron microscope (Figure 4C). Second, spheroplasts allowed to age, even for 2 h, exhibit little or no leakage of the cytoplasmic enzymes isocitrate dehydrogenase (IDH) or glucose-6-phosphate dehydrogenase (G6PDH) (Figure 6A). Third, when freshly isolated spheroplasts are biotinylated and then incubated with a monoclonal antibody that recognizes biotin, and subsequently with a secondary antibody that is conjugated with 12 nm gold particles, the resultant gold labeling appears almost exclusively in the region of the membrane, or within membranes that are in the process of vesiculating (Figure 6B). This is in sharp contrast to the almost exclusive cytosolic location of NBF1 described below upon modification of the N-terminal region (Figure 7C,D). Finally, and perhaps most importantly, Figure 6C shows that the strictly cytosolic protein of the *E. coli* glucose phosphotransferase system (Saffen et al., 1987), called Enzyme I, which contains 34 lysine residues, is not biotinylated in intact spheroplasts under exactly the same conditions that resulted in biotinylation of wild type [G404−N432]NBF1 and its ΔF508 mutant form (Figure 5A,B). In experiments not shown here, and as expected, Enzyme I was subjected to biotinylation when spheroplasts had been previously lysed.

Membrane-Bound [G404−N432]NBF1 Retains Its Capactiy To Bind ATP. In previous studies, we and others have demonstrated that NBF1 has the capacity to bind ATP (Thomas et al., 1991; Hartman et al., 1992; Ko et al., 1993). Therefore, if [G404−N432]NBF1 upon insertion into the *E. coli* membrane has folded properly, it should be possible to solubilize this domain in a nonionic detergent and demonstrate its capacity to bind ATP. Solubilization of [G404−N432]NBF1 was readily achieved using a buffer containg 0.1% Triton X-100 (v/v) (see Methods). After addition of this fraction to an ATP−agarose column followed by washing with column buffer, [G404−N432]NBF1 was retained but could be released by the addition of a buffer containing ATP. Significantly, this procedure resulted in homogeneous [G404−N432]NBF1 that could be visualized

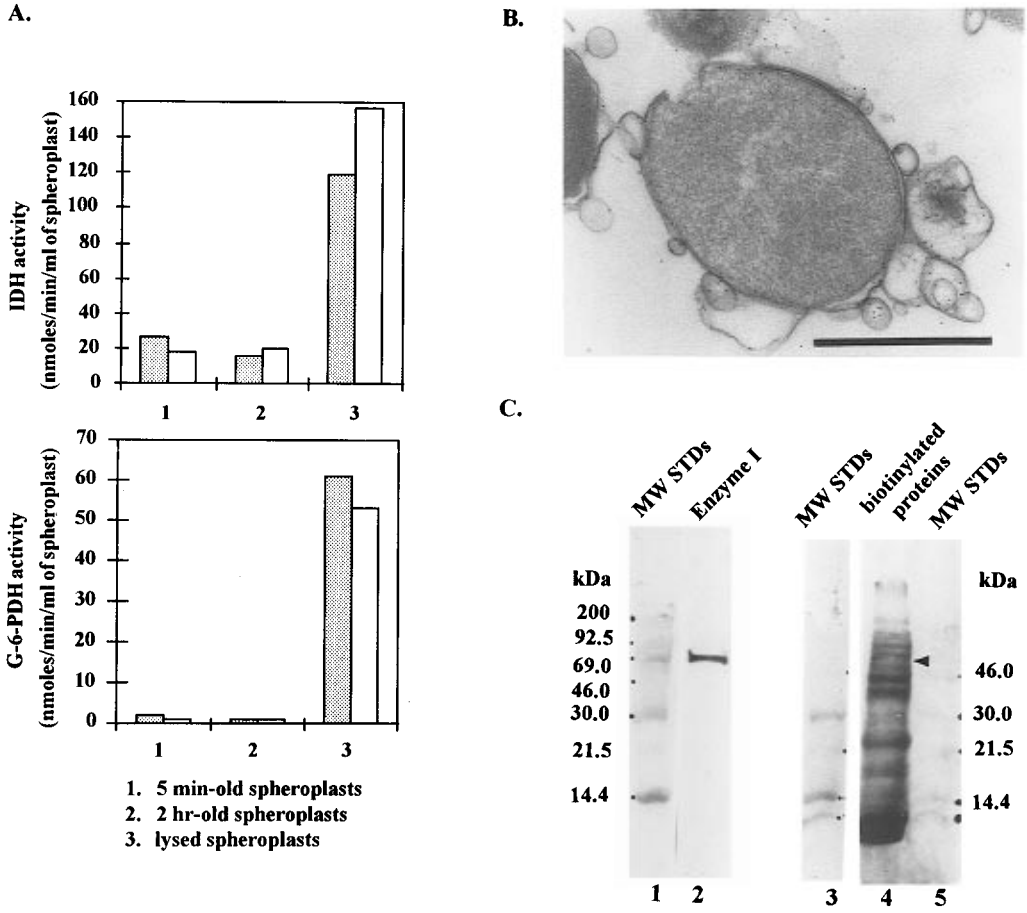

FIGURE 6: Controls demonstrating that the spheroplasts used for biotinylation studies were intact and that the probe used is impermeant to the membrane. (A) Inaccessibility of cytosolic enzymes. Spheroplasts were assayed for isocitrate dehydrogenase (upper panel) and glucose-6-phosphate dehydrogenase (lower panel), prior to and after lysis exactly as described in Methods. Dotted and clear bars represent enzyme activities derived, respectively, from spheroplasts containing [G404−N432]NBF1 and those in which this protein is absent. (B) Immunogold labeling of spheroplasts after biotinylation with Biotin-X-NHS. Spheroplasts were first biotinylated with 2 mM Biotin-X-NHS for 5 min on ice (see Methods) prior to fixation with glutaraldehyde and paraformaldehyde in a mirowave oven. Thin sections (90 nm) of the treated spheroplasts were then made. The biotinylated proteins were detected by reaction with a monoclonal antibody that recognizes biotin and subsequently treated with the secondary goat anti-mouse IgG antibody that is conjugated to 12 nm gold particles. The calibration bar is 1.0 μm in length. (C) Cytosolic Enzyme I of *E. coli* (see Methods) was not biotinylated by Biotin-X-NHS. The biotinylated spheroplasts (cells) as described were subjected to SDS−PAGE. Then, the separated proteins on the SDS−PAGE gel were electrophoretically transferred onto two layers of nitrocellulose membranes. The first layer of the membranes was used to detect biotinylated proteins colorimetrically by the avidin-conjugated alkaline phosphatase reaction (right panel). The second layer of the nitrocellulose was used to detect a cytosolic protein, Enzyme I, by use of chicken polyclonal antibody against this protein. Then, the immunoreactive band and biotinylated proteins were visualized colorimetrically by alkaline phosphatase conjugated to the secondary antibody against chicken IgG and by alkaline phosphatase conjugated to avidin, respectively.

as a single band following SDS−PAGE either by Coomassie dye staining (Figure 7A) or by Western blot analysis using a monoclonal antibody against NBF1 (Figure 7B). The yield of [G404−N432]NBF1 was quite good with ~300 μg of pure protein being derived from the membrane fraction of 100 mL of *E. coli* cell culture. These studies provide evidence that [G404−N432]NBF1 integrates into the *E. coli* membrane with a properly folded ATP binding domain or forms this domain upon interaction with the membrane.

Replacement of the 29 Amino Acid N-Terminal Extension, G404−N432, with the Maltose Binding Protein Results in a Cytosolic Location of NBF1. In a previous study (Ko et al., 1993), we showed that NBF1, as it is classically defined, i.e., as F433−S589, can be readily expressed in *E. coli* in fusion with the maltose binding protein (MBP). As this fusion protein (MBP−NBF1), in contrast to [G404−N432]NBF1, has a non-CFTR extension of amino acids at its N terminus (i.e., MBP), it was of interest to establish whether it preferentially localizes to the membrane or to the cytosol of *E. coli*. Using the immuno-gold labeling technique described in Methods, which involves reacting *in situ* the expressed MBP−NBF1 fusion protein first with a monclonal or polyclonal antibody to NBF1 and then with a secondary antibody conjugated to gold particles, it can be seen in panels C and D of Figure 7 that MBP−NBF1 localizes almost exclusively in the cytosol of *E. coli* with little or no propensity for interacting with the cytoplasmic membrane. This is consistent with our earlier finding (Ko et al., 1993) that MBP−NBF1 expressed in *E. coli* is readily solubilized upon cell lysis. These studies also indicate that the native 29-amino acid extension, G404−N432, within CFTR may be involved, directly or indirectly, in targeting NBF1 to the *E. coli* membrane.

DISCUSSION

Results presented here provide direct evidence that the first nucleotide binding fold (F433−S589) of the CFTR protein when expressed *in vivo* as [G404−N432]NBF1 preferentially

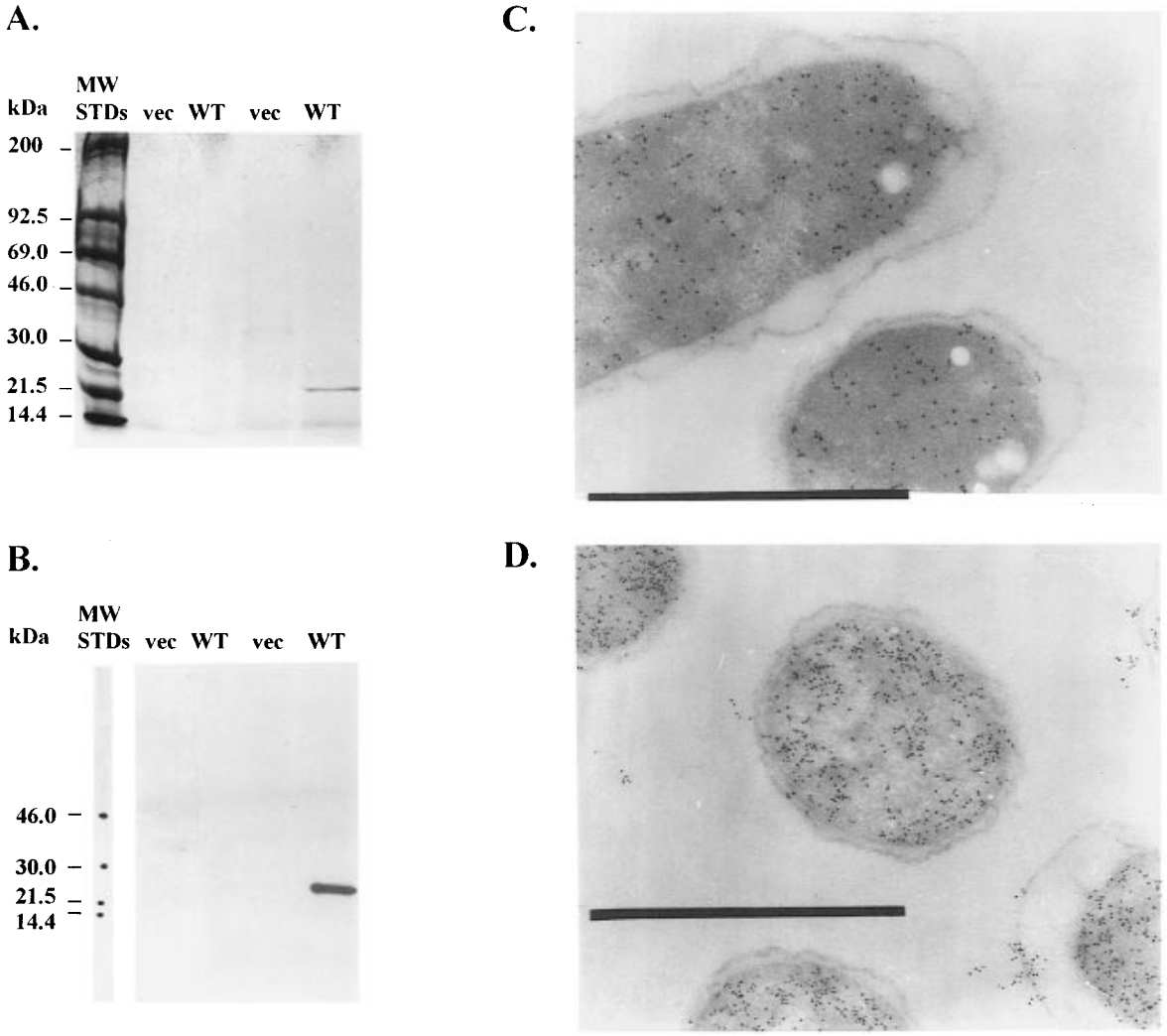

FIGURE 7: (A and B) Elution of [G404−N432]NBF1 with ATP from an ATP−agarose column. (A) SDS−PAGE gel stained with Coomassie blue dye to visualize [G404−N432]NBF1 eluted with ATP. Membrane fractions solubilized with Triton X-100 and prepared as described in Methods from 100 mL of *E. coli* culture, containing either [G404−N432]NBF1 or the vector only, were resuspended in an ATP column buffer and then loaded onto a 1 mL ATP−agarose column. The column was then washed with 12 mL of ATP column buffer. Finally, the bound protein was eluted with 100 mM ATP. Both the wash and eluted fractions (5 μg) were subjected to SDS−PAGE. (B) Western blot analysis of [G404−N432]NBF1 eluted with ATP. Both the wash and ATP-eluted samples as described in part A were subjected to Western blot analysis. After SDS−PAGE was conducted, the proteins were transferred electrophoretically onto a PVDF membrane and the presence of [G404−N432]NBF1 was confirmed by detection with a monoclonal antibody against NBF1 using an ECL detection system (see Methods) (C and D) Cytosolic localization of MBP−NBF1 analyzed by immuno-gold labeling of *E. coli* cells expressing the fusion protein. *E. coli* cells expressing the fusion protein MBP−NBF1 (Ko et al., 1993) were fixed with glutaraldehyde and paraformaldehyde as described in Methods. Thin sections (90 nm) of the fixed *E. coli* cells were made to visualize the localization of the expressed MBP−NBF1 protein by first interacting either with a monoclonal antibody against NBF1 (C) or with a polyclonal antibody having the epitope for MBP (D), and then with a secondary antibody conjugated to 5 nm gold particles. The calibration bar is 1 μm.

assumes a membrane location. In fact, even Western blot analysis failed to provide any evidence for a partial cytosolic location. In addition, the [G404−N432]NBF1 protein, which is the precise product of exons 9−12 of the CFTR gene, was found to be accessible from the outside of intact spheroplasts to the water soluble, membrane impermeant biotinylating agent Biotin-X-NHS. Accessibility to this labeling agent could not be ascribed to a small intrinsic permeability of the spheroplasts, as Enzyme I, a cytoplasmic protein in *E. coli* associated with the glucose phosphotransferase system, failed to undergo detectable labeling under identical conditions. Moreover, the spheroplasts used were shown to be intact by electron microscopy and by their failure to "leak" cytoplasmic enzymes even after 2 h. Significantly, membrane insertion of [G404−N432]NBF1 occurred with retention of its known ATP binding function, consistent with its final state in the membrane environment as a properly folded domain.

Studies described above implicate the 29-amino acid N-terminal segment, G404−N432, in targeting the NBF1 domain to the *E. coli* membrane. This is further supported by the finding that replacement of this sequence with the maltose binding protein results in a strictly cytosolic location of NBF1. Although the G404−N432 segment contains seven strongly hydrophobic amino acids, including four phenylalanine residues, further work will be necessary to establish whether it is this region, a region within NBF1, or both that insert into the *E. coli* membrane. It is of interest to note, however, that asparagine 432, which terminates the pre-NBF1 segment, resides within the sequence N-X-S(T) that conforms to the known consensus N-X-S(T) for N-glycosylation (Sharon & Lis, 1993).

Significantly, CFTR belongs to a superfamily of membrane proteins that are referred to as either "ABC transporters" (Higgins, 1992) or "traffic ATPases" (Doige & Ames, 1993). Although the functions of these transporters are all quite different, each contains two nucleotide binding domains, one or both of which are believed to bind and hydrolyze ATP. The novel studies reported here, taken together with other recent studies (Arispe et al., 1992; Biachwal et al., 1993; Loo & Clarke, 1995), represent a growing body of evidence that shows that these nucleotide domains can interact with biological membranes. Thus, Arispe et al. (1992) has shown *in vitro* that NBF1 of the CFTR protein readily incorporates into planar lipid bilayers consisting of palmityloleoylphosphatidylethanolamine and phosphatidylserine. Baichwal et al. (1993) demonstrated that the bacterial *His*P protein, an ATPase associated with the histidine permease, is accessible from the outside of the cytoplasmic membrane by performing exeriments involving proteolysis and biotinylation. Finally, Loo and Clarke (1995) have recently shown in coexpression/co-immunoprecipitation experiments, involving fragments of the multidrug resistant protein called "MDR" or "P-glycoprotein", that the two nucleotide binding domains are associated with the membrane.

Although the capacity of nucleotide binding domains of ABC transporters like CFTR to interact with biological membranes may at first appear to be at odds with programs that predict a cytosolic location (Riordan et al., 1989), it is important to realize that both locations may occur in the physiological state. For example, in yeast, the peripheral subunit of *E. coli* pre-protein translocase called "Sec A" alternates between a membrane-inserted and -deinserted state as part of a catalytic cycle of pre-protein translocation (Economau & Wickner, 1994). Here, hydrolysis of ATP drives a profound conformational change, leading to membrane insertion. Using a completely different mechanism, protein kinase C is recruited to the membrane from the cytosol in eukaryotic cells by diacylglycerol or phorbol esters, which plug a hydrophobic binding site giving a domain with a contiguous hydrophobic surface (Newton, 1995). Finally, in highly malignant tumors, hexokinase binds to the outer mitochondrial membrane in the absence of glucose 6-phosphate but is released into the cytosol in its presence (Parry & Pedersen, 1983).

As yet, the relevance of these studies to the membrane topology and trafficking of CFTR in eukaryotic membranes is not clear. Nevertheless, it seems likely that, if [G404−N432]NBF1 has the capacity to insert into a prokaryotic membrane, it has the capacity to insert into eukaryotic membranes. This possibility is underscored by the recent study of Geller et al. (1996) in which the topology of the N-terminal half of the yeast ABC transporter Ste6 was compared in its native environment in the yeast *Saccharomyces cerevisiae* and upon expression in a foreign environment, i.e., *E. coli*. In both cases, the final destination, i.e., membrane, and the resultant topology were identical.

Finally, the potential importance of the novel studies reported here to CFTR research in general and more specifically to the disease cystic fibrosis is several-fold. First, further study of [G404−N432]NBF1 expressed both in *E. coli* and in eukaryotic cells should help establish the precise nature of its membrane interaction, thus defining that amino acid segment associated with the membrane and establishing whether its release into the cytosol can be induced, e.g., by phosphorylation or ATP hydrolysis. Second, it may be possible in gene therapy experiments to competitively "force" ΔF508 CFTR out of the endoplasmic reticulum by using adeno-associated viral or other vectors expressing only wild type [G404−N432]NBF1. Third, work reported here provides a simple method for purifying in milligram quantities the complete modular unit encoded by exons 9−12 of the CFTR gene, namely [G404−N432]NBF1. This should facilitate structure and function studies of this important region of the CFTR protein, which upon deletion of F508 results in most cases of cystic fibrosis. Future work in this laboratory will include all of these avenues of research.

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