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Cell Biology

CFTR protein expression in primary and cultured epithelia

(cystic fibrosis transmembrane conductance regulator/chloride channel)

PAMELA L. ZEITLIN*†‡, ISABELLE CRAWFORD†, LUO LU†, SYBILLE WOEL*, MICHAEL E. COHEN§, MARK DONOWITZ§, MARSHALL H. MONTROSE§, ADA HAMOSH*, GARRY R. CUTTING*, DIETER GRUENERT¶, RICHARD HUGANIR‖, PETER MALONEY§, AND WILLIAM B. GUGGINO*§

Departments of *Pediatrics, †Physiology, §Medicine, and ‖Neurosciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; and ¶Cardiovascular Research Unit, University of California, San Francisco, CA 94143

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ABSTRACT **The gene responsible for the lethal disorder cystic fibrosis encodes a 1480-amino acid glycoprotein, CFTR. Using polyclonal antibodies directed against separate phosphorylation sites in the pre-nucleotide-binding fold (exon 9) and the R domain (exon 13), we have identified a 165-kDa protein in *Xenopus laevis* oocytes injected with recombinant CFTR cRNA transcribed from the full-length CFTR plasmid pBQ4.7. A protein of the same mobility was also detected with Western blotting techniques in whole cell extracts of cells that express CFTR mRNA (T84, FHTE, HT-29), including biopsied human nasal and bronchial tissue. Immunodetectable 165-kDa protein was concentrated in the apical membrane fraction of ileal villus tissue. We also report that the 165-kDa protein levels can be modulated pharmacologically, and these levels are appropriately correlated with second-messenger-regulated Cl^- efflux. Thus, native or recombinant CFTR can be recognized by these anti-CFTR peptide polyclonal antibodies.**

Cystic fibrosis (CF) is a lethal autosomal recessive disorder in which abnormal regulation of epithelial Cl^- channels is associated with the pathophysiology of the disease. The CF gene has been cloned and sequenced (1) and the amino acid sequence of the putative product (cystic fibrosis transmembrane conductance regulator, or CFTR) has been deduced (2). CFTR contains two membrane domains, each with six potential transmembrane segments, two nucleotide-binding folds (NBFs), and a highly charged cytoplasmic domain (R domain). The most common mutation associated with CF is the deletion of three nucleotides that would encode a phenylalanine at position 508 within the first NBF (exon 10). Both the NBFs and the R domain contain multiple potential phosphorylation sites for protein kinase A and protein kinase C (2). Protein kinases are important physiologic regulators of Cl^- secretion, and defective regulation of outwardly rectifying Cl^- channels by protein kinases A and C is one defect in CF patients (3, 4). Since it has been suggested that CFTR is either a Cl^- channel (5–7) or a regulatory protein closely associated with the channel, we reasoned that one or more phosphorylation sites in CFTR might be important functionally and detectable immunologically.

This report describes the identification of CFTR by the use of antibodies raised against peptides containing phosphorylation sites in the CFTR pre-NBF and R domains. We have confirmed that our antibodies detect a 165-kDa protein by heterologous expression in *Xenopus laevis* oocytes. With this information at hand, we report on the use of immunological and molecular biological techniques to study the expression and modulation of CFTR protein in established cell lines and human tissue biopsy specimens.

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METHODS

Production of Synthetic Peptide Antigens and Polyclonal Antisera. Peptide 181 (CFTR amino acid residues 415–427, exon 9, pre-NBF) and peptide 169 (residues 724–746, exon 13, R domain) were synthesized by the Applied Biosystems peptide synthesizer of the Johns Hopkins University Medical School Protein/Peptide Facility, Department of Biological Chemistry. Crude peptides were purified by HPLC to give >99% purity. Both peptides were linked to bovine serum albumin and to thyroglobulin (for affinity purification) by 1% glutaraldehyde crosslinking at a ratio of 1 mol of peptide per 50 amino acids in albumin (8). The efficiency of crosslinking, monitored by mobility in an SDS/10% polyacrylamide (Laemmli) gel, was virtually complete.

Polyclonal antibodies against the peptide-albumin conjugates were raised in New Zealand White female rabbits by an initial intradermal injection of 200 μg of antigen emulsified in Freund's complete adjuvant. This was followed by booster injections of 100–200 μg of antigen in Freund's incomplete adjuvant at biweekly intervals.

Cell Culture. T84 (human colon carcinoma line) and FHTE (human fetal tracheal epithelial line) (9) were grown to confluence as described (3). HT-29 (human intestinal cell line) clone 18 cells were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose, supplemented with human transferrin (10 μg/ml; Sigma), streptomycin sulfate (50 μg/ml), penicillin (50 units/ml), 4 mM glutamine, and 10% fetal bovine serum. For those experiments in which galactose was substituted for glucose, 5 mM galactose replaced 25 mM glucose and 1 μM pyruvate, as described (10). HT-29 cells were harvested 7 days after reaching confluence.

Western Blotting. Whole cell extracts were prepared in 10% SDS (100°C) and then protease inhibitors were added: *p*-aminobenzoic acid (1 mM), phenylmethylsulfonyl fluoride (0.5 mM), pepstatin (5 μg/ml), and chymostatin (10 μg/ml). Protein was quantitated by the Lowry assay (11).

Cell extracts were prepared for electrophoresis in 44 mM Tris·HCl, pH 8.9/4.5 mM EDTA/1.8% SDS/10% glycerol/1% 2-mercaptoethanol and heated to 95°C for 90 sec. Boiling for 90 sec in the absence of 2-mercaptoethanol led to aggregation of the CFTR protein at the top of the gel and was avoided. Extracts were electrophoresed in an SDS/5% PAGE (Laemmli) system, and then transferred to nitrocellulose in 0.04% SDS/25 mM Tris/192 mM glycine/20% methanol for 1 hr at 100 V and 4°C. All subsequent steps were performed at room temperature. The nitrocellulose was blocked with 1.3% gelatin (Baker) in wash buffer [0.15 M NaCl/10 mM Tris·HCl, pH 7.4/2% (vol/vol) Triton X-100] for 1 hr. The primary whole antiserum was diluted 1:1000 in

Abbreviations: CF, cystic fibrosis; NBF, nucleotide-binding fold.

‡To whom reprint requests should be addressed at: Department of Pediatrics, Park 316, Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21205.

0.4% bovine serum albumin in wash buffer and applied with shaking for 1 hr. The blots were rinsed in wash buffer and exposed to the secondary antibody (horseradish peroxidase-linked donkey anti-rabbit IgG; Amersham) at a 1:10,000 dilution for 1 hr. The blots were rinsed as described and exposed to enhanced chemiluminescence detection solution (ECL; Amersham). In some experiments, ^{125}I-protein G (150,000 cpm/ml) was substituted for the secondary antibody and was applied for 4 hr at 4°C. Autoradiograms were prepared with Kodak XAR film. Nitrocellulose blots were stripped in 2% SDS/62 mM Tris·HCl, pH 6.7/2 mM 2-mercaptoethanol at 60°C; stripping was confirmed by probing with secondary antibody before incubation with new primary antibody.

Oocyte Expression System. CFTR cRNA was transcribed from the full-length CFTR plasmid pBQ4.7 (kindly provided by J. Rommens, The Hospital for Sick Children, Toronto) using the T7 RNA polymerase promoter. Oocytes were removed from adult female *X. laevis* frogs (Xenopus I, Michigan), defolliculated, and stored as described (12). Oocytes were injected with 50 ng cRNA in 50 nl water with a positive-displacement micropipette (Drummond). Cell extracts were prepared 48 hr after injection.

Plasma Membrane Preparations. Rabbit (New Zealand White males) ileal villus cells were obtained by light scraping and brush-border membranes were prepared by magnesium precipitation (13). Basolateral membrane fractions were also prepared from rabbit ileal villus cells; in this preparation, isolated ileal cells were initially prepared by a modification of the Weiser technique in which the first three 10-min fractions represent villus cells (14). The cells were then homogenized by 20 strokes with a loose Dounce homogenizer and basolateral membranes were prepared using a Percoll gradient (15).

Characterization of Patient Genotype. Nasal polyps removed at surgery were isolated by protease digestion (16) and cultured as for FHTE. Cells from nasal brushings were obtained from the lateral nasal turbinate of human volunteers by gentle scraping with a bronchoscopy brush. The nucleotide sequence of CFTR was determined as described (17).

RESULTS

Identification of CFTR. Antibodies raised to peptides containing putative phosphorylation sites in the pre-NBF (181) and the R domains (169) were obtained in high titer and maintained by periodic booster injections (Fig. 1, lanes a–f). To prove the specificity of each of these anti-CFTR peptide antibodies, CFTR cDNA contained in pBQ4.7 was transcribed *in vitro* and the cRNA was injected into *Xenopus* oocytes. Both pre-NBF (Fig. 1, lanes a and b) and R (data not shown) antibodies detected a 165-kDa protein in oocytes microinjected with CFTR cRNA, but not in control (sham-injected) oocytes. The detection of the band at 165 kDa, a size consistent with predictions for CFTR, in samples from injected (but not sham-injected) oocytes provided convincing evidence that the antibodies can recognize CFTR in an *in vitro* expression system.

To assess whether the anti-CFTR peptide antibodies could recognize native CFTR, we examined T84, a human colon carcinoma cell line, and FHTE, a fetal human tracheal epithelial cell line, both known to secrete Cl^- and express CFTR mRNA. Both cell lines expressed the 165-kDa band as recognized by the two CFTR peptide antisera (Fig. 1, lanes c–f). Two other bands, at about 145 and 100 kDa, were also detected with pre-NBF antiserum in T84 cells, but not in the oocyte expression system.

To further investigate the nature of the crossreactivity of pre-NBF antiserum for T84 cell proteins, the immunizing CFTR peptide was used as a competitive blocker in Western blotting experiments. Inclusion of peptide at 1 mg/ml during the first antibody incubation reduced detection of the 165-kDa protein in T84 and FHTE (Fig. 2), as expected, and also eliminated staining of lower molecular mass bands in T84 cells. This suggests some crossreactivity of the immunizing peptide sequence with other proteins in this cell line. This might not be surprising, since there is extensive amino acid sequence conservation among members of the family of NBF-containing proteins to which CFTR belongs.

To identify the subcellular localization of CFTR from an *in vivo* polarized epithelium, rabbit ileal villus cell brush-border (enriched 21-fold in sucrase specific activity) and basolateral (enriched 10-fold in Na^+,K^+-ATPase specific activity) mem-

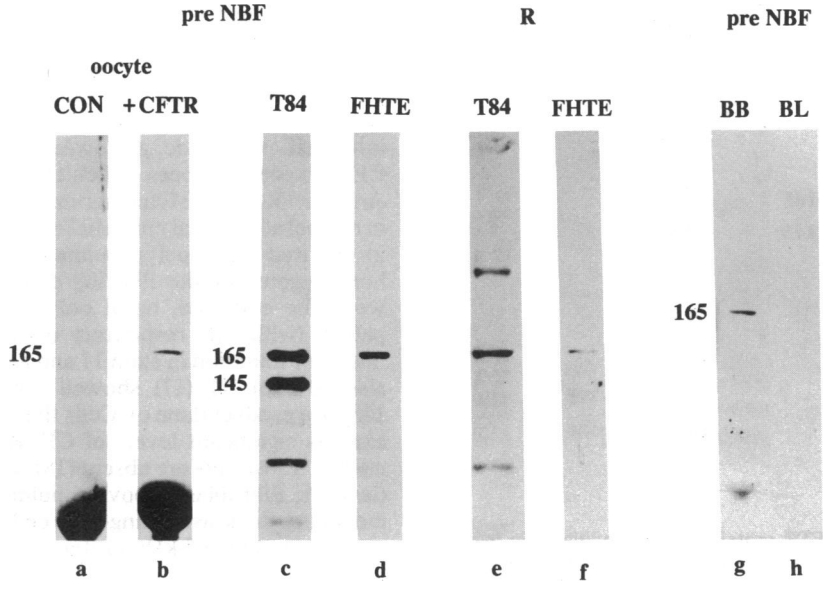

FIG. 1. Antisera to phosphorylation sites in CFTR in the pre-NBF and R domain detect a protein migrating at 165 kDa. *Xenopus* oocyte whole cell extract (200 μg of protein) was loaded in lanes a and b and detected with pre-NBF antisera. Control (CON) oocytes were punctured, but not injected. The oocytes in lane b (+CFTR) were injected with 50 ng of CFTR cRNA. T84 whole cell extract (lanes c and e) or FHTE whole cell extract (lanes d and f) (60 μg per lane) was probed with either pre-NBF or R antiserum. Rabbit ileal villus brush-border membranes (BB, lane g) or basolateral membranes (BL, lane h) (40 μg) were assessed using pre-NBF antiserum.

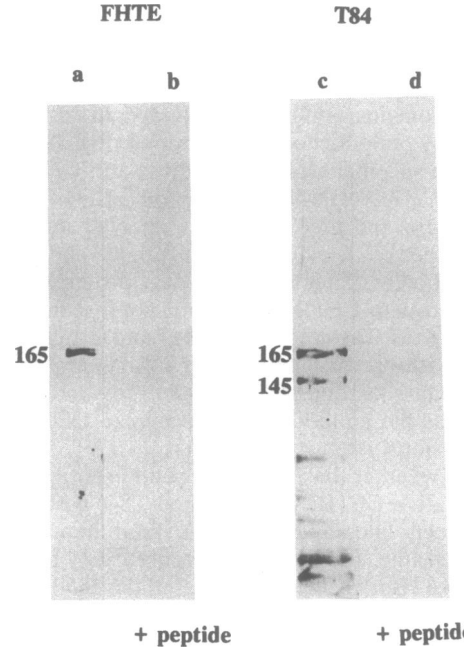

FIG. 2. Detection of the 165-kDa protein is eliminated by excess peptide. FHTE cell extract (lanes a and b) or T84 cell extract (lanes c and d) (60 μg per lane) was incubated with pre-NBF antiserum, and unconjugated peptide (1 mg/ml) was included for lanes b and d.

brane fractions were analyzed by Western blotting. Immunodetectable 165-kDa protein was present in the brush-border membrane fraction but not the basolateral fraction with pre-NBF antiserum (Fig. 1, lanes g and h). This experiment demonstrates that immunodetectable 165-kDa protein is localized normally to the apical membrane fraction of intestinal

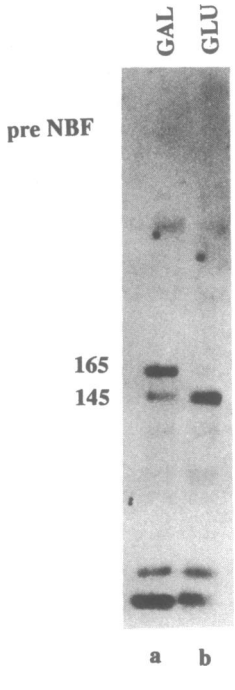

FIG. 3. Expression of CFTR protein can be modulated in intestinal secretory epithelia. HT-29 clone 18 was maintained in medium with either galactose or glucose as described (10) for 14 days. Whole cell extracts (40 μg per lane) were electrophoresed. Western blots were probed with pre-NBF antiserum and detected with ^{125}I-protein G. Note that HT-29 cells differentiated in galactose-supplemented (glucose-deficient) medium (lane a) express more 165-kDa protein than those in glucose-sufficient medium (lane b).

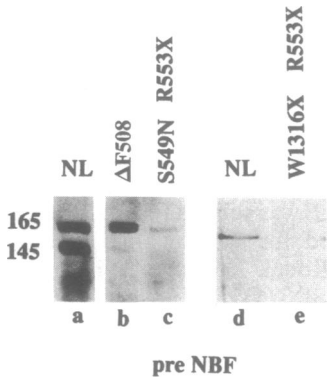

FIG. 4. Expression of CFTR in primary airway tissue. Lanes a and d, nasal epithelial cell extracts from two non-CF individuals; lane b, CF nasal polyp cells homozygous for ΔF508; lane c, cultured nasal polyps obtained from a CF patient with genotype R553X (Arg-553 codon changed to stop codon) and S549N; lane e, CF nasal epithelial cells with genotype W1316X and R553X. Sixty micrograms of protein was electrophoresed in lanes a–c, and 40 μg in lanes d and e. Pre-NBF antiserum was used to identify CFTR.

epithelial cells. This is consistent with the hypothesis that CFTR functions as an apical Cl^- channel.

Modulation of CFTR Expression in Secretory Epithelia. To assess the relationship between changes in CFTR gene expression at the mRNA level and those at the protein level, we evaluated CFTR-expressing cells under conditions presumed to exert control over CFTR mRNA levels.

HT-29 clone 18 is a clonal intestinal line that can be induced to differentiate by substitution of galactose for glucose in the medium (10). Under these conditions, CFTR mRNA abundance is increased 9-fold and second-messenger-regulated Cl^- transport is increased 5-fold (10). Immunodetectable CFTR was then evaluated in both differentiated and undifferentiated cells (Fig. 3). The 165-kDa band detected with pre-NBF antiserum was substantially increased in galactose (differentiated) conditions. In contrast, the 145-kDa component was increased in glucose (undifferentiated) medium. Furthermore, other, nonspecifically stained bands served as controls and were of equal intensity. Thus, within this particular cell line, only the amount of immunodetectable 165-kDa protein is correlated in a quantitative fashion with steady-state CFTR mRNA levels.

Association of CFTR Protein Levels with Genotype in CF Patients. Defective Cl^- transport occurs in CF in a number of secretory epithelia, specifically from tissues of respiratory, intestinal, pancreatic, and sweat duct origins. Nevertheless, CFTR protein has been difficult to identify in primary tissues. Anti-CFTR pre-NBF antiserum stained the 165-kDa protein in normal adult nasal epithelial cells (Fig. 4, lanes a and d) and in cultured nasal polyp epithelial cells from a CF patient homozygous for the Phe-508 deletion (ΔF508; lane b). As would be expected, nasal polyp cells isolated from a CF patient (with mild respiratory disease at age 14) carrying a missense mutation in exon 11 and a nonsense (stop) mutation also in exon 11 (17) showed lowered expression of the 165-kDa product (lane c). Cells that would not be expected to express significant levels of CFTR protein because CFTR mRNA transcripts are absent (18) were negative in this assay (lane e). Fibroblasts, bovine pulmonary endothelium, and medullary thick-ascending-limb cells did not have detectable amounts of the 165-kDa protein on Western blots (Table 1).

DISCUSSION

The anti-CFTR peptide antisera in this study detect a single protein at 165 kDa in immunoblot samples from oocytes injected with recombinant CFTR, but not from sham-injected

Table 1. Distribution of CFTR

CFTR-positive	CFTR-negative
T84 cells	MTAL cells
HT-29 cells	3T3 fibroblasts
FHTE cells	VP-79 fibroblasts
Trachea/bronchi	Endothelial cells
Nasal polyp	*Oxalobacter formigenes*
Rat liver microsomes	*Escherichia coli* P19

CFTR protein expression was assessed by analysis of 50 μg of whole cell extracts by Western blotting with pre-NBF anti-CFTR antiserum. MTAL is a rabbit line from the medullary thick ascending limb of the kidney. 3T3 is a mouse fibroblast line. VP-79 is a Chinese hamster fibroblast line. Endothelial cells were derived from a bovine aortic line. Membrane vesicles were prepared from *O. formigenes* and *E. coli* P19.

oocytes. These same antisera detect a 165-kDa protein in COS cells transfected with recombinant CFTR (19). Moreover, tissue localization studies by Crawford *et al.* (19) have demonstrated specific binding to the apical pole of sweat duct and pancreas, as would be expected for CFTR. Thus in two independent recombinant systems, anti-CFTR peptide antisera recognize a single 165-kDa protein. Because of the possibility that polyclonal antibodies may crossreact with other cellular proteins, experiments were designed to upregulate CFTR mRNA in HT-29 cells. Once again, the antisera detected a 165-kDa band that correlated with changes in CFTR mRNA levels.

As expected the anti-CFTR peptide antisera detected the 165-kDa protein in airway cells from normal individuals but revealed little or no 165-kDa protein in CF patients with genotypes S549N/R553X and W1316X/R553X. Our experiments suggest that the 165-kDa form of ΔF508 CFTR (in homozygous patients) is present in native airway epithelia. This observation suggests that at least some CFTR can be processed into a mature form by airway epithelial cells from CF patients. It is also possible that, similar to the recombinant CFTR expression in COS cells observed by Cheng *et al.* (20), a processing defect in native tissue prevents a significant portion of CFTR from being processed normally. Another possibility is that differences in glycosylation moieties between tissues and cell lines may account for the apparently mature form seen in nasal epithelial cells in our study. Our experiments cannot be interpreted to address whether the 165-kDa protein detected in CF patients homozygous for ΔF508 ever reaches the plasma membrane.

An additional implication from our studies arises from data on intestinal membrane fractionation. As demonstrated in Western blots of intestinal villus cell apical and basolateral membrane fractions, a significant proportion of immunodetectable protein is associated with the apical fraction, consistent with the hypothesis that CFTR functions to facilitate regulated Cl^- secretion by close association (or identity) with Cl^- channels. In a related paper (19), we show that CFTR can be localized in human tissues by using R-domain antibodies and indirect immunofluorescence methods. Those studies, too, clearly demonstrate that CFTR is normally present at the apical pole of the cell. Thus both membrane fractionation experiments and immunolocalization techniques with anti-CFTR antibodies suggest polarization of CFTR to the region of the epithelial cell involved in Cl^- channel function.

Studies of the regulation of CFTR protein levels in intestinal epithelial cell lines demonstrated a correspondence of CFTR mRNA level, CFTR protein level, and second-messenger-regulated Cl^- efflux (10). These data support the hypothesis that CFTR is a Cl^- channel. However, our studies of CFTR protein expression in CF patients demonstrated higher levels of CFTR in patients with the common ΔF508 mutation and severe respiratory phenotype than in patients with reduced expression of CFTR protein and a milder respiratory phenotype. In CF patients, more defective CFTR protein may be associated with worse disease than an absence of the protein. This is consistent with the idea that the ΔF508 mutation, in addition to disrupting Cl^- secretion, causes a processing or trafficking defect in the epithelial cell (20, 21), thus interfering with more cellular processes than just Cl^- secretion.

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