Biochemical Characterization of the Cystic Fibrosis Transmembrane Conductance Regulator in Normal and Cystic Fibrosis Epithelial Cells*

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Affinity-purified polyclonal antibodies, raised against two synthetic peptides corresponding to the R domain and the C terminus of the human cystic fibrosis transmembrane conductance regulator (CFTR), were used to characterize and localize the protein in human epithelial cells. Employing an immunoblotting technique that ensures efficient detection of large hydrophobic proteins, both antibodies recognized an approximately 180-kDa protein in cell lysates and isolated membranes of airway epithelial cells from normal and cystic fibrosis (CF) patients and of T84 colon carcinoma cells. Reactivity with the anti-C terminus antibody, but not with the anti-R domain antibody, was eliminated by limited carboxypeptidase Y digestion. When normal CFTR cDNA was overexpressed via a retroviral vector in CF or normal airway epithelial cells or in mouse embryonic fibroblasts, the protein produced had an apparent molecular mass of about 180 kDa. The CFTR expressed in insect (Sf9) cells by a baculovirus vector had a molecular mass of about 140 kDa, probably representing a nonglycosylated form. The CFTR in epithelial cells appears to exist in several forms. N-glycosidase treatment of T84 cell membranes reduces the apparent molecular mass of the major CFTR band from 180 kDa to 140 kDa, but a fraction of the T84 cell CFTR could not be deglycosylated, and the CFTR in airway epithelial cell membranes could not be deglycosylated either. Moreover, wheat germ agglutinin absorbs the majority of the CFTR from detergent-solubilized T84 cell membranes but not from airway cell membranes. The CFTR in all epithelial cell types was found to be an integral membrane protein not solubilized by high salt or lithium diiodosalicylate treatment. Sucrose density gradient fractionation of crude membranes prepared from the airway epithelial cells, previously surface-labeled by enzymatic galactosidation, showed a plasma membrane localization for both the normal CFTR and the CFTR carrying the Phe508 deletion (ΔF 508). The CFTR in all cases co-localized with the Na⁺,K⁺-ATPase and the plasma membrane calcium ATPase, while the endoplasmic reticulum calcium ATPase and mitochondrial membrane markers were enriched at higher sucrose densities. Thus, the CFTR appears to be localized in the plasma membrane both in normal and ΔF 508 CF epithelial cells.

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The gene containing the mutations responsible for the development of the lethal genetic disease, cystic fibrosis (CF), has been cloned (1-3), and the hypothetical protein product has been named the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (2). Based on hydropathy analysis and homologies with specific domains of other proteins, the gene product has been predicted to contain 12 membrane-spanning regions, two nucleotide binding domains, a large regulatory (R) domain with numerous sites for phosphorylation by protein kinases, and two sites for N-linked glycosylation in an extracellular segment between the seventh and eighth proposed membrane-spanning helices (2). In most CF cases, the mutation responsible for the disease, which probably stems from a defect in cAMP-dependent regulation of epithelial chloride channels (4-9), has been shown to be a deletion of the phenylalanine residue at position 508 (ΔF 508) (3). This information prompted the suggestion that the normal CFTR is a plasma membrane-localized nucleotide binding protein, which either regulates or forms protein kinase A-dependent chloride channels (2, 10, 11).

Recent reports have shown that the expression of a normal CFTR in ΔF 508 mutant cells corrects the abnormal chloride channel behavior (12-15). Moreover, by expressing the CFTR in insect Sf9 cells, the appearance of cAMP-dependent, protein kinase A-regulated chloride channels was demonstrated (16). Regarding the pathological effect of the Phe508 deletion, one report (17) indicated that the ΔF 508 CFTR expressed in heterologous cells had a smaller apparent molecular mass than the normal protein, and the suggestion was made that the mutant protein may not be folded and then glycosylated correctly, resulting in a failure to reach the plasma membrane to form or regulate chloride channels. However, no evidence was provided for the maturation and the localization of the endogenous CFTR protein.

In spite of the rapid progress in the expression of the normal

1 The abbreviations used are: CF, cystic fibrosis; Beas cells, SV 40-transformed normal human tracheal epithelial cells; BCIP, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio]-1-propane sulfonate; CFP1 cells, human papilloma virus E6 and E7 gene transformed human CF tracheal epithelial cells (homozygous for ΔF 508 mutation); CF-T43 cells, SV 40-transformed human CF nasal epithelial cells (homozygous for ΔF 508 mutation); CFTR, cystic fibrosis transmembrane conductance regulator; EGTA, ethylenebis(oxyethylenenitrito)tetraacetic acid; HBE cells, human papilloma virus E6 and E7 gene transformed normal human tracheal epithelial cells; KLH, keyhole limpet hemocyanin; NBT, nitro blue tetrazolium chloride; PMCA, plasma membrane calcium ATPase; PMSF, phenylmethylsulfonyl fluoride; PVPD, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SERCA, sarcoplasmic reticulum calcium ATPase; Sf9 cells, Spodoptera frugiperda cultured ovarian cells; T84 cells, human colonic carcinoma cell line; WGA, wheat germ agglutinin.
CFTR and the functional evaluation of this expression, relatively few data are available concerning the biochemical nature of the CFTR in normal and CF epithelial cells. The reports which have provided biochemical information about the CFTR (16, 17) have employed in vitro expression systems and/or the high CF-TR-expressor T84 colon carcinoma cell line. However, no data are currently available regarding the presence, glycosylation, and membrane localization of the normal and AF 508 CFTR in airway epithelial cells, where abnormal CFTR function results in the lethal consequences of the CF disease.

In the present report, using antibodies specific for two different regions of the CFTR and an immunodetection technique with high efficiency and sensitivity, we describe a variety of biochemical features of this protein in normal and CF (AF 508 homozygote) airway epithelial cells as well as T84 colon carcinoma cells which are of epithelial origin. We also compare the characteristics of these proteins to those over-expressed via viral vectors in human airway epithelial cells, mouse NIH 3T3 fibroblasts, and insect Sf9 cells. In addition to establishing several fundamental properties of the CFTR in normal, CF, and overexpressing cell systems, these studies may contain tools for future studies of the structure, localization, and function of this newly recognized protein so essential for normal epithelial cell function.

**EXPERIMENTAL PROCEDURES**

**Reagents**—N-Glycosidase F from Flavobacterium meningosepticum, recombinant endoglycosidase H, 0-glycosidase from Diplococcus pneumoniae, and neuraminidase from Vibrio cholerae were obtained from Boehringer Mannheim. 

| Reagent | Source |
|---------|--------|
| PHUDP galactose, pretailed molecular mass standards, and enhanced chemiluminescence Western blotting reagents | Amersham |
| Carboxypeptidase Y, lithium diiodosalicylate, galactosyltransferase, lectin-agarose beads, NBT, BCIP, PMSF, aprotinin, and leupeptin | Sigma |
| Synthetic peptides prepared in the Protein Chemistry Laboratory of UNCCH-NIEHS. | |
| Anti-CFTR antibodies were diluted 100-fold, while other polyclonal and monoclonal antibodies were diluted 500-fold |
| SDS-PAGE and Immunoblotting—Membrane suspensions were either directly mixed with disaggregation buffer (50 mM Tris, pH 8.5, with H2PO4, containing 2% SDS (w/v), 15% glycerol (w/v), 2% β-mercaptoethanol (v/v), 1 mM EDTA, and 0.02% (w/v) bromophenol blue) or precipitated with trichloroacetic acid (4% (w/v) final concentration) and centrifuged for 5 min at 5,000 × g, and then suspended in the disaggregation buffer. Samples were disaggregated at room temperature for 20 min at a protein concentration of 1-3 mg/ml. Electrophoresis of the samples was usually carried out using Bio-Rad Mini-Protean ready gels (4-15% acrylamide gradient, 0.375 M Tris-HCl, pH 8.3, 0.2% (w/v) SDS) at room temperature. Polyclonal anti-CFTR antibodies were diluted 100-fold, while other polyclonal and monoclonal antibodies were diluted 500-fold |

**Cell Lines and Membrane Preparation**—Epithelial cell lines used in this study were: BEAS, a line of SV40 large T antigen immortalized normal human tracheal epithelial cells (19); CF-T45 cells, a line of similarly immortalized nasal epithelial cells from a homozygous AF 508 CF patient, retain the CF genotype and phenotype (20); HEBe cells, normal human bronchial epithelial cells immortalized by human papilloma virus E6 and E7 genes; and CFT1 cells, human tracheal epithelial cells from a homozygous AF 508 CF patient, immortalized by human papilloma virus E6 and E7 genes. T84 cells, derived from a human colon cancer, and HeLa cells, from human cervical cancer origin, were obtained from the American Type Tissue Culture Collection, Rockville, MD. The special culturing conditions for the airway cells are described in Refs. 19 and 20.

Cell extracts and crude membrane fractions were prepared from the various cultured cells as follows: confluent cell cultures were washed in phosphate-buffered saline and the cells were scraped into phosphate-buffered saline, pH 7.0, containing 0.2% (w/v) sodium deoxycholate, 0.5 M NaCl and 0.5 mM PMSF. When the proteins of whole cells were to be assayed, the cell suspensions were precipitated with trichloroacetic acid (4% (w/v) final concentration) and centrifuged for 5 min at 5,000 × g, and the pellet was dissolved in SDS-PAGE disaggregation buffer (see below). For membrane preparations, the cells were lysed and homogenized in a glass-Te]mTing tissue homogenizer in TEMP (50 mM Tris, pH 7.0, with HCl, containing 50 mM mannitol, 2 mM EGTA, 10 μg/ml leupeptin, 8 μg/ml aprotinin, 0.5 mM PMSF, and 2 mM β-mercaptoethanol), and the undissolved nuclei and nuclear debris were removed by centrifugation at 500 × g for 10 min. The supernatant fluid was then centrifuged for 60 min at 100,000 × g, and the pellet containing the membranes was resuspended in TEME at a protein concentration of 5–10 mg/ml. All procedures were carried out at 4 °C, and the membranes were stored at −70 °C. Human red cell membrane vesicles and lymphoblast (Jurkat) membranes were prepared as described previously (21, 22).

**SDS-PAGE and Immunoblotting**—Membrane suspensions were either directly mixed with disaggregation buffer (50 mM Tris, pH 8.5, with H2PO4, containing 2% SDS (w/v), 15% glycerol (w/v), 2% β-mercaptoethanol (v/v), 1 mM EDTA, and 0.02% (w/v) bromophenol blue) or precipitated with trichloroacetic acid (4% (w/v) final concentration) and centrifuged for 5 min at 5,000 × g, and then suspended in the disaggregation buffer. Samples were disaggregated at room temperature for 20 min at a protein concentration of 1-3 mg/ml. Electrophoresis of the samples was usually carried out using Bio-Rad Mini-Protean ready gels (4-15% acrylamide gradient, 0.375 M Tris-HCl, pH 8.3, 0.2% (w/v) SDS) at room temperature. Polyclonal anti-CFTR antibodies were diluted 100-fold, while other polyclonal and monoclonal antibodies were diluted 500-fold. After electrophoresis in SDS-PAGE gels, proteins were transferred to nitrocellulose paper using a Tris buffer containing 20% methanol and 0.2% acrylamide (w/v) or Tris-glycine buffer. The membranes were then blocked in a 5% (w/v) Carnation nonfat dry milk, for 60 min at room temperature. Polyclonal anti-CFTR antibodies were diluted 100-fold, while other polyclonal and monoclonal antibodies were diluted 500-fold. After incubation for 60 min at room temperature, the blots were washed twice for 15 min in TBS-Tween solution and incubated with the appropriate second antibody solution.
tions (peroxidase-conjugated antibodies diluted 20,000-fold, alkaline phosphatase-conjugated antibodies diluted 750-fold) for 60 min. The blots were then washed three times (15 min each) in TBS-Tween solution. The alkaline phosphatase-labeled blots were developed in color reaction buffer (100 mM NaCl and 5 mM MgCl2 in 100 mM Tris, pH 9.5, with HCl), containing 0.5 mg/ml NBT and 0.15 mg/ml BCIP (2'). The PVDF blots were then washed three times (15 min each) in the same buffer without detergents. The solution was then centrifuged for 10 min at 1,000 × g, and the supernatant fluid was used for lectin binding studies. Lectin agarse beads, preincubated and washed with buffer 0.1 X S (containing the same ingredients as buffer S but only 0.2% (w/v) CHAPS and 0.1% (v/v) Triton X-100 in a final packed volume of 0.2 ml, were mixed with 0.8 ml of the 1000 X g supernatant fluid. After 2 h of incubation (4 °C, with gentle shaking), the lectin beads were sedimented by centrifugation for 1 min at 200 X g, and the supernatant fluid was collected and analyzed for the presence of CFTR. After three washes of the beads with 5 ml of buffer 0.1 X S, the bound glycoproteins were eluted by incubating the beads for 20 min with 1 ml of buffer 0.1 X S, containing a 0.5 M concentration of the appropriate sugar. The mixture was centrifuged again for 1 min at 1,000 × g, and the proteins in the supernatant fluid were precipitated by adjusting the solutions to 4% (w/v) trichloroacetic acid. Protein precipitation was facilitated by the addition of 100 μg/ml (final concentration) bovine serum albumin. The mixture was centrifuged for 5 min at 5,000 × g, and the pellet was dissolved in disaggregation buffer for SDS-PAGE analysis. The pH of the samples was neutralized by the addition of small amounts of Tris base.

Surface Labeling of Cells with [3H]UDP-galactose—Labeling of cell surface glycoproteins by tritiated galactose was carried out according to the method described in Ref. 25. In brief, approximately 106 airway cells or 5 × 105 T84 cells, collected as described above, were resuspended in 6 ml of a buffer containing 160 mM NaCl, 25 mM Tris, pH 7.0, with 1 mM MgCl2, 0.5 units of neuraminidase, and 5 μCi of UDP-[3H]galactose and incubated at 4 °C for 40 min. The labeled cells were then washed with Tris mannitol buffer, and membranes were prepared as described above.

Sucrose Density Gradient Separation of Membranes—Crude membranes were resuspended in Tris KCl buffer (50 mM Tris, pH 7.0, with 1 mM MgCl2, 0.5 mM PMSF, 2 mM d-mercaptoethanol) at a protein concentration of about 0.5 mg/ml, and 2-ml aliquots were layered on top of a discontinuous sucrose gradient consisting of 2-ml aliquots of 50%, 45%, 35%, and 25% (w/v) sucrose in Tris KCl buffer in a 12-ml centrifuge tube. The gradients were centrifuged in a Beckman SW 40 Ti rotor (150,000 × g average) at 4 °C for 3 h, and the supernatant fluids and the membrane layers at the top of each sucrose solution (0.6 ml) were collected and assayed for protein content and tritium content when appropriate. Additionally, the proteins in 0.5-ml aliquots of the collected samples were mixed with 100 μg of bovine serum albumin (final concentration), precipitated with trichloroacetic acid (4% (w/v)) final concentration, and the precipitates were collected by centrifugation. The trichloroacetic acid pellets were then dissolved in the amounts of disaggregation buffer necessary to obtain equal membrane protein concentrations, and the samples were analyzed by SDS-PAGE as described above.

Other Methods—Membrane protein concentrations were determined by a modified Lowry method (28). *H* measurements were performed in PG 2:1 (29) scintillation fluid in a Packard Tri-Carb 1500 liquid scintillation counter.

**RESULTS**

Fig. 1 shows Western blots of several whole epithelial cell and membrane preparations immobilized by two different polyclonal antibodies (K770 and K858) generated against polypeptide regions of the R domain (anti-CFTR-R) and the C terminus (anti-CFTR-C) of the CFTR, and affinity-purified by binding to the respective synthetic peptides as described under "Experimental Procedures." In the trichloroacetic acid-precipitated, SDS-solubilized epithelial cells (panel A) or crude membrane preparations thereof (panels B and C), both antibodies recognized several proteins including an approximately 180-kDa protein, which, as will be elaborated upon below, corresponds to the CFTR. This recognition was absent when the antibodies were preincubated with the relevant peptides (panels A and C, lane 5). Although shown only for the T84 cells, immunostaining of all of the bands in the other cells was likewise eliminated by preincubation of the antibodies with their specific peptides.

Consistent with the results shown in Fig. 1, in experiments carried out with more than 10 different cell and membrane preparations, the 180-kDa bands were present with about equal densities in both normal (Beas, HBE) and CF (ΔF 508
homzygote CF-T43 and CFT1) cells or in their isolated membranes. In T84 cells or membranes, the band appeared to be broader and more diffuse (see below), and the intensity of immunostaining in the 180-kDa region was always 20-50 times greater than in the airway cells, whether compared on the basis of cell number or membrane protein. As demonstrated in Fig. 1A, lane 4, the 180-kDa band was also recognized by the anti-CFTR antibody in cells of a primary culture of an airway tissue sample obtained from a AF 508 CF patient. A similar recognition of the 180-kDa band was observed in nasal scrapes of normal individuals and the CF (AF 508 homozygote) patients (data not shown). In HeLa cells and in Jurkat lymphoblast membrane preparations, a 180-kDa band was also observed, albeit with less intensity than in the airway cells, while no protein staining by either antibody could be detected in isolated human red blood cell membranes (not shown).

Fig. 1 also clearly shows that the two anti-peptide antibodies recognized several higher and lower molecular mass proteins in addition to the 180-kDa protein. In epithelial cells and membranes, the K770 anti-R antibody recognized proteins at about 350 kDa, 140 kDa, 105 kDa, and an intensive triplet between 45 and 55 kDa. The anti-C antibody K858 recognized bands at 140 kDa, 105 kDa, 65 kDa, and 40 kDa. The fact that all of these bands disappeared in the peptide competition experiments (Fig. 1, lanes 5) indicates the presence of similar epitopes in these proteins. Two other antibodies, generated against the R domain and the C terminus of the CFTR (K50 and K82), recognized the same 180- and 140-kDa bands and weakly the 105-kDa bands in all the epithelial cell types, while the recognition pattern for the lower molecular weight bands was different (see below, Fig. 3). Although a protein with about the expected molecular mass of the CFTR (168,000 + possible glycosylation) is clearly recognized by both types of our anti-CFTR polyclonal antibodies in the epithelial cells and their isolated membranes, the obvious presence of several other antibody-reactive proteins in these cells necessitated a further investigation of the identity of the 180-kDa band. In order to determine whether or not the epitope recognized by the anti-C terminus antibody is indeed at the C terminus of the various proteins, a limited carboxypeptidase Y digestion of the isolated cell membranes was performed, and the products were analyzed in Western blots. Incubations were carried out at room temperature with or without 0.5% Triton X-100 in the medium. As shown in Fig. 2, panel B, lane 3, a 5-min carboxypeptidase Y digestion of the isolated T84 membranes in the presence of 0.5% Triton X-100 eliminated the recognition of the 180-kDa band, as well as the lower molecular mass proteins, by the anti-C terminus antibody. In the absence of detergent, only about a 50% decrease in the intensity of the 180-kDa immunoreactive band was observed, even though the digestion was carried out for 60 min (lane 2). The detergent effect suggests that some of the antibody-reactive proteins are present in sealed vesicles with their C terminus inaccessible to the enzyme. In similar experiments with an anti-R domain antibody (panel A), there

FIG. 1. Recognition of epithelial cell proteins by the anti-CFTR antibodies. Intact cells (A) or crude membrane preparations (B,C) precipitated with trichloroacetic acid and dissolved in SDS-PAGE disaggregation buffer were electrophoresed in 4-15% acrylamide gradient SDS-PAGE gels and blotted onto PVDF membranes as described under “Experimental Procedures.” The binding of anti-CFTR R domain (K770, panel A and B) and of anti-CFTR C terminus (K858, panel C) anti-peptide antibodies was detected by a peroxidase-conjugated second antibody using the chemiluminescence method. Samples containing 50μg of protein were deposited in each well. Lanes 1, Beas cells or membranes; lanes 2, CF-T43 cells or membranes; lanes 3, T84 cells or membranes; lane 4, cells from a primary culture of airway epithelial cells from a CF (ΔF 508 homozygote) patient; lanes 5, T84 cells or membranes, immunodetection performed after preincubation of the anti-CFTR antibodies with the appropriate peptides (20μg/ml) for 2 h. The numbers on the scales or arrows in each figure show Mr, + 1,000.

FIG. 2. Effect of carboxypeptidase Y digestion on the recognition of the epithelial cell membrane proteins by the anti-CFTR antibodies. T84 cells membranes were treated with carboxypeptidase Y, precipitated with trichloroacetic acid, and dissolved in SDS-PAGE disaggregation buffer as described under “Experimental Procedures.” Samples were electrophoresed and immunoblotted, and the immunostaining was visualized as described in the legend for Fig. 1. Each lane contained 50μg of membrane protein. Panel A, detection with anti-CFTR R domain (K770) anti-peptide antibody; panel B, detection with anti-CFTR C terminus (K858) anti-peptide antibody. Lanes 1, membranes incubated for 60 min without carboxypeptidase Y in the presence of 0.5% Triton X-100; lanes 2, membranes incubated for 60 min with carboxypeptidase Y in the absence of 0.5% Triton X-100; lanes 3, membranes incubated for 5 min with carboxypeptidase Y in the presence of 0.5% Triton X-100.
was no change in the recognition of the immunoreactive bands over a 60-min period, irrespective of the presence or absence of detergent. Identical results were obtained with isolated membranes of Beas and CF-T43 airway epithelial cells (data not shown). These findings support the identity of the CFTR with the 180-kDa band recognized by the anti-C terminus antibody.

To further explore the identity of the 180-kDa protein, we examined the expression of the CFTR by introducing its cDNA into several host cells via viral vectors. For these experiments, the antibody-reactive proteins were separated in longer, 6% Laemmli-type gels, which provide a better resolution for the 180-kDa region than the minigels. As shown in Fig. 3A, lanes 1, in T84 cells, both the R domain (K770 and K50) and the C terminus (K858) antibodies recognize a broad, multiple, and/or fuzzy band between 165- and 185 kDa, and a sharp band at the top of it (about 185 kDa), slightly overlapping the fuzzy band. In CFTl (AF 508 homozygote) cells, a band at 140 kDa is also recognized by both the R domain and the C terminus antibodies. A similar picture was observed in the case of all the airway epithelial cell types examined (not shown).

Retroviral introduction of the normal CFTR into CFTl (Fig. 3A, lanes 3) airway epithelial cells induced a dramatic increase in the immunoreactive bands in the 180-kDa region, including increases in the intensities of the sharp upper band, the broad fuzzy band, and the 140-kDa band. Importantly, these increases were seen with all three anti-CFTR antibodies. Although not shown, similar results were obtained with control and CFTR cDNA-infected normal (HBE) airway epithelial cells. Collectively, these data strongly suggest that the immunoreactive bands in the 180-kDa and 140-kDa regions represent various forms of the CFTR protein.

Fig. 3B demonstrates expression of the CFTR in mouse NIH 3T3 fibroblasts and in insect SF9 cells, again using appropriate viral vectors. In the control (mock-infected) mouse NIH 3T3 fibroblasts (lanes 1), the anti-CFTR antibody; reported a low level of the 180- and 140-kDa proteins. The CFTR expressed in these cells via a retroviral vector (lanes 2) was clearly recognized as a broad band at an apparent molecular mass of about 180 kDa by both the anti-R domain and anti-C terminus antibodies. When expressed in the cultured insect ovarian SF9 cells via a baculovirus containing the CFTR cDNA (lanes 3), the CFTR had an apparent molecular mass of about 140 kDa, as reported by both antibodies. In the control SF9 cells, with the small amount of protein applied in the electrophoresis wells, no endogenous antibody reactivity was detected. The lower molecular mass of the CFTR in SF9 cells presumably results from inefficient glycosylation in this system (16, and see below).

It is important to note that in the CFTR-overexpressing epithelial cells or fibroblasts there was no major change in the amount of another characteristic membrane protein, the plasma membrane calcium ATPase, and no significant differences could be observed in the protein pattern of the Coomassie blue-stained blots (not shown). These latter data indicate that the CFTR, even when overexpressed, is a minor protein component of the mammalian cell membranes. In CFTR-expressing SF9 cells, a visible, although low intensity protein band, at 140 kDa could be detected by Coomassie Blue staining.

Taken together, all of the above data suggest that the approximately 180-kDa proteins recognized in the epithelial cells by the anti-CFTR-peptide polyclonal antibodies, indeed represent the CFTR. They also point to a polydispersity of the CFTR, probably caused by glycosylation and/or other post-translational modifications.

In the following experiments we intended to examine the nature of the possible glycosylation of the CFTR in the epithelial cells. Again using 6% gels for higher resolution, we found that, when isolated, detergent-treated T84 membranes were incubated with N-glycosidase, the molecular mass of the major, broad CFTR band decreased from about 180 kDa to 140 kDa (Fig. 4A, lanes 1 and 2). However, a sharp band near the top (about 185 kDa), and another band, near to the lower
FIG. 4. A, N-glycosidase digestion of T84 and CFTR-expressing Sf9 cell membranes. Detergent-solubilized membranes were treated with N-glycosidase as described under “Experimental Procedures.” Samples were electrophoresed and immunoblotted, and the immunostaining was visualized as described in the legend for panel A. Immunodetection was carried out with anti-CFTR C terminus antibody, K858. Lane 1, T84 cell membranes (50 μg of protein), incubated overnight without N-glycosidase; lane 2, T84 cell membranes (50 μg of protein) incubated overnight with 2 units of N-glycosidase; lane 3, 2 μg of protein from Sf9 cells expressing the CFTR, incubated overnight without N-glycosidase; lane 4, 2 μg of protein from Sf9 cells expressing the CFTR, incubated overnight with 2 units of N-glycosidase. B, wheat germ agglutinin-agarose absorption of the CFTR. T84 cell membranes were treated with the lectin agarose, precipitated with trichloroacetic acid and dissolved in SDS-PAGE disaggregation buffer as described under “Experimental Procedures.” Samples were electrophoresed and immunoblotted, and the immunostaining by the anti-CFTR antibodies was visualized as described in the legend for Fig. 1. Immunoblots with anti-PMCA and anti-SERCA antibodies were developed with alkaline phosphatase-conjugated secondary antibodies. Panel I, detection with anti-CFTR C domain (K858) antipeptide antibody; panel II, detection with the anti-PMCA monoclonal antibody; panel III, detection with the anti-sarcoendoplasmic reticulum calcium ATPase (SERCA) monoclonal antibody. Lanes 1, 50 μg of T84 membrane protein solubilized in 2% (w/v) CHAPS + 1% (v/v) Triton X-100; lanes 2, 5 μg of WGA-agarose-isolated T84 membrane proteins plus 50 μg of bovine serum albumin (see “Experimental Procedures”).
the enzyme, and the monoclonal anti-PMCA antibody detected a 140-kDa protein, which is the monomer size of this ATPase. The anti-SERCA monoclonal antibody reacted with the 100-kDa calcium ATPase present in the endoplasmic reticulum, while the polyclonal antibody against the mitochondrial inner membrane complex III detected five prominent bands (two core proteins, two cytochromes, and iron-sulfur protein) in the range of about 45–24 kDa.

Pilot experiments with various epithelial cells indicated that the surface and intracellular organelle membranes in our crude membrane preparations could be effectively separated in sucrose step gradients at sucrose concentrations between 25 and 50%. The plasma membrane markers had the highest intensities at the 35% sucrose shelf, while the endoplasmic reticulum marker was greatly enriched at 50% sucrose. The mitochondrial markers were found to be present in each density gradient fraction, but the highest levels were found at 50% sucrose. The subcellular distribution of the various markers is presented below as part of Fig. 6.

Fig. 5 demonstrates the distribution of the anti-CFTR-C reactive bands of the Beas (normal) and the CF-T43 (AF 508 homozygote) cell membranes in the sucrose gradient fractions. For both membrane preparations, the 180-kDa CFTR band had the highest intensity at the 35% sucrose shelf, where the plasma membrane markers are enriched (Fig. 6), while most of the lower molecular mass antibody-reactive proteins were concentrated in the lower density fractions. This is in good agreement with the fact that these proteins are easily removed from the membranes by high salt or lithium diiodosalicylate treatments. When detected with the anti-R domain antibodies, the CFTR showed the same localization in the sucrose fractions, and the lower molecular mass bands were likewise enriched in the lower density fractions (data not shown).

Fig. 6 shows a quantitative compilation of the data obtained by densitometry of the CFTR, the PMCA, the SERCA, the Na⁺,K⁺-ATPase, and the Complex III immunoreactive materials, and by radioactivity measurements for the distribution of the [³H]galactose surface label, in membrane fractions from Beas (normal) and CF-T43 (AF 508 CFTR) airway epithelial cells after sucrose density gradient centrifugation. Clearly, in both cell membranes, the 180-kDa CFTR co-localizes with the plasma membrane marker enzymes and with the peak of the ³H activity. The CFTR is much less abundant in the lighter fractions and at the higher sucrose densities, where the endoplasmic reticulum and mitochondrial membranes are enriched. As shown in panels C and D, in contrast to the integral plasma membrane markers, some [³H]galactose label is also present near to the top of the gradient, presumably reflecting the surface labeling of nonintegral membrane glycoproteins or glycolipids. This interpretation is supported by the fact that about 30% of the [³H]galactose label is removed from the membranes by extraction with 1 M KCl (data not shown). Although not shown, in T84 cell membrane preparations, the CFTR and the different membrane markers exhibited the same distribution pattern as demonstrated for the airway cells.

Fig. 7 shows subcellular fractionation data for the CFTR in the sibling airway epithelial cell lines, HBE (normal) and CFT1 (AF 508 homozygote), as well as in the CFT1 cells infected with CFTR cDNA-containing retroviral vector and thus expressing large amounts of the normal CFTR. Similar to the results obtained with the Beas, CF-T43, and T84 cells, for all these cells the CFTR was enriched in the sucrose density fractions corresponding to the plasma membrane markers and not in the heavier fractions enriched in endoplasmic reticulum and mitochondrial membranes.
The cloning of the gene responsible for cystic fibrosis and the determination of the gene sequence (1-3) opened up many new avenues of approach to the investigation of this severe genetic disease. In the present study, we have used this sequence information to prepare immunoreagents to be used in experiments to detect the CF gene product and determine its biochemical features and cellular localization, with the future aim to purify it, and eventually ascertain its physiological function in a reconstituted system.

Although not elaborated upon under “Results,” several obstacles encountered in these studies are worth noting briefly. In order to avoid nonspecific reactions of the antisera on Western blots, the antibodies had to be affinity-purified using the appropriate peptides immobilized on a Sulfolink column. We also found that standard SDS-PAGE and blotting techniques did not efficiently transfer the high molecular mass integral membrane proteins, rendering a quantitative evaluation of the amounts of these proteins on Western blots impossible. However, the use of a high glycine, no methanol disaggregation buffer as described under “Experimental Procedures.” Samples were electrophoresed and immunoblotted, and the immunostaining by anti-CFTR C terminus antibody (KS58) was visualized as described in the legend for Fig. 1. Equal amounts of protein (10 μg) were loaded in each well. The intensity of the immunostained bands was quantitated by densitometry and computer-assisted integration of peak areas. Data are from a representative experiment.  

**Figure 7.** Distribution of the CFTR after sucrose density gradient separation of membrane fractions of CFT1, HBE, and CFT1 cells overexpressing the normal CFTR. Membranes were fractionated on a sucrose step gradient, and aliquots of different fractions were precipitated with trichloroacetic acid and dissolved in SDS-PAGE disaggregation buffer as described under “Experimental Procedures.” Samples were electrophoresed and immunoblotted, and the immunostaining by anti-CFTR C terminus antibody (KS58) was visualized as described in the legend for Fig. 1. Equal amounts of protein (10 μg) were loaded in each well. The intensity of the immunostained bands was quantitated by densitometry and computer-assisted integration of peak areas. Data are from a representative experiment.

The most compelling evidence that the approximately 180-kDa band is the CFTR came from the CFTR expression experiments. The CFTR overexpressed in human epithelial cells and in mouse fibroblasts via viral vectors, albeit with a somewhat different appearance in the different cell types, was identical to the CFTR antibodies at about 180 kDa. In the airway cells, overexpression increased the intensity of the sharp CFTR bands and also produced a broad, fuzzy band similar to that seen in the T84 tumor cells (see Fig. 3). In NIH 3T3 mouse fibroblasts, the CFTR overexpressed by the human CFTR cDNA showed a broad fuzzy band in the 180-kDa region (Fig. 3B). When the CFTR was expressed in insect S9 cells, similar to an earlier report (16), a 140-kDa, probably un- or underglycosylated form of the protein, was detected by both the anti-C and anti-R CFTR antibodies. This 140-kDa immunoreactive band, with relatively low intensity in the control cells, but increasing with overexpression of the CFTR, was also observed in the human epithelial and in the NIH 3T3 cells. The smaller molecular mass proteins in various cells, recognized differently by the different antibodies, are most probably not related to the CFTR, inasmuch as the amounts of these proteins do not change detectably when cells are overexpressing the CFTR.

All the above findings strongly support the conclusion that our polyclonal antibodies recognize the CFTR and that the endogenous, mature CFTR in human epithelial cells has an apparent molecular mass of about 180 kDa. Nevertheless, a molecular heterogeneity of the mature CFTR is suggested by the present experiments. In the 165-185-kDa region of the immunoblots, multiple immunoreactive bands can be resolved which, although similarly recognized by the different antibodies, are clearly different. N-Glycosidase treatment of the CFTR in T84 cell membranes reduced the molecular mass of the major CFTR band from about 180 kDa to 140 kDa, equivalent to the molecular mass of the underglycosylated CFTR in S9 cells (Fig. 4A). However, certain T84 cell CFTR bands found in the airway epithelial cell CFTR could not be enzymatically deglycosylated. Detergent-solubilized CFTR of T84 cell membranes was shown to be specifically bound to wheat germ agglutinin agarose and then eluted with the lectin-specific sugar N-acetylgalcosamine (Fig. 4B), while no such lectin agarse binding was found in the case of the airway epithelial cell CFTR. These findings suggest a different pattern of glycosylation and/or other post-translational modifications of this protein in the different cells. The biosynthesis of heterogeneous forms and various glycosylation patterns of the multidrug resistance protein (P-glycoprotein), a protein apparently similar to the CFTR, has already been described (32). An alternative explanation for the differences observed here could be an incomplete solubilization of the
epithelial cell membranes in the detergents used and the formation of membrane protein clusters which may prevent glycosidase and/or specific lectin interactions. Thus, the nature of the glycosylation and post-translational modification of the CFTR in the various cell types requires further investigation.

Three other important biochemical features of the CFTR are also apparent from these studies. First, treatment of isolated membranes by high salt concentrations and lithium diodosalicylate, agents which remove loosely attached proteins (30), did not solubilize the CFTR, indicating that it is an integral membrane protein. Second, the fully mature, 180-kDa form of the CFTR is present both in normal and in ΔF 508 mutant airway cells, indicating that the Phel00 deletion does not interfere in any major way with the normal biosynthesis of the endogenous CFTR. Third, in all of our experiments, it was observed that the CFTR is a minor membrane component, unrecognizable by Coomassie Blue protein staining even in the CFTR-overexpressing mammalian cells or in lectin-isolated membrane glycoprotein fractions.

A major question related to the function of the CFTR is its subcellular localization, as in CF a possible error in the cellular processing and transport of this protein has been suggested (17). Immunocytochemical techniques could be decisive in this respect. However, such studies have made uncertain by the presence of lower molecular mass immunoreactive proteins, possibly unrelated to the CFTR, in the epithelial cells. We therefore employed a biochemical approach to establish the subcellular localization of the CFTR. Because culturing of the airway epithelial cells in large quantities is time-consuming, laborious, and expensive, only submilligram quantities of membranes could be used for the fractionation studies. We therefore developed a relatively simple sucrose step gradient technique which proved to be useful in differentiating between cell surface and internal membranes in small samples of crude membranes. Moreover, the use of specific antibodies against the relevant membrane marker enzymes significantly improved the sensitivity of the detection over most conventional marker enzyme assays. The significant enrichment of the plasma membrane markers at the 35% sucrose shelf and the enrichment of the endoplasmic reticulum and mitochondrial markers at higher sucrose densities indicates a reasonably efficient separation of the different cellular membranes by this technique.

The most important findings in the subcellular localization experiments are that the CFTR is a plasma membrane protein and that it is localized in the plasma membranes of both normal and ΔF 508 homozygote airway epithelial cells. The experiments were carried out with two different normal (Beas and HBE) and two different ΔF 508 mutant (CF-T43 and CFT) cell lines, and identical results were obtained in all cases (Figs. 6 and 7). A similar localization of the CFTR was found for the airway cells expressing large amounts of the CFTR (Fig. 7) and for the T84 cells as well. These results render it unlikely that the major problem in the ΔF 508 CFTR cells is defective maturation and/or transport of the mutant endogenous CFTR to the plasma membrane. Recent work by Gregory et al. (33), involving expression of CFTR mutants which lack glycosylation sites, clearly demonstrates that carbohydrate addition per se is not necessary for CFTR to reach its site of action or to generate cAMP-activated Cl− channels. Thus, although in a heterologous expression system the maturation of ΔF 508 CFTR seems to be impaired (17), our results suggest that explanations for the CF pathogenesis other than the absence of the CFTR in the plasma membrane are more likely to be correct.

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