Localization of the Cystic Fibrosis Transmembrane Conductance Regulator in Pancreas

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Abstract

Cystic fibrosis (CF) is characterized by an abnormality in cAMP-regulated chloride transport that results from a primary defect in the protein product of the CF gene, the CF transmembrane conductance regulator (CFTR). In this report, antibodies against CFTR peptides were used to localize the CFTR protein in human pancreas. An affinity purified antibody (α-1468) raised against a synthetic CFTR peptide identified a 155–170-kD protein on immunoblot. Cytochemical studies with α-1468 localized CFTR to small branching, tubular structures. The same structures were recognized by two other antibodies raised against different regions of the CFTR molecule. To identify the cells being stained, double-label immunofluorescence studies were performed using α-1468 and a monoclonal antibody which stains pancreatic centroacinar and intralobular duct cells. Both antibodies localized to the same population of cells, with α-1468 being confined to the apical domain of these cells. No conclusive staining of acinar cells was evident. These findings suggest that proximal duct epithelial cells play a key role in the early events leading to pancreatic insufficiency in CF, and imply that apical chloride transport by these cells is essential for normal pancreatic secretory function. (*J. Clin. Invest.* 1991. 88:712–716.) Key words: CFTR • pancreatic secretion • duct cell • epithelial cell polarity • immunocytochemistry • chloride

Introduction

Cystic fibrosis (CF)[1] is a genetic disease in which progressive pancreatic and pulmonary insufficiency occurs in association with abnormalities in epithelial chloride transport (1). Within epithelial cells, CF is characterized by a defect in cyclic AMP-regulated membrane chloride conductance (2, 3). The gene responsible for CF was recently identified (4–6) and its protein product, designated the CF transmembrane conductance regulator (CFTR), has been expressed in transfected cells (7). Such transfected cells have been used to show that the normal form of CFTR differs from that commonly found in CF in both its ability to restore cAMP-regulated chloride transport in CF cells (8, 9) and its glycosylation and intracellular localization (10). Nonetheless, the role of CFTR in regulating chloride transport in secretory epithelia remains unknown.

Understanding the function and pathophysiologic significance of CFTR will require information about its cellular and subcellular distribution in affected secretory organs. Among chloride-secreting tissues, the pancreas was selected for cytochemical study because it expresses CFTR mRNA at high levels (5). Within the exocrine pancreas, both acinar and duct cells exhibit regulated chloride transport (11–13). Accordingly, both cell types can be implicated as potential sites of the primary pancreatic defect in CF. In the present study, antibodies to CFTR peptides were used to demonstrate that CFTR is primarily associated with pancreatic duct cells. This finding supports a model of CF pathogenesis which is based on a primary defect in proximal duct epithelial cell function.

Methods

Generation of antibodies. CFTR(1468–1480), sequence KEETEEEVQDTRL, and CFTR(26–39), sequence KGYRQRLELSDIYQ, were synthesized and HPLC-purified by Multiple Peptide Systems (San Diego, CA). Peptide (3 mg in ~ 200 μl water) was conjugated to thyroglobulin (60 mg in 4 ml of 100 mM phosphate buffer, pH 7.4) using glutaraldehyde (final concentration, 0.1% in 100 mM phosphate buffer, pH 7.4). The solution was quenched with sodium borohydride (10 μM final concentration), dialyzed against 100 mM NaCl, 10 mM phosphate buffer, pH 7.4, and concentrated with Aquacide II (Calbiochem Behring Corp., La Jolla, CA). The resulting peptide conjugate preparations (~ 50 mg/ml) were used to immunize rabbits. For antibody purification, 3 mg of peptide (dissolved in water) was coupled to 300 mg of epoxy-activated Sepharose beads (Pharmacia Fine Chemicals, Vineland, NJ). The affinity columns were washed with 100 mM NaCl, 10 mM Tris, pH 7.8, and 0.01% NaN_3 (TBS buffer) supplemented with 5 mM benzamidine and 0.1% BSA, and then incubated with antiserum. The CFTR(1468–1480) peptide column was washed with 500 mM NaCl in TBS buffer before eluting the antibody (designated α-1468) with 4.9 M $MgCl_2$, pH 6.5. Antibody bound to the CFTR(26–39) peptide column was eluted with 1 M acetic acid (pH 2) containing 100 mM NaCl and immediately neutralized with 2 M Tris, pH 7.4 to yield α-26. Both antibodies were subsequently desalted by dialysis against TBS buffer with 1 mM benzamidine, concentrated with Aquacide II, and stored at a final concentration of ~ 0.5 mg/ml in 50% glycerol at −20°C.

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1. *Abbreviations used in this paper:* CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator.

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Immunoblot. To determine the electrophoretic mobility of antigens detected by α-1468, detergent-solubilized proteins from normal human pancreas (obtained from an organ donor under a protocol approved by the Yale University Human Investigations Committee) were separated by SDS-PAGE (14) and transferred to nitrocellulose (15). Nitrocellulose strips were quenched with 5% nonfat dry milk in TBS buffer, pH 7.4, containing 0.1% Triton X-100 (quench buffer) and reacted overnight at 4°C with α-1468 diluted 1:50 in quench buffer supplemented with protease inhibitors (2.5 μg/ml of soybean trypsin inhibitor and 1 μg/ml each of leupeptin, chymostatin, pepstatin, and aprotinin). To determine the specificity of labeling, α-1468 (final concentration, ~ 0.25 mg/ml) was preincubated overnight at 4°C with CFTR(1468–1480) peptide (0.5 mg/ml). The samples were then diluted and used for immunoblotting as described above. After primary antibody reaction, nitrocellulose strips were reacted with ^{125}I-goat anti-rabbit IgG (New England Nuclear, Boston, MA) and immunoreactive bands were visualized by autoradiography.

Immunocytochemistry. Normal human pancreas was obtained from organ donors and immediately frozen in liquid nitrogen. Frozen sections (2–4 μm) were fixed in acetone for 10 min at −20°C, air dried, and rehydrated in PBS (100 mM NaCl, 10 mM sodium phosphate, pH 7.4) before cytochemical analysis. For indirect immunofluorescence studies, fixed tissue sections were rinsed in PBS containing 1% BSA and the protease inhibitors described above. Tissue sections were incubated with α-1468 at room temperature for 1 h. To determine the specificity of labeling, α-1468 was preincubated with peptides using the procedure described above. Some sections were labeled with a mouse monoclonal antibody against a β-galactosidase-CFTR exon 13 fusion protein (Genzyme Corp., Cambridge, MA) and used at a final concentration of 2 μg/ml. The basolateral membranes of ductular cells were labeled using a mouse monoclonal antibody (7-2M) raised against the β-subunit of the Na^+/K^+-ATPase (provided by J. D. Jamieson, Yale University School of Medicine) at a dilution of 1:50. For double-labeling experiments, primary antibody incubations were performed simultaneously. Tissue sections were subsequently washed with PBS containing 1% BSA and protease inhibitors, then incubated at room temperature for 1 h with dilutions (see figure legends) of either TRITC- or FITC-conjugated goat $F(ab')_2$ fragments (affinity isolated and human Ig absorbed; Tago Inc., Burlingame, CA). Images were photographed on a Zeiss Axiophot fluorescent microscope (provided by P. DeCamilli, Yale University School of Medicine).

Immunoperoxidase studies were performed on either acetone-fixed, paraffin-embedded tissue or on frozen, unfixed tissue. Frozen tissue was briefly fixed in acetone as described above. Tissue sections were then quenched with PBS containing 1.5% vol/vol of goat serum and incubated for 1 h at room temperature with α-1468 or α-26 diluted 1:50 in PBS with 1.5% vol/vol of goat serum and protease inhibitors. Immunoreactivity was detected using biotinylated goat anti-rabbit IgG, followed by horseradish peroxidase-conjugated avidin (Vectastain ABC kit; Vector Laboratories, Burlingame CA) and 0.2% diaminobenzidine with H_2O_2.

Results

Immunoblots of human pancreatic homogenates using α-1468 detected a predominant protein of 155–170 kD (Fig. 1, lane *a*). This broad band has a molecular mass similar to that predicted for CFTR (5) and resembles that reported for the protein immunoprecipitated from transfected cells expressing CFTR (7, 10). Binding was specific in that the 155–170-kD band was eliminated when α-1468 was preincubated with excess CFTR(1468–1480) peptide (Fig. 1, lane *b*). Extensive washing of the peptide affinity column with 30–40 ml of 500 mM NaCl in TBS buffer before antibody elution resulted in a preparation of α-1468 exhibiting minimal nonspecific binding (Fig. 1, lane *c*). This preparation of α-1468, which detected only the 155–170-kD protein, was used to stain tissue sections.

In immunocytochemical studies of pancreas, α-1468 localized to small tubular structures, some of which exhibited a branching pattern (Fig. 2 *a*). Immunofluorescence was confined to the cells lining these structures and was eliminated by preincubation of α-1468 with CFTR(1468–1480) peptide (Fig. 2 *b*). Staining was not eliminated by preincubation of α-1468 with CFTR(26–39) peptide or with a second unrelated peptide, sequence CLGEESEERDDHLLPH (data not shown). The same α-1468 staining pattern was obtained using biotinylated IgG and avidin-conjugated horseradish peroxidase for detection (Fig. 2 *c*). No specific staining was observed with secondary antibodies alone.

To confirm that α-1468 was recognizing CFTR, similar studies were performed using two other antibodies raised against different regions of the CFTR molecule. α-26, an affinity purified rabbit antibody to a peptide near the NH_2-terminal region of CFTR, localized to structures indistinguishable from those identified with α-1468 (Fig. 2 *d*). Identical results were also obtained with a mouse monoclonal antibody raised against a β-galactosidase-CFTR exon 13 (R-domain) fusion protein. This monoclonal antibody (designated 13-1) has been previously shown to immunoprecipitate recombinant and en-

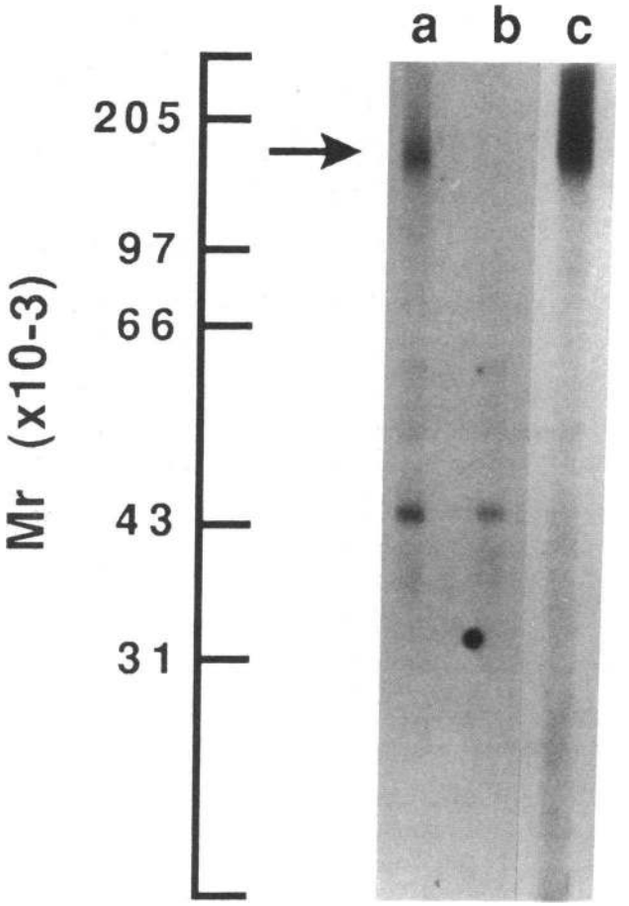

Figure 1. Immunoblots of human pancreas using α-1468. (Lane *a*) α-1468; (lane *b*) α-1468 preincubated with CFTR(1468–1480) peptide; (lane *c*) α-1468, obtained after washing the affinity column with 30–40 ml of 500 mM NaCl in TBS buffer (autoradiograph overexposed to show absence of minor contaminants). Molecular mass markers are labeled and the arrow identifies a band of 155–170 kD.

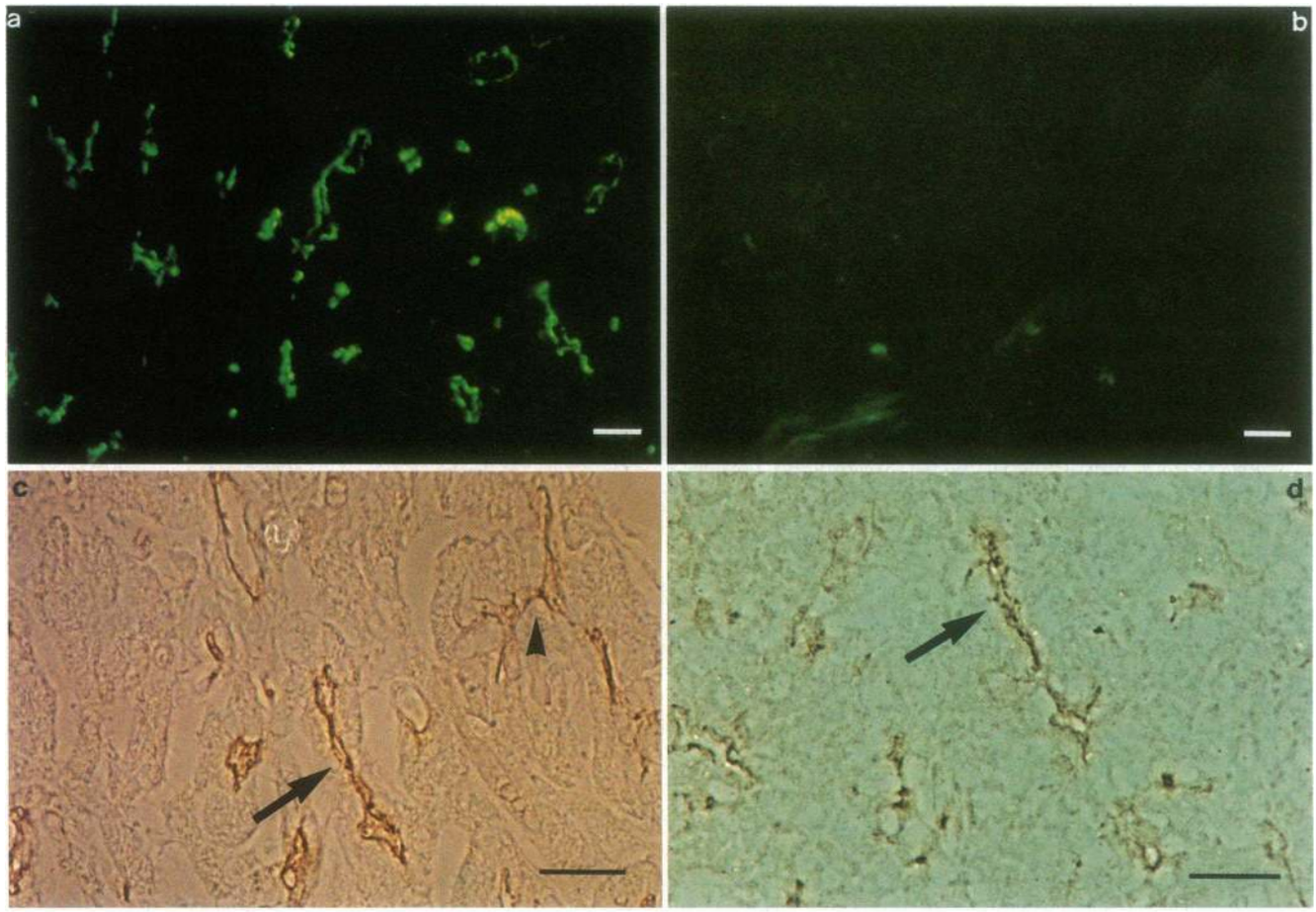

Figure 2. Immunocytochemical localization of α-1468 (final dilution, 1:50) and α-26 (final dilution, 1:50) in human pancreas. (*a* and *b*) Indirect immunofluorescence with α-1468 alone (*a*) or with α-1468 preincubated with peptide (*b*). Staining detected with FITC-goat anti-rabbit $F(ab')_2$ fragments diluted 1:200. (*c* and *d*) Immunoperoxidase localization of α-1468 (*c*) or α-26 (*d*). Arrows identify the tubular structures. Arrowhead denotes branching. Bar, 10 μm.

dogenous CFTR in transfected cell lines (7, 10). Double-label immunocytochemical studies with α-1468 and 13-1 demonstrated colocalization of both antibodies to the same tubular structures (Fig. 3). No staining of other structures was noted with either antibody. Thus, antibodies raised against three different regions of CFTR recognized the same structures. This indicates that the immunoreactive species being identified is CFTR.

The tubular structures identified using α-1468 appeared to be lined by cuboidal epithelial cells, consistent with the morphology of small (intralobular) ducts in the pancreas. Epithelia of larger ducts did not stain as intensely or as consistently as those of smaller ducts (data not shown). No prominent staining of either very large (lobular) ducts or acini was noted. To further characterize the tubular structures stained by α-1468, double-label experiments were performed using a monoclonal antibody against the β-subunit of Na^+-K^+-ATPase. This antibody, also known as 7-2M, has been shown to intensely stain the basolateral membranes of centroacinar and intralobular duct cells in pancreas (16). When human pancreas was double-labeled with both α-1468 and 7-2M, the two antibodies bound to the same population of cells (Fig. 4). This finding confirms that the cells stained with α-1468 are epithelial cells lining the proximal ducts.

At high magnification, it was evident that α-1468 and 7-2M were associated with different membrane domains of these duct cells (Fig. 4). The rhodamine signal (red) was associated with the basolateral domain, reflecting the polarized expression of Na^+-K^+-ATPase in intralobular duct cells of pancreas (16). By contrast, fluorescein staining (green) of α-1468 was detected only at or near the apical plasma membrane of these cells. No staining for CFTR was identified along the basolateral membrane. Thus, CFTR immunoreactivity in human pancreas exhibits polarized expression and is associated with the apical domain of proximal duct epithelial cells.

Discussion

In the present study, an affinity-purified antibody (α-1468) has been used to identify CFTR immunoreactivity in human pancreas. By Western blot, this antibody identifies a single protein species with a molecular mass consistent with that predicted for CFTR. In immunocytochemical studies, this antibody localizes to the apical domain of epithelial cells lining the proximal ducts. Because the same staining pattern is detected in localization studies using two additional antibodies raised against different regions of the CFTR molecule, we conclude that this staining pattern reflects the distribution of CFTR in this tissue.

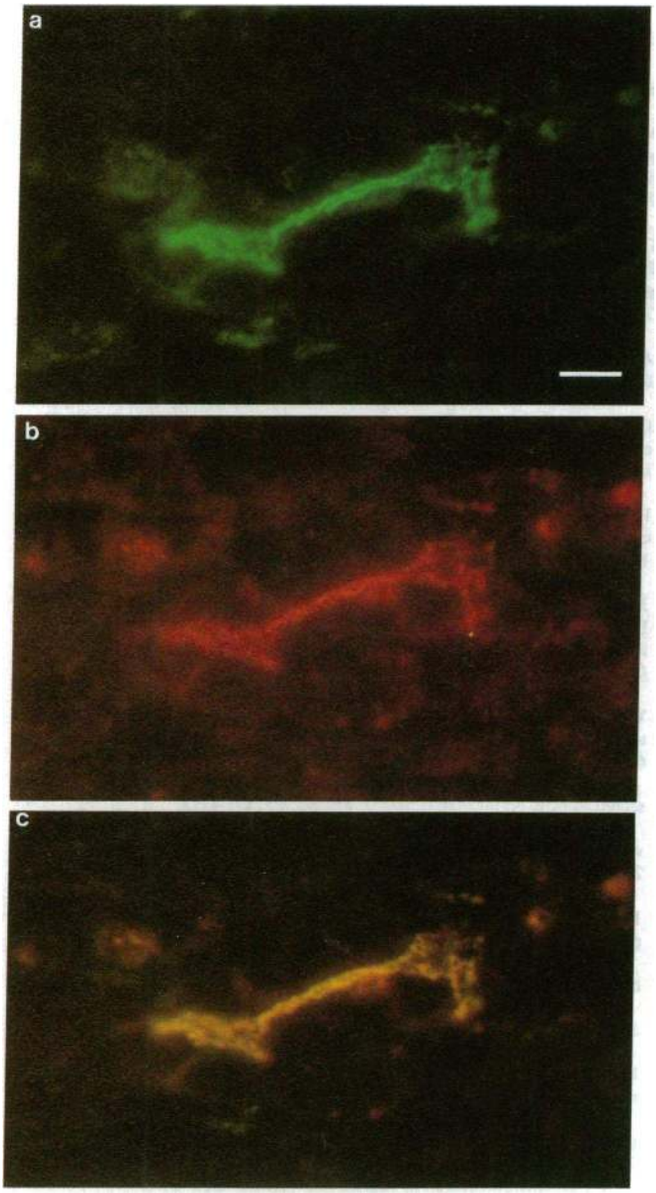

Figure 3. α-1468 and 13-1 stain the same tubular structures in human pancreas by double-label indirect immunofluorescence. α-1468, diluted 1:30, is detected with FITC-goat anti-rabbit $F(ab')_2$ fragments, diluted 1:200. 13-1, at 2 μg/ml concentration, is detected with TRITC-goat anti-mouse $F(ab')_2$ fragments, diluted 1:200. (*a*) α-1468 staining in the fluorescein channel; (*b*) 13-1 staining in the rhodamine channel; (*c*) the combined fluorescence in both channels. Bar, 10 μm.

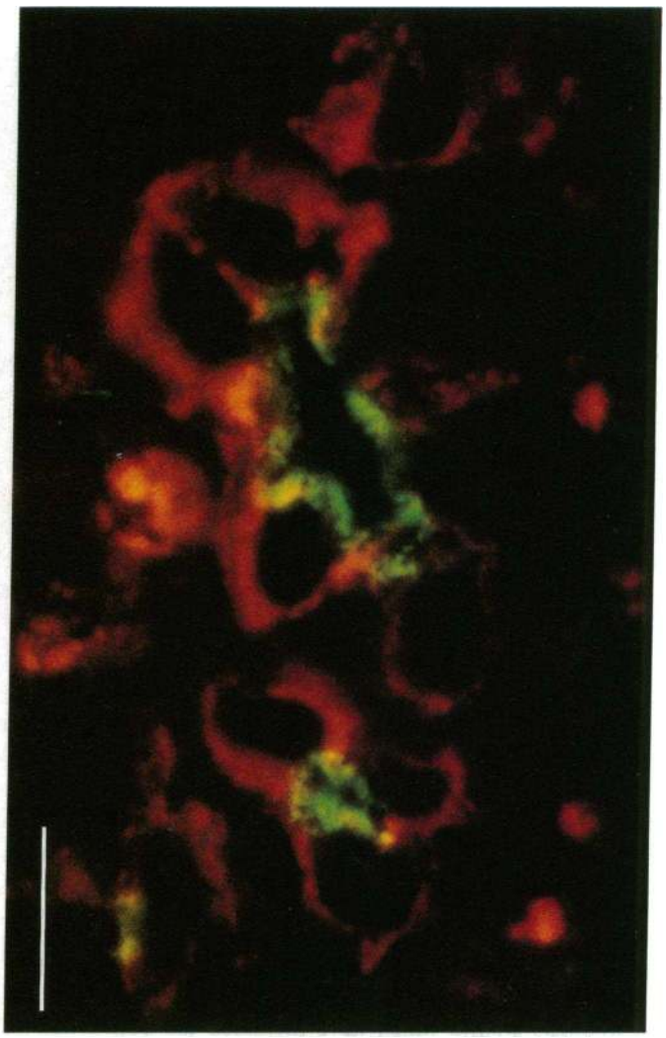

Figure 4. Double-labeling of human pancreas with α-1468 and 7-2M by indirect immunofluorescence. α-1468, diluted 1:50, is detected with FITC-goat anti-rabbit $F(ab')_2$ fragments, diluted 1:200, and 7-2M, diluted 1:50, is detected with TRITC-goat anti-mouse $F(ab')_2$ fragments, diluted 1:500. 7-2M generates a prominent red (rhodamine) signal localized to the basolateral domain of centroacinar and intralobular duct cells (16). α-1468 generates a green (fluorescein) signal localized to the apical domain of the same cells. Bar, 10 μm.

No prominent staining of either very large (lobular) duct cells or acinar cells was identified in this study. This finding does not, however, exclude the possibility that small quantities of CFTR may exist in these cell populations. The absence of significant acinar cell staining does support the conclusion that the intracellular mechanisms which regulate chloride transport in pancreatic acinar cells differ from those in duct cells. Thus, acinar cell chloride secretion may be predominantly regulated by changes in intracellular Ca^{2+} levels (11, 17) whereas duct cells, which are more responsive to secretagogues acting through cAMP, may mediate chloride secretion predominantly through a CFTR-dependent, cAMP-regulated pathway.

Within the duct epithelial cell, CFTR appears to occur at the apical plasma membrane. However, the methodology employed in this study cannot resolve staining of the plasma membrane from that of a membrane-associated subapical compartment. In either case, the localization of CFTR to the apical domain of these duct cells is in agreement with electrophysiological data which has demonstrated cAMP-regulated chloride channels on the apical membrane of human duct epithelial cells (13).

The localization of CFTR to the proximal segments of the pancreatic ductular system is consistent with current knowledge of duct cell physiology and CF pathology. The primary function of the ductular system is to enhance pancreatic flow and alkalinize the pancreatic juice (18). Both processes are associated with duct cell bicarbonate secretion (19). In CF, pancreatic secretions are less alkaline (20) and more concentrated (21), suggesting that diminished bicarbonate secretion is funda-

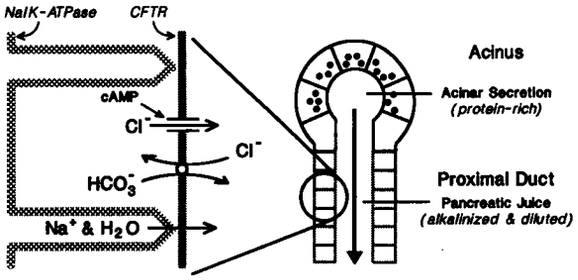

Figure 5. Model summarizing how CFTR may function during pancreatic secretion. As pancreatic juice flows through the duct, the protein-rich acinar secretions are diluted and alkalinized by the duct epithelial cells. CFTR, localized to the apical domain of these cells, exists near the site of the cAMP-regulated chloride channel which has been implicated in the control of bicarbonate secretion (13, 23). If CFTR is required for normal regulation of this channel, then failure of the channel to open properly in CF could lead to a decrease in bicarbonate secretion resulting in impaired fluid secretion. Thus, a defect in CFTR function in the duct cell may promote the formation of protein plugs and thereby contribute to the ductal obstruction and progressive pancreatic insufficiency which occur in CF.

mental to this disease. Because bicarbonate and chloride secretion are equally affected in CF, a functional relationship between these two transport processes has been proposed. This hypothesis postulates that the observed decrease in bicarbonate secretion in CF results from a primary defect in duct cell chloride transport (22). Although a cAMP-regulated chloride channel has been identified on the apical membrane of duct cells (13, 23), evidence for its involvement in the CF disease process has been lacking. The finding that CFTR is localized to the apical domain of the proximal duct cells provides independent support for this hypothesis. Further support is provided by pathologic studies which have identified the obstruction of proximal intralobular ducts by inspissated protein plugs as the initial pancreatic manifestation of CF (24, 25). Thus, a model can be proposed (Fig. 5) which implicates a defect in apical chloride transport by proximal pancreatic duct cells in the pathogenesis of CF. Through such a defect, duct cell bicarbonate secretion is decreased and the subsequent alkalinization and hydration of pancreatic secretions is compromised. The end result is inadequate dilution of protein-rich acinar secretions which remain viscous and eventually form protein plugs in the ducts.

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