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CFTR: Development of High- Affinity Antibodies and Localization in Sweat Gland

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Rabbit antisera were raised to six synthetic peptides corresponding to amino acid sequences contained in the protein product of the cystic fibrosis gene, CFTR. For two peptides, [Lys[102]]CFTR(102-116) and CFTR(1468-1480), antibody-peptide binding was of high affinity in that half-maximal binding occurred at peptide concentrations below 10 nM. Monospecific antibodies were prepared using these peptides, and these antibodies were used to stain human skin. Specific staining was detected in the cells lining the reabsorptive duct of the sweat gland. Within these lumenal cells, staining was most prominent at the apical domain but was also detected near the basolateral surface. This finding agrees well with predictions based on the effects of cystic fibrosis on sweat gland function, and suggests that these antibodies will be useful for studying CFTR in other human tissues.

In 1989, the cystic fibrosis (CF) gene was identified and its protein product was assigned the acronym, CFTR, for CF transmembrane conductance regulator [1,2]. Initial efforts to understand the function of CFTR were based both on knowledge of the ion transport abnormalities associated with CF [3,4], and on predictions about the structure of CFTR deduced from the gene's cDNA sequence [2].

Since that time, there has been rapid progress towards defining the properties of CFTR in transfected cells. Early studies established that the CF ion transport defect could be corrected by expressing the normal CFTR gene in CF cell lines [5-7]. More recent studies have led to two findings with profound implications concerning the function and regulation of CFTR: First, many CF-causing mutations affect the glycosylation and localization

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Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; α-102 and α-1468, monospecific antibodies against the CFTR peptides, [Lys[102]]CFTR(102-116) and CFTR(1468-1480), respectively.

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of CFTR in transfected cells [8,9]. And second, expression of CFTR in transfected cells is closely associated with the appearance of a specific type of chloride channel [10-12].

By contrast, relatively little information has been available concerning the localization and properties of CFTR in human tissues [13]. We have recently reported the localization of CFTR in human pancreas using α-1468, a monospecific antibody raised to a C-terminal CFTR peptide [14]. The present study presents additional information about α-1468, and describes the development of a second monospecific antibody, α-102, for use in cytochemical studies. After confirming that α-102 and α-1468 stain the same structures in human pancreas, these antibodies are used to determine the localization of CFTR in human sweat duct.

METHODS

CFTR peptides and generation of antisera. Antisera were raised to six synthetic peptides which were designed based on the CFTR deduced structure using four criteria: 1. Sequences showing homology to other proteins were avoided to minimize binding to proteins other than CFTR. 2. Hydrophobic sequences were avoided. 3. Peptides were designed to contain lysine as the N-terminal amino acid to permit oriented peptide coupling during preparation of thyroglobulin conjugates, peptide-Sepharose affinity columns and iodopeptides. 4. Sites of post-translational modification were avoided to increase the likelihood that antibodies would recognize naturally occurring forms of CFTR. Peptides were synthesized, HPLC-purified, conjugated to thyroglobulin, and used to immunize rabbits as described [14].

Peptide binding. The development of peptide-specific antibodies was monitored by ELISA using the appropriate unconjugated peptide. Antibody affinities were estimated by radioimmunoassay using iodinated [Lys^{102}]CFTR(102-116) and CFTR(1468-1480) prepared using [^{125}I]Bolton-Hunter reagent (ICN Biomedicals, Inc., Irvine, CA). Peptide-antiserum binding was performed overnight in 1 ml 100 mM Tris (pH 8.0) containing 0.05% Triton X-100, 0.1% BSA, 1 mM EGTA, 1 mM EDTA, 0.5 μl antiserum, plus 2 nCi ^{125}I-labeled iodopeptide. Bound vs. free iodopeptides were separated using 2% charcoal/1% dextran.

Generation of monospecific antibodies. Antibodies to [Lys^{102}]CFTR(102-116) were purified by affinity chromatography using epoxy-Sepharose (Pharmacia, Vineland, NJ) coupled to 3 mg peptide. The column was loaded with 3 ml of antiserum and washed with column buffer (100 mM NaCl, 0.1% BSA, 5 mM benzamidine, 0.01% NaN_3, 10 mM Tris, pH 7.8) followed by column buffer containing 500 mM NaCl. Monospecific antibodies, designated α-102, were eluted using 100 mM glycine, pH 2.5. The eluate was dialyzed against 100 mM NaCl, 1 mM benzamdine, 10 mM Tris, pH 7.8, and concentrated using Aquacide II (Calbiochem Behring Corp, La Jolla, CA), and stored at -20^{o} in 50% glycerol containing 0.02% NaN_3. Based on ELISA titers, the final recovery of peptide-binding antibodies was roughly 50%. Protein assays were performed using a protein assay kit (Protein Gold) obtained from Integrated Separation Systems, Hyde Park, MA using BSA as a standard.

Immunocytochemistry. Cell staining was performed using snap-frozen specimens of human pancreas and skin obtained at autopsy. Skin specimens were obtained with the assistance of B.J. Kerns of the Duke Comprehensive Cancer Center. Cryosections (4 μm) were fixed in acetone for 10 min at room

temperature, incubated in PBS containing 5% goat serum, and then incubated for 45 min at 40° C with α-102 or α-1468 in PBS (1-5 μg/ml, final concentration). Immunoperoxidase staining was performed using biotinylated goat anti-rabbit IgG (1:200) and a Vectastain Elite ABC kit (Vector Labs, Inc., Burlingame, CA). Specimens were counterstained with modified Harris Hematoxylin.

RESULTS AND DISCUSSION

The six peptides used for antiserum development are listed in table 1. Peak ELISA titers for each peptide were at least 1/3700, indicating that the peptides were immunogenic. Highest titers occurred with [Lys^{102}]CFTR(102-116) and CFTR(1468-1480), and additional studies focused on antisera raised against these peptides.

To estimate the affinities of these antisera, radioimmunoassays were developed using iodinated [Lys^{102}]CFTR(102-116) and CFTR(1468-1480). Figure 1 shows the purification of one of these iodopeptides by reverse-phase HPLC, as detected using in-line radioactivity (upper tracing) and UV (lower tracing) monitors. In this chromatogram, unlabeled peptide eluted at 34 min (arrow, lower tracing) and unreacted [^{125}I]Bolton-Hunter reagent eluted at 35-38 min (upper tracing). Binding studies were performed using the iodopeptide which eluted at 40 min (arrow, upper tracing). CFTR(1468-1480) was iodinated and HPLC-purified using the same procedure (not shown).

Table 2 summarizes calibration data for radioimmunoassays developed using antisera raised to [Lys^{102}]CFTR(102-116) and CFTR(1468-1480). Binding of each labeled iodopeptide was prevented by the addition of unlabeled peptide at low concentrations. In each case, half-maximal displacement of the iodopeptide occurred at peptide concentrations of approximately 5 nM. Because these antisera bind their respective peptides with high affinity, we anticipated that these sera would contain anti-peptide antibodies which could be used to examine CFTR immunoreactivity. In the case of CFTR(1468-1480), we have recently used such a monospecific antibody, α-1468, to determine the localization of CFTR in human pancreas [14]. To further

Table 1. CFTR Peptides

Peptide	Sequence	Titer	CFTR domain
CFTR(26-39)	KGYRQRLELSDIYQ-NH_2	8300	cytoplasmic
[Lys^{102}]CFTR(102-116)	KGRIIASYDPDNKEE-amide	20000	extracellular
CFTR(254-268)	KISERLVITSEMIEN-amide	3700	cytoplasmic
CFTR(716-730)	KTPLQMNGIEEDSDE-amide	6900	cytoplasmic
[Lys^{814}]CFTR(814-828)	KETGLEISEEINEED-amide	4100	cytoplasmic
CFTR(1468-1480)	KEETEEEVQDTRL-acid	10000	cytoplasmic

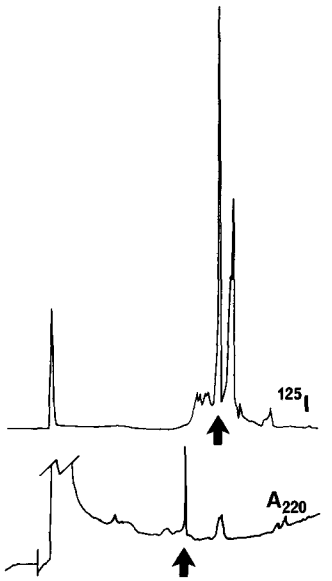

Figure 1. Purification of iodinated [Lys^{102}]CFTR(102-116) by HPLC. The iodopeptide was eluted from a C-18 column using a linear acetonitrile gradient in 0.1% TFA. The elution of iodopeptides was detected based on radioactivity (^{125}I, upper tracing) and absorbance at 220 nm (A_{220}, lower tracing). Unlabeled peptide eluted at 34 min (arrow, lower tracing); the labeled iodopeptide eluted at 40 min (arrow, upper tracing).

characterize the antiserum raised to [Lys^{102}]CFTR(102-116), it was therefore of interest to determine whether α-102 (the monospecific antibody to this peptide) resembled α-1468 with respect to cell staining in pancreas.

Figure 2 shows the localization of CFTR immunoreactivity in human pancreas using α-102. Staining was most prominent at or near the lumenal surface of tubular, branching structures. This staining pattern was

Table 2. Peptide Binding by Radioimmunoassay

Peptide (nM)	CFTR(1468-1480) (% Iodopeptide bound)	[Lys^{102}]CFTR(102-116)
0	57.7 ± 0.5[1]	65.5 ± 0.3
0.5	53.5 ± 0.7	61.9 ± 0.5
1	50.8 ± 0.7	55.5 ± 0.6
2	38.8 ± 0.5	45.4 ± 0.3
4	31.4 ± 0.9	30.4 ± 0.3
8	18.4 ± 2.5	15.5 ± 0.8
16	7.6 ± 1.2	9.2 ± 0.5
32	2.3 ± 0.4	3.8 ± 0.1
50% binding	5 nM	4 nM

[1] Background values were measured in the absence of antibody and were 13% for CFTR(1468-1480) and 15% for [Lys^{102}]CFTR(102-116).
Each value is the mean ± SEM for 3 determinations.

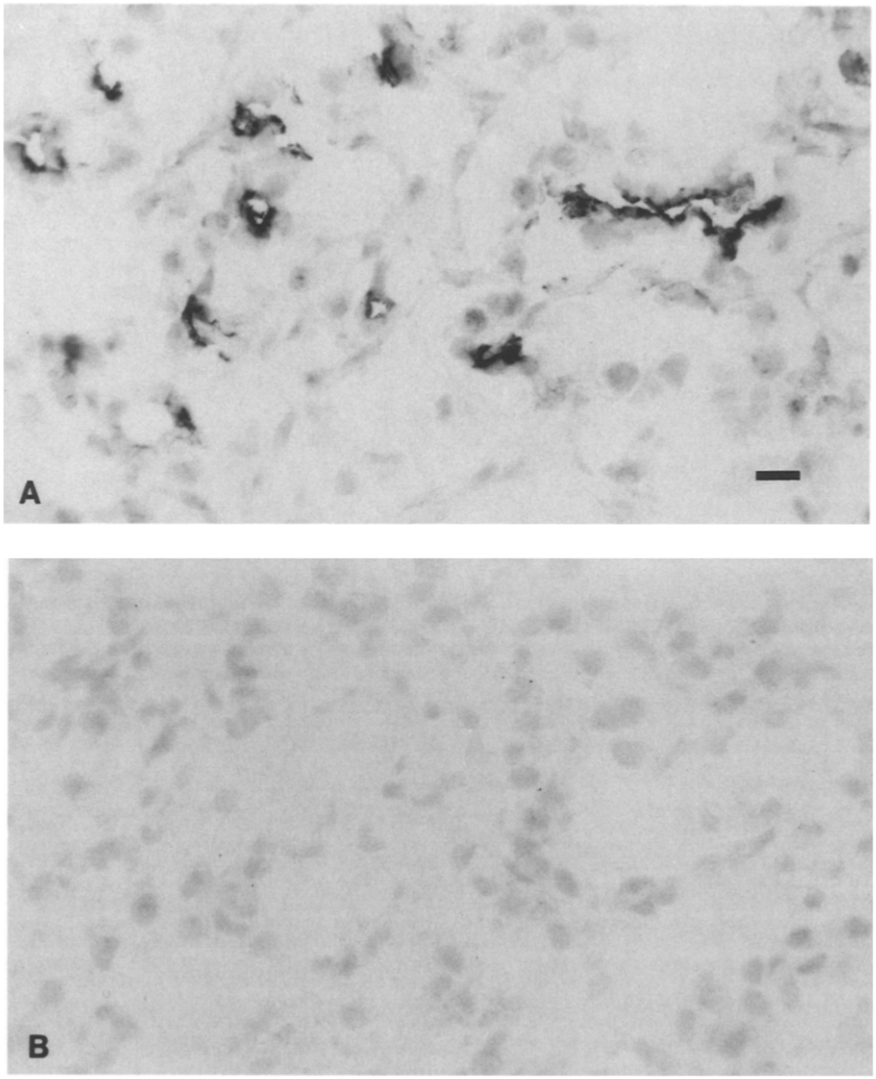

Figure 2. Peroxidase staining of CFTR immunoreactivity in human pancreas using α-102. (A) Staining with α-102. (B) Control showing elimination of staining by α-102 after incubating the antibody with competing peptide. Bar = 16 μm.

specific in that it did not occur using either non-immune rabbit serum (not shown) or α-102 preincubated with an excess of [Lys^{102}]CFTR(102-116). In addition to this prominent staining pattern, weaker staining of endothelial cells occurred in some sections. Such endothelial staining differed from the more prominent peroxidase signal in that it occurred diffusely throughout the cell and did not appear to be polarized. In additional studies, staining with α-102 and α-1468 was directly compared to confirm that equivalent structures were recognized by both antibodies (not shown).

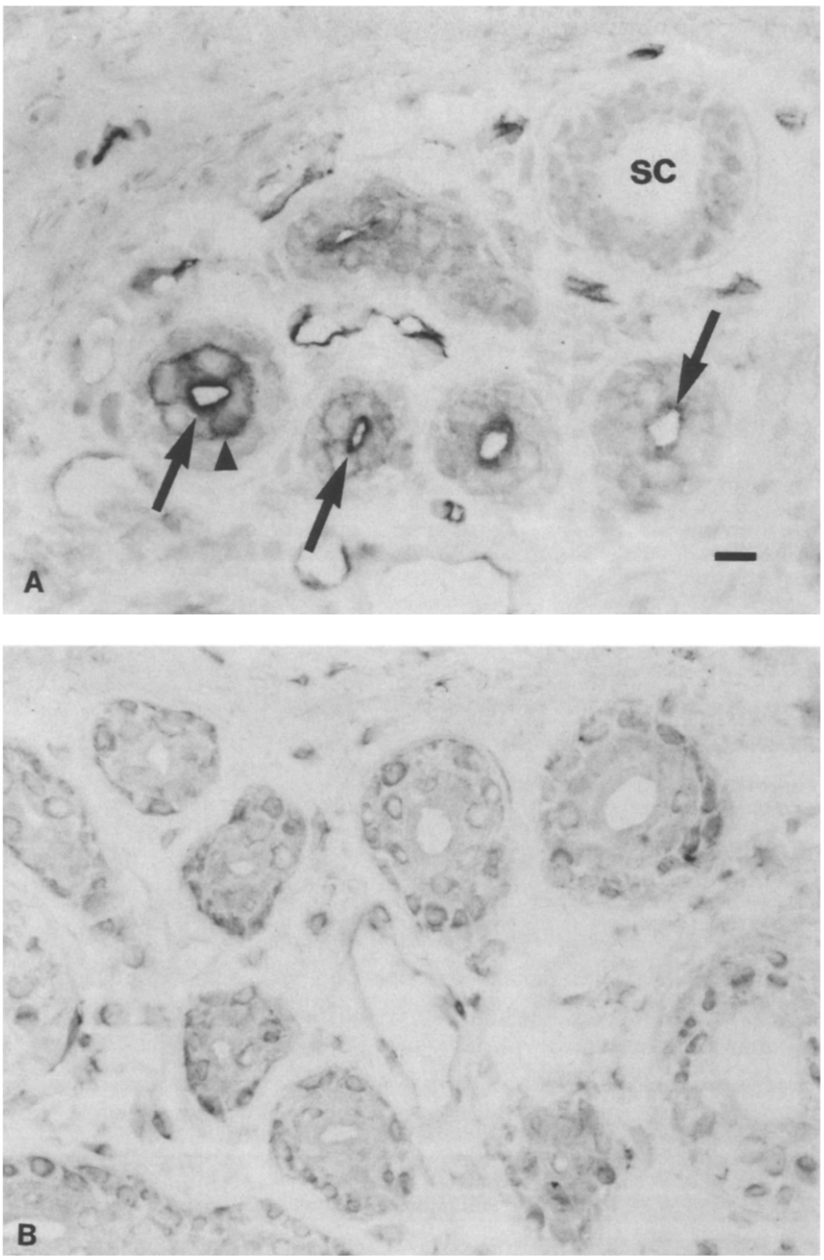

Figure 3. Peroxidase staining of CFTR immunoreactivity in human sweat gland using α-1468. (A) Staining with α-1468. (B) Control showing elimination of staining by α-1468 after incubating the antibody with competing peptide. Staining is most prominent at the apical surface of the lumenal cells lining the reabsorptive ducts (arrows). Staining of the basolateral domain of these cells is also detected (arrowhead). An unstained secretory coil is labeled, sc. Bar = 12 μm.

In all examined sections, the observed staining was consistent with the conclusion that the proximal pancreatic duct is the predominant site of CFTR [14].

The distribution of CFTR immunoreactivity in human sweat gland is shown in Figure 3. With each antibody, staining was most prominent in the cells lining the reabsorptive ducts. Within these duct-lining epithelial cells, also known as lumenal or cuticular cells, staining was most prominent near the apical surface but was also detected near the basolateral membrane. Staining also occurred with the same pattern in the acrosyringium. By contrast, staining was much less prominent in the secretory coil.

Thus, antisera have been raised to a total of six CFTR peptides. High-affinity monospecific antibodies were prepared from two of these antisera, and these antibodies were shown to detect CFTR immunoreactivity in cytochemical studies of human pancreas and sweat gland. It seems likely that the staining patterns observed with these antibodies reflect the distribution of CFTR for four reasons: First, antibodies to two unrelated CFTR domains stain the same structures. Second, preliminary reports indicate that several other CFTR antibodies also stain these structures, both in pancreas [15-17] and in sweat duct [15-18]. Third, the ion transport properties of both the exocrine pancreas [14,19,20] and the eccrine sweat duct [3] are consistent with the observed cellular and subcellular localization of CFTR based on cell staining. And fourth, Western blots using one of these antibodies (α-1468) detect a single broad band at 155-170 kDa, a signal which agrees well with the prediction that CFTR is a glycoprotein containing 1480 amino acids [14]. Additional studies are in progress aiming to identify the protein recognized by these antibodies and to use these antibodies to determine the distribution of CFTR immunoreactivity in other tissues.

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