Altered Binding of ^{125}I-Labeled Calmodulin to a 46.5-Kilodalton Protein in Skin Fibroblasts Cultured from Patients with Cystic Fibrosis

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Abstract

The levels of calmodulin and calmodulin-binding proteins have been determined in cultured skin fibroblasts from patients with cystic fibrosis (CF) and age- and sex-matched controls. Calmodulin ranged from 0.20 to 0.76 μg/mg protein; there was no difference between calmodulin concentration in fibroblasts from CF patients and controls. Calmodulin-binding proteins of 230, 212, 204, 164, 139, 70, 59, 46.5, and 41 kD were identified. A protein with a mobility identical to the 59-kD calmodulin-binding protein was labeled by antiserum against calmodulin-dependent phosphatase. Although Ca^{2+}/calmodulin-dependent phosphatase activity was detected, there was no difference in activity between control and CF fibroblasts or in the level of phosphatase protein as determined by radioimmunoassay. Lower amounts of ^{125}I-calmodulin were bound to the 46.5-kD calmodulin-binding protein in CF fibroblasts as compared with controls. The 46.5-kD calmodulin-binding protein may be reduced in CF fibroblasts or its structure may be altered resulting in a reduced binding capacity and/or affinity for calmodulin and perhaps reflecting, either directly or indirectly, the genetic defect responsible for cystic fibrosis.

Introduction

Cystic fibrosis, an autosomal recessive genetic disease, is the most common genetic disease affecting Caucasians, occurring with a frequency of 1 in 2,000 births. Classified clinically as a generalized disease of the exocrine glands, it is characterized by disturbances in the electrolyte composition of secretory fluids (1). Although the biochemical basis for the disease is unknown, evidence indicates that the defect may involve a reduced epithelial permeability to chloride (2–7). This could result from a reduction in the number of chloride channels, a reduction in their ability to transport chloride, or an alteration in their regulatory mechanisms. Chloride channel activity in epithelial cells from trachea (6, 8) and intestine (9–15) is regulated by both Ca^{2+} and cyclic AMP (cAMP). Recently, Frizzell et al. (16) have shown that airway epithelia of cystic fibrosis patients contain chloride channels biophysically similar to those from normal tissue but which appear to be defective in their regulatory properties. These results suggest that the defect is not in the chloride channel itself but in its regulation by intracellular messengers.

Calmodulin, a Ca^{2+}-binding protein found throughout the eukaryotes, serves as a primary intracellular receptor for Ca^{2+}. The Ca^{2+}/calmodulin complex interacts with various intracellular proteins, many of which are enzymes and structural proteins of known physiological function (17). Studies using calmodulin antagonists suggest that calmodulin may be a component of the Ca^{2+}-dependent regulation of chloride secretion in intestinal tissue (14, 18, 19). Furthermore, rats chronically treated with reserpine to induce a cystic fibrosis–like hypersecretion of mucus seem to be deficient in a calmodulin-dependent regulatory mechanism that controls mucous glycoprotein secretion (20). Using a calmodulin-dependent phosphodiesterase assay system to quantitate calmodulin, Gnegy et al. (21) reported that the calmodulin concentration in fibroblasts from cystic fibrosis patients was elevated over that of cells from controls. We have reinvestigated the level of calmodulin in cystic fibrosis and normal fibroblasts with a specific radioimmunoassay; in addition, we have identified calmodulin-binding proteins in both cystic fibrosis and control fibroblasts and compared their levels. We find no significant difference in the average levels of calmodulin in cystic fibrosis vs. control fibroblasts. However, we did find that the binding of ^{125}I-calmodulin to a protein of 46.5 kD is significantly depressed in fibroblasts from cystic fibrosis patients. This observation may be important regarding identification of the primary genetic defect responsible for cystic fibrosis.

Methods

Chemicals. [^{125}I]NaI (13–17 mCi/μg) was purchased from Amersham Corp., Arlington Heights, IL. Tris buffer, calcium chloride, nonidet P-40, EGTA, EDTA, Triton X-100, and Protein A were purchased from Sigma Chemical Co., St. Louis, MO. Phenyl-Sepharose was purchased from Pharmacia, Inc., Piscataway, NJ. Electrophoresis reagents were purchased from Bio-Rad Laboratories, Richmond, CA. Nitrocellulose sheets (0.45 μM pore) were purchased from Sartorius Inc., Hayward, CA. Trifluoperazine was provided free of charge by SmithKline Laboratories, Philadelphia, PA.

We prepared calmodulin from bovine brain by phenyl-Sepharose chromatography (22) followed by gel filtration chromatography on Sephadex G-100. Calmodulin-dependent protein phosphatase (calcineurin) was purified from bovine brain (23, 24).

Cell culture. Human fibroblast cultures derived from forearm skin biopsies were provided by Dr. Jeffery B. Smith and Lucinda Smith of the Tissue Culture Core Laboratory of the Gregory Fleming James Cystic Fibrosis Research Center at the University of Alabama at Birmingham. Skin biopsies from cystic fibrosis patients and age- and sex-matched control individuals were provided by Raymond Lyrene, M.D., Pulmonary Division, University of Alabama at Birmingham Department of Pedi-

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Received for publication 13 October 1986.

J. Clin. Invest.

0021-9738/87/02/0643/06 $1.00
Volume 79, February 1987, 643–648

atrics. Cultures were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C. Each experiment was conducted with a matched pair of cell lines of the same passage number. Upon reaching confluence, cells were scraped into an ice-cold phosphate-buffered saline (PBS) solution (20 mM sodium phosphate, pH 7.2, 0.15 M NaCl), collected by centrifugation at 2,000 *g* for 5 min and resuspended in 50 mM Tris-HCl, pH 7.8, 3 mM EGTA. For radioimmunoassays and gel electrophoresis, the resuspended cells were solubilized by the addition of sodium dodecyl sulfate (SDS) to 0.25% and were incubated for 2 min in boiling water. For the determination of phosphatase enzyme activity, the cells were homogenized in a glass/teflon tissue homogenizer, lysed by two cycles of freezing in dry ice/methanol and rapid thawing, and assayed immediately for activity.

Radioimmunoassays. We used a detergent-modified radioimmunoassay for the calmodulin-dependent protein phosphatase to measure phosphatase levels (24); bovine brain phosphatase was used as a standard (23). The calmodulin radioimmunoassay as described by Wallace and Cheung (25) was modified in a manner analogous to the phosphatase radioimmunoassay to allow the inclusion of 0.2% SDS from the detergent-solubilized fibroblast samples (unpublished data) and used to measure the calmodulin concentration; bovine brain calmodulin was used as a standard.

Identification of calmodulin-binding proteins. Human fibroblast calmodulin-binding proteins were identified by Western blotting of fibroblast proteins from SDS-polyacrylamide gels onto nitrocellulose, followed by overlaying the nitrocellulose with ^{125}I-calmodulin. Fibroblast proteins were separated on linear gradients of 5–15% polyacrylamide in the presence of 0.1% SDS, using the buffer system of Laemmli (26). The separated proteins were transferred onto nitrocellulose at 60 V for 5 h according to procedure of Towbin et al. (27). The nitrocellulose sheets were quenched for 1 h in 20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 3% nonfat dry milk (Carnation), and 0.02% sodium azide. Calcium chloride (1 mM), the detergent Nonidet P-40 (0.05%) and ^{125}I-calmodulin (10^6 cpm/ml) were then added to the quenching solution and incubation with constant shaking was continued for 4 h. The nitrocellulose sheet was then washed (1 h with one change in 250 ml of 20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 1 mM $CaCl_2$ and 0.05% Nonidet P-40), dried and autoradiographed on XAR-5 film (Eastman Kodak Co., Rochester, NY). The amount of ^{125}I-calmodulin bound to individual bands was quantitated by excising the bands from the nitrocellulose sheet and gamma counting, and by scanning the autoradiograph with a densitometer and integrating the area under the peaks. ^{125}I-labeled calmodulin for Western blots was prepared by the lactoperoxidase-glucose oxidase procedure (28). Affinity chromatography on phenyl-Sepharose (22) was used to separate the ^{125}I-calmodulin from the unreacted ^{125}I-NaI and from the ^{125}I-lactoperoxidase and ^{125}I-glucose oxidase that are produced during the iodination reaction. A typical preparation of ^{125}I-calmodulin had a specific activity of 1.2 μCi/μg.

Immunoblotting procedures. Using previously described immunoblotting techniques (29), we identified fibroblast proteins that crossreact with antibodies against the 60-kD calmodulin-binding subunit (subunit A) of bovine brain calmodulin-dependent phosphatase. We used polyclonal antibodies against subunit A of the phosphatase that were prepared by injecting rabbits with phosphatase subunit A excised from SDS-polyacrylamide gels (unpublished data). The antigen–antibody complex was detected with ^{125}I-protein A, iodinated by the chloramine T procedure (30).

Calmodulin-dependent phosphatase assay. Phosphatase activity was determined in crude homogenates of fibroblast samples by measuring the release of ^{32}P from ^{32}P-labeled casein (29). Calmodulin-dependent phosphatase activity was calculated as the difference between total phosphatase activity and calmodulin-independent activity. Total phosphatase activity was determined in the presence of 0.1 mM $CaCl_2$ and 1.1 μM exogenous calmodulin; calmodulin-independent phosphatase activity was measured in the presence of the calmodulin antagonist trifluoperazine (100 μM) as well as 1 mM EGTA, a Ca^{2+} chelator.

Protein determination. Protein was determined as described by Lowry et al. (31) after the proteins were precipitated with 10 vol of 10% perchloric acid and 1% phosphotungstic acid. Bovine serum albumin (Bio-Rad Laboratories) was used as a standard.

Results

The concentration of calmodulin was determined in fibroblasts from patients with cystic fibrosis and from age- and sex-matched controls using a specific radioimmunoassay. The fibroblast samples from both groups effectively competed with ^{125}I-calmodulin for binding to the anticalmodulin serum, with binding inhibition curves that were parallel to the standard curve obtained with purified bovine brain calmodulin (data not shown). The calmodulin concentration, measured in eight sets of matched cell lines, ranged from 0.20 to 0.76 μg/mg protein. There was no significant difference in the average concentration of calmodulin in cystic fibrosis as compared with control cell lines (0.38±0.12 vs. 0.37±0.15 μg calmodulin/mg protein; mean±SD; $P > 0.1$).

Calmodulin-binding proteins were identified in fibroblasts from cystic fibrosis patients and their matched controls by Western blotting techniques and overlays with ^{125}I-calmodulin. As shown in Fig. 1, nine distinct calmodulin-binding proteins were detected in fibroblasts when the overlays were conducted in the presence of Ca^{2+} (panel *B*); no calmodulin-binding proteins were detected when EGTA and EDTA were added to chelate metal ions or in the presence of Ca^{2+} and an excess amount of nonradiolabeled calmodulin (data not shown). Fibroblast proteins that bound calmodulin in both the control and cystic fibrosis cells had molecular weights ($\times 10^{-3}$) of 230, 212, 204, 164, 139, 70, 59, 46.5 and 41. The 46.5-kD calmodulin-binding region is a doublet, consisting of two distinct calmodulin-binding proteins; a darker exposure of the same ^{125}I-calmodulin overlay indicated that the 139-kD and 41-kD calmodulin-binding proteins are also doublets (data not shown). Fractionation of a fibroblast homogenate into crude particulate and soluble fractions revealed that all of the larger molecular weight calmodulin-binding proteins (230 kD–139 kD) are associated with the particulate fraction, the 70-kD protein is predominantly in the soluble fraction (data not shown), the 59-kD and 41-kD proteins are largely in the soluble fraction, and the 46.5-kD protein is exclusively in the particulate fraction (Fig. 1).

The identification of a 59-kD calmodulin-binding protein in fibroblasts suggested that these cells might contain a calmodulin-dependent protein phosphatase similar to the well-characterized phosphatase from bovine brain that contains a 60-kD calmodulin-binding subunit (32, 33). A polyclonal antibody specific for the 60-kD subunit of bovine brain calmodulin-dependent phosphatase bound to a fibroblast protein with a mobility identical to the 59-kD calmodulin-binding protein (Fig. 1, panel *C*). Moreover, fibroblast homogenates, when assayed with a radioimmunoassay specific for the phosphatase, competed with ^{125}I-phosphatase for binding to the antiphosphatase serum with inhibition curves parallel to a standard curve generated with purified bovine brain phosphatase (data not shown). In eight sets of matched cell lines, the amount of the phosphatase was the same for the cystic fibrosis as for the control samples (0.17±0.04 vs. 0.15±0.03 μg phosphatase/mg protein; mean±SD; $P > 0.1$). We also assayed fibroblast homogenates for Ca^{2+}/calmodulin-dependent phosphatase activity, using ^{32}P-labeled casein as a substrate and the calmodulin antagonist trifluoperazine as well as the Ca^{2+} chelator EGTA to inhibit Ca^{2+}/

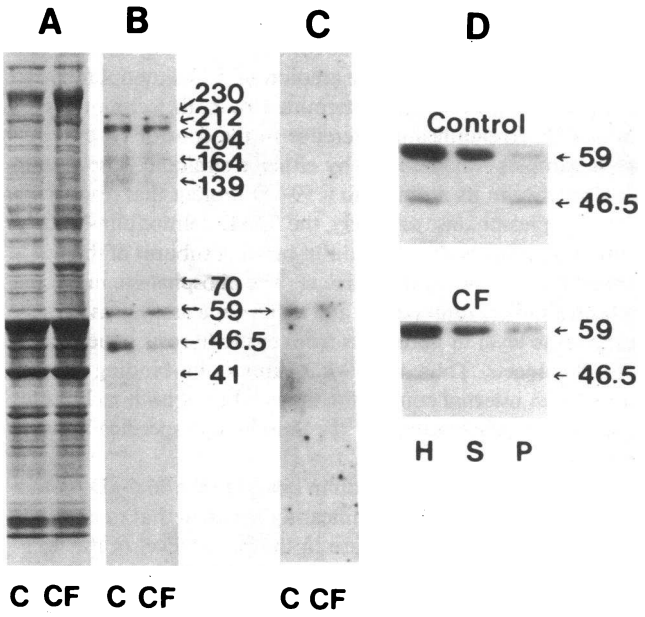

Figure 1. Identification of calmodulin-binding proteins and the calmodulin-dependent phosphatase in control and cystic fibrosis (*CF*) fibroblasts. Fibroblast proteins (150 μg/lane) were resolved by SDS-gel electrophoresis and either stained for total protein with Coomassie brilliant blue (*A*) or transferred to nitrocellulose, overlaid with ^{125}I-calmodulin in the presence of 1 mM $CaCl_2$ (*B*), or overlaid with antisera against subunit A of the calmodulin-dependent phosphatase followed by ^{125}I-protein A (*C*) and autoradiographed. The numbers refer to the molecular weight ($\times 10^{-3}$) of each calmodulin-binding protein and the immunoreactive protein. (*D*) is an enlargement showing the subcellular fractionation of the 46.5-kD and 59-kD calmodulin-binding proteins. Freeze-thawed fibroblast homogenates (*H*) were fractionated into soluble (*S*) and particulate (*P*) fractions by centrifugation at 15,000 *g* for 30 min at 4°C. The supernatants were used as the soluble fractions; the particulate fractions were washed one time in 50 mM Tris-HCl, pH 7.8, 3 mM EGTA and dispersed in the same buffer. Both soluble and particulate fractions were made 0.25% SDS and incubated for 2 min in boiling water. The proteins were resolved by SDS-gel electrophoresis, Western blotted with ^{125}I-calmodulin, and autoradiographed. The amount of protein applied per lane for the homogenates was 110 μg; aliquots equivalent to the original homogenate were applied for the soluble and particulate fractions.

Table I. Comparison of the Relative Amounts of Calmodulin-binding Proteins in Control and Cystic Fibrosis Fibroblasts

	Calmodulin-binding protein (cystic fibrosis/control)						
Set	230 kD	204–212 kD	164 kD	139 kD	59 kD	46.5 kD	41 kD
1	0.87	0.92	0.83	0.94	0.88	0.55	0.91
2	0.73	1.02	0.93	1.22	1.22	0.32	1.08
3	0.56	0.39	0.37	0.19	0.96	0.40	1.45
4	1.26	1.92	1.29	1.48	1.24	0.79	1.51
5	0.76	1.00	0.90	1.00	0.95	0.98	1.57
6	0.78	1.03	0.78	0.83	1.00	0.85	0.77
7	0.63	0.84	0.64	0.68	0.94	0.68	0.84
Mean	0.80	1.02	0.82	0.91	1.03	0.65	1.16
±S.D.	0.23	0.46	0.28	0.41	0.14	0.24	0.34
P	<0.05	>0.10	>0.10	>0.10	>0.10	<0.005	>0.10

Bands corresponding to ^{125}I-calmodulin–binding proteins from matched sets of control and cystic fibrosis fibroblasts were excised from the nitrocellulose sheets and counted in a gamma counter. The mean and SD of the ratio of each protein was determined and statistical analysis was by Student's *t* test for matched pairs.

calmodulin-dependent activity. The fibroblast homogenates did have Ca^{2+}/calmodulin-dependent phosphatase activity; we found no significant difference in the average enzyme activity between the eight sets of normal and cystic fibrosis cell lines (0.051±0.028 vs. 0.060±0.025 nmol ^{32}P released/mg protein/min; mean±SD; $P > 0.1$).

To determine the relative amount of ^{125}I-calmodulin bound to each calmodulin-binding protein in both control and cystic fibrosis fibroblasts we excised the areas of ^{125}I-calmodulin from the nitrocellulose sheets and quantitated by gamma counting. As shown in Table I, the amount of ^{125}I-calmodulin bound to the 46.5-kD polypeptide was depressed in six of the seven cystic fibrosis samples as compared with their respective control samples; the average ratio was 0.65±0.24 ($P < 0.005$). The ^{125}I-calmodulin bound to the 230-kD protein from cystic fibrosis samples was also slightly depressed compared with its control; however, the difference was not as great (CF/control = 0.80±0.28) or significant ($P < 0.05$) as that observed for the 46.5-kD protein. In addition, the 230-kD calmodulin-binding protein is adjacent to the much larger signal from the 204–212 kD band (Fig. 2) that makes accurate quantitation of the 230-kD band difficult. This was not a problem for the 46.5-kD protein that is well resolved from the other calmodulin-binding proteins. There was no statistically significant difference in the amount of ^{125}I-calmodulin bound to the other calmodulin-binding proteins. In a separate experiment, we scanned the autoradiographs with a densitometer, as shown in Fig. 2, and integrated the peaks corresponding to the 59-kD and 46.5-kD calmodulin-binding proteins. Utilizing eight sets of matched cell lines, the average ratio of ^{125}I-calmodulin associated with the 59-kD and 46.5-kD proteins was 1.00±0.38 ($P > 0.1$) and 0.52±0.19 ($P < 0.005$), respectively, which is in agreement with the results obtained by cutting and counting the 59-kD and 46.5-kD protein bands. Both quantitation techniques indicated that ^{125}I-calmodulin

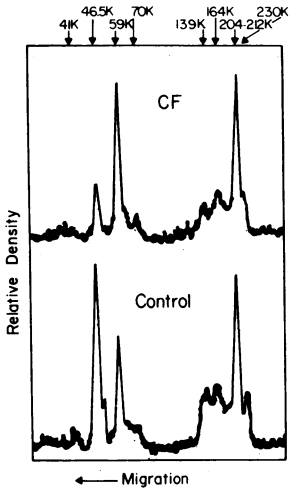

Figure 2. Densitometer scans of an autoradiograph depicting calmodulin-binding proteins in control and cystic fibrosis (*CF*) fibroblasts. The calmodulin-binding proteins from Fig. 1, panel *B* were scanned with an LKB Zeineh Soft Laser Densitometer. The relative position of each calmodulin-binding protein is indicated by its molecular weight.

bound to the 59-kD protein equivalently in control and cystic fibrosis fibroblasts. Assuming that the 59-kD calmodulin-binding protein is subunit A of a calmodulin-dependent phosphatase, these data are consistent with the radioimmunoassay and enzyme assay data described above.

Discussion

Although the calmodulin concentration in human fibroblasts ranged from 0.20 to 0.76 μg/mg protein, we found no significant difference between its average concentration in fibroblasts from cystic fibrosis patients and controls. These results contrast markedly to those of Gnegy et al. (21), who found a 60% increase in the level of calmodulin in cystic fibrosis fibroblasts over that of control fibroblasts. Our cell lines have been carefully matched with regard to the age and sex of the donor, the number of passages in culture, and the growth stage at which the cells were harvested. Variations in any of these factors could account for the differences observed by Gnegy et al. (21). In addition, we found significantly lower levels of calmodulin than reported by Gnegy et al. (21), a difference which is most likely due to differences in the techniques used to measure calmodulin. While Gnegy et al. (21) used a calmodulin-dependent phosphodiesterase system that measures calmodulin based upon the degree of enzyme stimulation by a heat-treated extract, we used a specific radioimmunoassay. The enzyme-based assay is subject to interferences by both lipids and proteolytic enzymes, which stimulate phosphodiesterase and result in anomalous overestimations of the calmodulin concentration (34); these interferences are not a problem in the radioimmunoassay. An alteration in calmodulin does not appear to be the genetic defect responsible for cystic fibrosis. Instead, evidence obtained by a genetic linkage approach indicates that the locus for the defective gene in cystic fibrosis is on a specific region of chromosome seven (35–37); the calmodulin gene is not located on chromosome seven (unpublished data).

The nine fibroblast calmodulin-binding proteins interact with calmodulin in a specific manner; the binding was Ca^{2+} dependent and could be blocked by the addition of nonradiolabeled calmodulin. These proteins are most likely fibroblast enzymes and structural proteins regulated by Ca^{2+} through a calmodulin-dependent mechanism. These nine proteins represent the minimum number of calmodulin-dependent enzymes and proteins in human fibroblasts; some binding proteins may not survive the denaturing effects of SDS or be renatured effectively during the blotting process. In addition, some binding proteins may not be detected due to inadequate binding to the nitrocellulose; however, the Amido black protein staining pattern of fibroblast proteins transferred to nitrocellulose appeared to be identical to the Coomassie blue protein staining pattern of the gel before transfer (data not shown).

The relative amount of ^{125}I-calmodulin associated with each of the calmodulin-binding proteins was determined by counting excised bands from the nitrocellulose and by densitometric analysis of the autoradiographs. These techniques cannot be used to compare different calmodulin-binding proteins quantitatively because of possible variable rates of transfer to nitrocellulose and renaturation during blotting. It is possible, however, to compare the amount of ^{125}I-calmodulin bound to a particular protein in different lanes of the same gel because these proteins have been exposed to identical conditions during transfer and renaturation. For example, the amount of ^{125}I-calmodulin bound to the 59-kD protein was determined by both techniques and compared; no significant difference in the control vs. cystic fibrosis samples was detected by either technique. Based upon the similarity in its mobility to a 59-kD protein that reacts with the brain phosphatase antibody, the 59-kD calmodulin-binding protein is probably the calmodulin-binding subunit of the Ca^{2+}/calmodulin-dependent phosphatase. The phosphatase, measured by both a radioimmunoassay and an enzyme assay, was present at the same level in fibroblasts from cystic fibrosis patients and control subjects. Thus, the 59-kD calmodulin-binding protein serves as an internal control for the validity of both techniques for comparing the binding of ^{125}I-calmodulin to specific fibroblast proteins.

The amount of ^{125}I-calmodulin bound to the 46.5-kD protein in cystic fibrosis cells was significantly less than that in controls. This could represent a decrease in the actual level of the 46.5-kD protein due to an alteration in the level of its expression or an increase in the activity of a protease. In platelets we have found that certain calmodulin-binding proteins in vivo are susceptible to degradation by a Ca^{2+}-dependent protease (unpublished data). Alternatively, the decreased amount of ^{125}I-calmodulin bound could represent a reduction in the affinity for and/or capacity of the 46.5-kD protein to bind calmodulin. Either of these factors could be affected by a mutation in the gene that codes for the 46.5-kD calmodulin-binding protein or by an alteration in its degree of phosphorylation. Phosphorylation has been shown to decrease the affinity of several calmodulin-binding proteins for calmodulin (38, 39).

Although the biochemical defect in cystic fibrosis is unknown, patients exhibit alterations in the electrolyte compositions of their secretory fluids that may result from a reduction in the epithelial permeability of chloride (1–7). Frizzell et al. (16) demonstrated that epithelial cells from patients with cystic fibrosis contain chloride channels that are biophysically similar to channels from unaffected individuals and are regulated by Ca^{2+}, possibly through a calmodulin-dependent mechanism, but have lost their ability to be regulated by cAMP. An alteration in the response of cystic fibrosis tissues to agonists whose affects are mediated by cAMP has also been demonstrated in the secretory coils of sweat glands (40), nasal epithelia (41), and cultured airway cells (42). However, normal increases in the levels of cAMP were observed in all these systems. Thus, the cystic fibrosis genetic defect may lie in the pathway connecting cAMP to the regulation of the chloride channel. Cyclic AMP functions as an intracellular regulator by activating a cAMP-dependent protein kinase that in turn phosphorylates various proteins and alters their activities. Several calmodulin-dependent enzymes—myosin light chain kinase (38) and a Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (39)—are phosphorylated by the cAMP-dependent kinase with subsequent decreases in their affinities for calmodulin. Thus, the decreased binding of ^{125}I-calmodulin to a 46.5-kDa protein in cystic fibrosis fibroblasts could result from an alteration in the level of its phosphorylation by the cAMP-dependent kinase. Phosphorylation of the 46.5-kD calmodulin-binding protein (and of several of the other fibroblast calmodulin-binding proteins) could also account for the appearance of doublets; the phosphorylated and dephosphorylated forms of

the proteins may exhibit slight differences in mobility on SDS gels. Moreover, since the genetic defect could be in the primary structure of the phosphorylation site, the altered level of ^{125}I-calmodulin binding to the 46.5-kD protein could be a direct indication of the genetic defect responsible for cystic fibrosis. Alternatively, the altered binding may be due to a generalized secondary effect of the defective cystic fibrosis gene or a specific secondary effect present only in skin fibroblasts. The identity of the 46.5-kD calmodulin-binding protein, its presence or absence in epithelial secretory tissues, and its possible involvement in cystic fibrosis are currently under investigation in our laboratory.

Acknowledgments

Mrs. Diane Lee provided expert assistance in the preparation of this manuscript. We thank Bill Weaver, Ph.D., and Lyn Stafford for a critical reading of the manuscript.

This research was supported by the National Institutes of Health, NHLBI grant HL-29766 and by a Research Center Grant from the Cystic Fibrosis Foundation.

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