E3KARP Mediates the Association of Ezrin and Protein Kinase A with the Cystic Fibrosis Transmembrane Conductance Regulator in Airway Cells*

Fei Sun‡, Martin J. Hug‡‡, Christopher M. Lewarchik‡, C.-H. Chris Yun‡, Neil A. Bradbury‡, and Raymond A. Frizzell‡

From the ‡Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261 and the ‡Department of Medicine, Division of Gastroenterology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Although it is generally recognized that cystic fibrosis transmembrane conductance regulator (CFTR) contains a PSD-95/Disc-large/ZO-1 (PDZ)-binding motif at its COOH terminus, the identity of the PDZ domain protein(s) that interact with CFTR is uncertain, and the functional impact of this interaction is not fully understood. By using human airway epithelial cells, we show that CFTR associates with Na+/H+ exchanger (NHE) type 3 kinase A regulatory protein (E3KARP), an EBP50/NHE regulatory factor (NHERF)-related PDZ domain protein. The PDZ binding motif located at the COOH terminus of CFTR interacts preferentially with the second PDZ domain of E3KARP, with nanomolar affinity. In contrast to EBP50/NHERF, E3KARP is predominantly localized (>95%) in the membrane fractions of Calu-3 and T84 cells, where CFTR is located. Moreover, confocal immunofluorescence microscopy of polarized Calu-3 monolayers shows that E3KARP and CFTR are co-localized at the apical membrane domain. We also found that ezrin associates with E3KARP in vivo. Co-expression of CFTR with E3KARP and ezrin in Xenopus oocytes potentiated cAMP-stimulated CFTR Cl− currents. These results support the concept that E3KARP functions as a scaffold protein that links CFTR to ezrin. Since ezrin has been shown previously to function as a protein kinase A anchoring protein, we suggest that one function served by the interaction of E3KARP with both ezrin and CFTR is to localize protein kinase A in the vicinity of the R-domain of CFTR. Since ezrin is also an actin-binding protein, the formation of a CFTR-E3KARP ezrin complex may be important also in stabilizing CFTR at the apical membrane domain of airway cells.

The cystic fibrosis transmembrane conductance regulator (CFTR)† is a member of the ATP-binding cassette protein family, and it accounts for the cAMP-dependent anion conductance at the apical membranes of epithelial cells (1). Mutations of CFTR cause cystic fibrosis, one of the most common fatal genetic diseases (2). To date, more than 800 mutations of the CFTR gene have been identified in patients with cystic fibrosis (CFTR data base, available on the World Wide Web). The primary step in CFTR channel activation involves protein kinase A (PKA)-mediated phosphorylation of the regulatory (R) domain, which contains nine dibasic PKA consensus phosphorylation sites. Phosphorylation at multiple R-domain sites is thought to release its block of the CFTR conductance pathway and to facilitate interactions between the nucleotide-binding domains, where sequential binding and hydrolysis of ATP elicit channel opening and closing (gating) activity (3).

Regulation of the CFTR Cl− channel via protein-protein interactions has been the subject of several recent studies. For example, the NH2 terminus of CFTR has been shown to bind syntaxin 1A, which inhibits cAMP-stimulated Cl− currents by reducing the number of open CFTR Cl− channels present in the plasma membrane (4, 5). In addition, a tyrosine-based motif in the COOH terminus mediates association of CFTR with the AP-2 adapter complex, which governs the preferential endocytosis of CFTR into clathrin-coated membrane vesicles (6, 7). Finally, the COOH terminus of CFTR corresponds to a PDZ-binding motif (TRL), which has been proposed to mediate an interaction of CFTR with EBP50 (ezrin-binding phosphoprotein 50), also known as NHERF (Na+/H+ exchanger type 3 regulatory factor) (8–10). Through its PDZ domains, NHERF has been shown to interact with several membrane receptors and ion channels/transporters (10). For example, NHERF confers cAMP/PKA-mediated inhibition of Na+/H+ exchange activity in renal cells (11), and its human homologue, EBP50, has been shown to bind to members of the ERM (ezrin-radixinmoeinsin) family of proteins (12). The interaction of EBP50/NHERF with ezrin, which contains a binding site for the regulatory type II (RII) subunit of PKA, may permit ezrin to function as a protein kinase A anchoring protein, or AKAP (13, 14).

Because PDZ domain proteins generally mediate associations of target proteins with various regulatory factors, CFTR-PDZ domain interactions could have important consequences for the regulation of CFTR Cl− channel activity and perhaps for other CFTR functions (15).

We demonstrated recently (14) that immunoprecipitates

* This work was supported by National Institutes of Health Grants DK56490 and DK44484 and from the Cystic Fibrosis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ Permanent address: Physiologisches Institut, Der Albert-Ludwigs Universität, D-79104 Freiburg 1, Germany.
‖ To whom correspondence should be addressed: Dept. of Cell Biology and Physiology, University of Pittsburgh School of Medicine, S362 BST, 3500 Terrace St., Pittsburgh, PA 15261. Tel.: 412-648-9498; Fax: 412-648-2004; E-mail: frizzell+@pitt.edu.

† The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; PKA, protein kinase A; AKAP, protein kinase A anchoring protein; PDZ, PSD-95/Disc-large/ZO-1; EBP50, ezrin-binding phosphoprotein 50; NHERF, Na+/H+ exchange regulatory factor; E3KARP, NHE3 kinase A regulatory protein; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; PVDF, polyvinylidene difluoride; IPs, immunoprecipitates; BSA, bovine serum albumin; NT, NH2 terminus; RD, regulatory domain; CT, COOH terminus.
(IPs) of CFTR contain the PKA regulatory and catalytic subunits and that both CFTR and model PKA substrate peptides could be phosphorylated in the CFTR IP by addition of cAMP and ATP. Ezrin was also present in CFTR IPs, and it was expressed at high levels in Cl−-secreting epithelia that express CFTR. Ezrin bound the PKA RII subunit in protein overlay and co-IP assays, indicative of AKAP activity. Finally, conditions that disrupt the ezrin-RII association markedly attenuated stimulation of CFTR Cl− currents by cAMP. These findings suggest that PKA anchoring is physiologically important for CFTR activation, and, based on the above observations, a model for PKA anchoring is depicted. An intact ezrin-RII complex could selectively regulate CFTR by sequestering PKA in proximity to the R-domain.

EcoRI from R&DSystems. Monoclonal GST and ezrin antibodies were obtained by agarose gel electrophoresis.

A cAMP-dependent inhibition of NHE3 requires co-expression of either E3KARP or NHERF in renal cell lines (17). Since this arrangement is analogous to the manner in which CFTR is regulated by cAMP/PKA, we examined the potential for E3KARP to provide a physical link between ezrin and CFTR. We report that the PDZ-binding motif at the COOH terminus of CFTR associates with E3KARP at high affinity. In turn, the interaction of E3KARP with ezrin assembles a regulatory complex at the apical membranes of human airway cells that brings CFTR into interactions with PKA and the cytoskeleton.

Experimental Procedures

Cell Culture—Calu-3 cells were cultured in Dulbecco’s modified Eagle’s/Ham’s F-12 media (Sigma) supplemented with 15% fetal bovine serum. Cells were maintained in a humidified atmosphere containing 5% CO2 at 37 °C. T84 cells were cultured under similar conditions, except that the medium was supplemented with 10% fetal bovine serum. For confocal microscopy, Calu-3 cells were seeded onto Costar Transwell cell culture inserts coated with fibronectin and vitrogen. The apical medium bathing Calu-3 cells was removed after several days in culture, and the cells were maintained at an air interface until use. Confocal microscopy (see below) was performed after 14–21 days in culture.

Antibodies—Generation of polyclonal E3KARP and NHERF antibodies was described previously (17). Monoclonal CFTR antibodies were obtained from R & D Systems. Monoclonal GST and ezrin antibodies were obtained from Sigma. Antibody dilutions are given below.

The indicated domains of CFTR (A) and E3KARP (B) were constructed and expressed in bacteria as GST fusion proteins. The numbers indicate the amino acid residues at the beginning and end of each CFTR or E3KARP construct. d indicates the number of amino acids deleted at the CFTR COOH terminus.

**FIG. 1.** Schematic diagram of CFTR and E3KARP domain structures. The indicated domains of CFTR (A) and E3KARP (B) were constructed and expressed in bacteria as GST fusion proteins. The numbers indicate the amino acid residues at the beginning and end of each CFTR or E3KARP construct. d indicates the number of amino acids deleted at the CFTR COOH terminus.

**A** CFTR

| CFTR | NT | TMD1 | NBD1 | RD | TMD2 | NBD2 | NT-CT |
|------|----|------|------|----|------|------|-------|
|      | 1-579 |       |      | 60-320 |       | 321-572 |       |

**B** E3KARP

| E3KARP | PDZ1 | PDZ2 | E3CT |
|--------|------|------|------|
| GST-F3 |       |      |      |
| GST-PDZ1 | 9-29 |      |      |
| GST-PDZ2 |      | 40-231 |      |
| GST-E3CT |      |      | 221-337 |

The cAMP/PKA construct was confirmed by DNA sequencing. Glutathione S-transferase (GST) fusion proteins were expressed in the Escherichia coli strain BL21 (DE3, Novagen). Optimal expression of fusion proteins was achieved by adding a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside to the bacterial culture, with incubation for 4 h at 37 °C. The bacteria were harvested by centrifugation and resuspended in phosphate-buffered saline with protease inhibitors. After sonication, GST fusion proteins or GST were purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech).

Immunoprecipitation—Pre-cleared Calu-3 cell lysates (~1 mg of protein) or membrane fractions (~500 μg of protein) were mixed with the appropriate primary antibodies for 1.5 h at 4 °C in lysis buffer. Twenty five μl of washed protein A- or G-Sepharose beads were added to each sample and incubated 1 h at 4 °C with gentle rotation. Immunocomplexes with protein A- or G-Sepharose beads were washed with lysis buffer four times and precipitated by centrifugation at 12,000 × g for 10 s. The immunocomplexes were resuspended in SDS sample buffer and subjected to immunoblotting.

Immunoblot Analysis—Equal amounts of protein from the membrane and cytosolic fractions were prepared from either forskolin-treated or non-treated Calu-3 and T84 cells. These samples, or the immunoprecipitates described above, were resolved by SDS-PAGE and transferred to PVDF membranes. Unbound sites were blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBST (10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20). The blots were incubated with primary antibodies (anti-E3KARP, 1:8000; anti-NHERF, 1:5000; and anti-CFTR-C, 1:1000) at room temperature for 1 h. The blots were then washed four times for 5 min each with TBST and incubated for 1 h with 2 μg/ml horseradish peroxidase-conjugated secondary antibodies (Sigma) in TBST with 10% fetal bovine serum, followed by five TBST washes. The reactive bands were visualized by Renaissance Chemiluminescence (NEN Life Science Products). Samples were exposed to x-ray film (Eastman Kodak Co.).

**Full-down Assays**—Confluent Calu-3 cells were lysed in lysis buffer and centrifuged at 12,000 × g at 4 °C for 1 min. The supernatant was incubated with 10 μg of recombinant GST fusion protein beads in lysis buffer at 4 °C for 1.5 h with gentle rotation. Bound proteins were washed five times with lysis buffer and resuspended in SDS sample buffer. The samples were resolved by SDS-polyacrylamide gel electrophoresis and were immunoblotted with appropriate antibodies (anti-CFTR) at 1:2000; anti-CFTR-C and anti-ezrin, 1:2000; other dilutions as above.

**In Vitro Binding Assays**—Ten μg each of GST-CFTR-NT, GST-CFTR-RD, GST-CFTR-CT, and GST were resolved by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane, and blocked with blocking buffer (5% nonfat milk, 10 mM Tris (pH 8.0), 150 mM NaCl) containing 1% BSA at room temperature for 3 h. The membrane was then overlaid with [35S]methionine-labeled full-length E3KARP (generated using the TNT in vitro transcription-translation system (Promega)) in blocking buffer containing 0.1% BSA for 4 h. The mem-

**EXPERIMENTAL PROCEDURES**

**Cell Fractionations**—Fractionation of Calu-3 cells was performed as described previously (14). Briefly, confluent Calu-3 and T84 cells, pre-treated with or without 10 μM forskolin for 15 min under the culture conditions, were scraped into buffer (10 mM Tris HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, with protease inhibitors (Roche Molecular Biochemicals)) and homogenized. Postnuclear supernatants were obtained by centrifugation (14,000 × g for 1 min). Cytosolic and membrane fractions were obtained by centrifugation of the postnuclear supernatants at 100,000 × g for 60 min. Cytosolic proteins were prepared by concentrating the supernatants in lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol). The membrane fraction was obtained by resuspension of the pellet in lysis buffer.

**Plasmid Constructs and Fusion Protein Expression**—Various domains of E3KARP and CFTR were amplified by polymerase chain reaction, as shown in Fig. 1. An EcolI site at the NH2 terminus and an XhoI site at the COOH terminus were generated for CFTR-NT, CFTR-CT, E3KARP-PDZ1, and PDZ2 constructs. An EcoRI site at the NH2 terminus and a SalI site at the COOH terminus were created for the full-length E3KARP and E3KARP-CT plasmids. CFTR-RD was generated by insertion of a Smal site at the NH2 terminus and an XhoI site at the COOH end. The COOH terminus deletion constructs, CFTR-CTd4 and CFTR-CTd23, were generated using the ExSite Polymerase Chain Reaction-Based Site-Directed Mutagenesis kit (Stratagene) from

**FIG. 1.** Schematic diagram of CFTR and E3KARP domain

| CFTR | NT | TMD1 | NBD1 | RD | TMD2 | NBD2 | NT-CT |
|------|----|------|------|----|------|------|-------|
|      | 1-579 |       |      | 60-320 |       | 321-572 |       |

| E3KARP | PDZ1 | PDZ2 | E3CT |
|--------|------|------|------|
| GST-F3 |       |      |      |
| GST-PDZ1 | 9-29 |      |      |
| GST-PDZ2 |      | 40-231 |      |
| GST-E3CT |      |      | 221-337 |

...
using synthetic resin and subjected to confocal microscopy. Collected incubation with fluorescein isothiocyanate (green) or rhodamine (red)-antibodies (anti-ezrin, anti-E3KARP, and anti-CFTR, dilutions as serum, the monolayers were incubated in the appropriate primary pH 7.4 in phosphate-buffered saline). After blocking with purified goat then washed three times with buffer A (0.5% BSA and 0.15% glycine at mixture of 2% paraformaldehyde and 0.1% Triton X-100. The cells were fixed in 2% paraformaldehyde and permeabilized with a Calu-3 cells was performed as described previously (14). Briefly, Calu-3 cell lysates provided the E3KARP-positive control (+ Cont.). B, Calu-3 membrane fractions were incubated with anti-E3KARP, and the blot was probed with anti-CFTR-C. Other procedures are as in A. The results are typical of two (A) or three (B) experiments. In this and subsequent figures, molecular mass is indicated, in kDa.

brane was washed three times in TBST, and the label was visualized by autoradiography. For in vitro competition assays, the blot containing 10 μg of GST-CFTR-CT fusion protein was overlaid with [35S]methionine-labeled E3KARP in the presence of different concentrations of E3KARP peptide (generated by digesting GST-E3KARP with PreScission protease (Amersham Pharmacia Biotech)). The blot was then washed three times in TBST, and the signal was visualized by autoradiography. Quantitation of the individual bands was achieved using a Bio-Rad PhosphorImager analysis system.

Confocal Microscopy—Immunofluorescence staining of filter grown Calu-3 cells was performed as described previously (14). Briefly, Calu-3 cells were fixed in 2% paraformaldehyde and permeabilized with a mixture of 2% paraformaldehyde and 0.1% Triton X-100. The cells were then washed three times with buffer A (0.5% BSA and 0.15% glycine at pH 7.4 in phosphate-buffered saline). After blocking with purified goat serum, the monolayers were incubated in the appropriate primary antibodies (anti-ezrin, anti-E3KARP, and anti-CFTR, dilutions as above) for 1 h followed by three washes in buffer A and subsequent incubation with fluorescein isothiocyanate (green) or rhodamine (red)-labeled secondary antibodies (Molecular Probes) for another hour. After washing with buffer A, the filters were mounted on glass coverslips using synthetic resin and subjected to confocal microscopy. Collected images were exported to ImageSpace (Molecular Dynamics) for subsequent reconstruction and processing.

Electrophysiology—The ND-96 solution utilized for the current measurements contained 96 mM NaCl, 1 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, and 5 mM HEPES. Xenopus oocytes expressing CFTR (1 ng) and the β2-adrenergic receptor (1 ng) or these cRNAs plus E3KARP (5 ng) and ezrin (5 ng) were used to record CFTR-dependent Cl- currents (I(Cl)) as described previously (5). Briefly, cells were impaled with two 3 M KCl-filled electrodes connected to a GeneClamp 500 amplifier (Axon Instruments) via Ag-AgCl pellet electrodes and referenced to the bath by Ag-AgCl pellets. Oocytes were held at −30 mV, and membrane currents were recorded during repetitive pulses to −60 mV for 500-ms duration; these inward currents reflect outward Cl− flow through CFTR during stimulation by 10 μM isoproterenol to raise intracellular cAMP. Values of I(Cl) were obtained at 2-s intervals; steady-state values are given in Fig. 9.

RESULTS

E3KARP Interacts with CFTR in Vivo—We performed co-immunoprecipitation experiments to determine whether CFTR interacts with E3KARP in intact cells. In these experiments, Calu-3 cell lysates were mixed with monoclonal antibodies against the CFTR regulatory domain (anti-CFTR-RD) or the COOH terminus (anti-CFTR-C). After SDS-PAGE, the immunoprecipitates were probed with a polyclonal E3KARP antibody (18). As shown in Fig. 2A, the immunoprecipitate obtained using anti-CFTR-RD contained E3KARP, the band detected at 50-kDa molecular mass. This result indicates that E3KARP interacts with CFTR in airway cells in vivo. Interestingly, when a similar immunoprecipitation was performed using anti-CFTR-C, we were unable to detect E3KARP in the precipitate. This result suggests that the region mediating the CFTR-E3KARP interaction might be located at the COOH terminus of CFTR (see below).

To evaluate further the CFTR-E3KARP interaction, the reciprocal immunoprecipitation was performed, in which an E3KARP antibody was mixed with the membrane fraction from Calu-3 cells. The immunocomplexes were precipitated with protein A-agarose beads. After SDS-PAGE and transfer to PVDF membrane, protein was blotted with anti-CFTR-C (denaturing conditions). As shown in Fig. 2B, E3KARP co-immunoprecipitated CFTR, whereas a control IgG did not. This further demonstrates that an interaction between E3KARP and CFTR occurs in vivo.

E3KARP Binds at the COOH Terminus of CFTR—Next, we addressed two questions regarding this protein-protein interaction. (i) Does CFTR interact directly with E3KARP or is this interaction mediated by another protein? (ii) Where is the E3KARP-binding site on CFTR? To approach the first question, we used in vitro protein overlay assays. We generated three CFTR cytoplasmic domains as follows: the NH2 terminus (NT), the regulatory domain (RD), and the COOH terminus (CT). These three CFTR cytoplasmic domains were expressed as GST fusion proteins in bacteria (GST-NT, -RD, and -CT). In these assays, 10 μg each of GST-CFTR fusion protein was resolved by SDS-PAGE, transferred to PVDF membrane, and probed with [35S]methionine-labeled E3KARP generated by in vitro transcription-translation. The results shown in Fig. 3A reveal that E3KARP interacts with the COOH terminus of CFTR. No interaction of E3KARP with CFTR-NT or CFTR-RD or with GST alone was detected. Because components other than labeled E3KARP were not present in the overlay assay, we conclude that the interaction between CFTR and E3KARP is direct.

To evaluate this interaction in epithelial cells, we performed pull-down assays in which GST and GST fusion proteins (-NT, -RD, and -CT) were bound to glutathione beads, mixed with Calu-3 cell lysates, and precipitated by centrifugation. The precipitates were resolved by SDS-PAGE and probed with an E3KARP antibody. When GST-CT protein beads were incubated with Calu-3 cell lysates, E3KARP was detected in the precipitate (Fig. 3B). In contrast, when beads containing GST-NT, GST-RD, or GST alone were mixed with these lysates, E3KARP was not found in the precipitates. These results confirm that the interaction between CFTR and E3KARP occurs at the COOH terminus of CFTR and that it occurs in airway epithelial cells.

In view of the above finding, we attempted to further resolve the E3KARP-binding site by deleting the COOH terminus of CFTR. Two different COOH terminus deletion mutants were made and expressed as GST fusion proteins in bacteria as follows: deletion of last 4 CFTR residues (GST-CTd4) and deletion of last 23 residues (GST-CTd23). By using these deletion mutants along with the complete GST-CT, we performed pull-down assays with Calu-3 cell lysates. As shown in Fig. 3C, the GST-CT precipitates E3KARP. However, deletion of the last 4 residues at the COOH terminus of CFTR was sufficient to abolish this interaction. These results are also consistent with the immunoprecipitation results of Fig. 2A, which show that E3KARP was co-precipitated by anti-CFTR-RD but not by anti-CFTR-C. The epitope recognized by anti-CFTR-C resides in the last 4 residues at the COOH terminus of CFTR; therefore, protein interactions at this site would be expected to mask antibody binding. Taken together, these results further demonstrate that E3KARP interacts with CFTR in human airway epithelial cells and that this interaction is mediated by the last.
probed with \(^{35}\text{S}\)methionine-labeled E3KARP generated by in vitro transcription-translation. In pilot experiments, we used different amounts of the in vitro labeled E3KARP translation product to determine the amount of labeled protein needed to saturate CFTR-CT binding (see legend, Fig. 5). To evaluate the affinity of E3KARP binding under these conditions, we generated full-length unlabeled E3KARP by digestion of the GST-E3KARP fusion protein. Seven different concentrations of purified E3KARP were then co-incubated with \(^{35}\text{S}\)-E3KARP in the GST-CFTR-CT overlay assays. As shown in Fig. 5, upper panel, incubation with 10 μM unlabeled E3KARP protein in the overlay assay completely abolished the binding of E3KARP to the CFTR-CT. Decreasing concentrations of unlabeled E3KARP in the overlay assay led to graded increases in the binding of \(^{35}\text{S}\)-E3KARP to the CFTR-CT. The half-maximal inhibitory concentration (IC\(_{50}\)) was 72 nM (Fig. 5, lower panel) when the data were fit with a single binding site algorithm (SigmaPlot). This suggests a high affinity interaction between E3KARP and CFTR, which is comparable to the affinity with which PDZ1 of EBP50/NHERF binds to CFTR (8).

**E3KARP Is Predominantly Expressed in the Membrane Fractions of Epithelial Cells**—We investigated the endogenous distribution of E3KARP and EBP50/NHERF in Calu-3 and T84 cells between soluble and membrane-associated compartments. As shown in Fig. 6, E3KARP is predominantly expressed (>95%) in the membrane fraction of both Calu-3 and T84 cells. In contrast, EBP50/NHERF is about equally distributed between these compartments. It has been demonstrated that NHERF is phosphorylated in intact cells under stimulated and non-stimulated conditions (17). To determine whether stimulation by cAMP/PKA would alter the distribution of E3KARP and EBP50/NHERF between membranes and cytosol, we treated Calu-3 and T84 cells with forskolin (10 μM) for 15 min prior to cell fractionation. The E3KARP and EBP50/NHERF expression patterns were not affected by forskolin pretreatment. The protein doublet detected by the NHERF antibody reflects phosphorylation of the protein, as reported previously (18). However, this phosphorylation pattern was not affected by forskolin pretreatment. In Calu-3 and T84 cells, EBP50/NHERF is expressed as a phosphoprotein in both basal and stimulated conditions, but phosphorylated EBP50/NHERF (shown by the slower migrating band in Fig. 6) is mainly present in the cytosolic fraction. It was difficult to detect phosphorylated EBP50/NHERF in the membrane fraction of T84 cells.
Ezrin Interacts with E3KARP in Human Airway Epithelial Cells—We have demonstrated previously that CFTR could co-immunoprecipitate ezrin, a protein kinase A and cytoskeleton-binding protein that is expressed in human airway epithelia (8). However, when we performed protein overlay assays to determine whether ezrin directly binds to CFTR, we did not detect a direct interaction between these proteins (data not shown). These findings led us to examine the possibility that E3KARP serves as a linker between ezrin and CFTR in Calu-3 cells (Fig. 2). Our findings demonstrate that the PDZ domain protein, E3KARP, interacts directly with CFTR in Calu-3 cells (Fig. 2).

![Fig. 5. Affinity of CFTR binding to E3KARP PDZ2.](image)

**Fig. 5.** Affinity of CFTR binding to E3KARP PDZ2. Equal amounts of GST-CT fusion protein (10 μg) were run on SDS-PAGE, blotted, and overlaid with [35S]methionine-labeled PDZ2 of E3KARP in the presence of seven different concentrations of purified unlabeled E3KARP protein, as shown. Signals were visualized by autoradiography (upper panel). Densitometric intensity of the bands is plotted on the ordinate (lower panel) relative to the intensity of binding observed at zero unlabeled E3KARP. Each point is the mean ± S.E. of three determinations. The amount of labeled E3KARP needed to saturate CFTR-CT binding was determined in pilot experiments, which is 1 μl of labeled E3KARP in 1 ml of blocking buffer.

![Fig. 6. E3KARP is primarily membrane-associated in epithelial cells.](image)

**Fig. 6.** E3KARP is primarily membrane-associated in epithelial cells. Equal amounts of protein from membrane (M) and cytosolic (C) fractions of Calu-3 and T84 cells were resolved on SDS-PAGE and transferred to PVDF membrane. In forskolin (FSK) stimulation experiments, Calu-3 and T84 cells were treated with 10 μM FSK for 15 min at 37 °C before cell fractionation. The membranes were probed with E3KARP or NHERF antibodies as indicated.

![Fig. 7. Interaction of E3KARP with ezrin in Calu-3 cells. A](image)

**Fig. 7. Interaction of E3KARP with ezrin in Calu-3 cells.** A, immunoprecipitates obtained with ezrin antibody from Calu-3 cell lysates were separated on SDS-PAGE, transferred to PVDF membrane, and probed with a polyclonal anti-E3KARP (anti-E3). In the negative control, the immunoprecipitation was performed using anti-GST. The results represent two different experiments. B, GST-E3KARP (GST-E3) and GST proteins (10 μg) were immobilized on beads, incubated with Calu-3 cell lysates, and pelleted. Bound proteins were resolved on SDS-PAGE and probed with ezrin antibody. Calu-3 cell lysates provided the ezrin-positive control. The results are typical of three experiments.

Co-localization of Ezrin and E3KARP with CFTR at the Apical Membranes of Calu-3 Cells—The results from these protein interaction assays suggest that E3KARP should be found in proximity to ezrin and CFTR, which are localized at the apical membrane domain of polarized airway epithelia (8, 19). By using laser-scanning confocal immunofluorescence microscopy, we found that ezrin and CFTR are expressed predominantly at the apical membrane domain of polarized Calu-3 cells (Fig. 8, A and D, respectively). Interestingly, CFTR expression at the apical membrane was not homogenous in Calu-3 epithelia (Fig. 8D), which is consistent with the previous findings of Loffing et al. (19). E3KARP was present also at the apical membranes, although lower levels appeared also to be present in the lateral membranes (Fig. 8, B and E). Fig. 8, C and F, shows reconstructed vertical (xz plane) sections of polarized Calu-3 epithelia. The findings that E3KARP is present in the apical membranes (Fig. 8, B and E) and that it colocalizes with ezrin (Fig. 8C) and CFTR (Fig. 8F) are consistent with the observed physical interactions among these proteins.

Co-expression of E3KARP and Ezrin with CFTR Potentiates CFTR Cl− Currents—In an attempt to identify functional consequences of these protein interactions, we measured CFTR Cl− currents (I_{Cl}) in Xenopus oocytes expressing CFTR with or without E3KARP and ezrin. To mimic physiological stimulation conditions, we also expressed the β2-adrenergic receptor in these cells and stimulated cAMP production by adding isoproterenol to the bath. As shown in Fig. 9, CFTR Cl− currents were increased in oocytes co-expressing CFTR with E3KARP and ezrin during isoproterenol stimulation; this current was inhibited by the β2-adrenergic receptor antagonist, propranolol (data not shown). On average, the CFTR Cl− currents observed during co-expression with E3KARP and ezrin were increased by approximately 45% over control values. We do not know the extent to which these cells express ezrin or ezrin-binding PDZ domain proteins endogenously, which would diminish the effects of their exogenous expression. Nevertheless, these results suggest that formation of a CFTR-E3KARP-ezrin complex enhances the efficacy of cAMP-mediated CFTR activation during cAMP stimulation.

**DISCUSSION**

Our findings demonstrate that the PDZ domain protein, E3KARP, interacts directly with CFTR in Calu-3 cells (Fig. 2).
inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange activity by cAMP-mediated agonists (18). The physical interaction of E3KARP with NHE3 involves the COOH-terminal portion of E3KARP, which includes the second PDZ domain. This is also the high affinity site for CFTR binding. Thus, in principle, NHE3 and CFTR could compete for binding at PDZ2 of E3KARP, but the extent of co-expression of NHE3 in cystic fibrosis target tissues is not completely known. NHE3, at least, does not express in lung (21).

Our immunoprecipitation data show that E3KARP interacts with CFTR in human airway epithelial cells. By using pull-down and in vitro protein binding (overlay) assays, recent studies of an E3KARP related protein, EBP50/NHERF, have shown that EBP50/NHERF can associate with CFTR via a PDZ domain interaction that has a similar affinity to that for E3KARP (8–10). Presumably, E3KARP and EBP50 could compete for CFTR binding because both proteins are expressed in human airway cells (Fig. 5). The co-expression, in the same cell type, of different PDZ proteins that have the capacity for associating with the same target protein is not a unique finding. For example, chapsyn-110 and PSD-95 are two membrane-associated guanylate kinase homologues that are expressed at postsynaptic membranes in the central nervous system. The PDZ domains from either PSD-95 or chapsyn-110 are equally effective in binding to the COOH terminus of Shaker-type K\textsuperscript{+} channels, but how selectivity of PDZ domain binding is regulated in vivo is unclear at present (22).

This remains a question also for CFTR-PDZ domain interactions. Our findings demonstrate that the CFTR-E3KARP association can be competed by purified E3KARP peptide (2) and that the affinity of PDZ2 for the CFTR-CT was approximately three times higher than that of PDZ1 (2). Similarly, intramolecular specificity for PDZ domain binding is observed for the interaction of EBP50/NHERF with CFTR and also for the COOH terminus of Shaker-type K\textsuperscript{+} channels, which interact preferentially with the second PDZ domain of PSD95 (22). Selectivity among PDZ protein interactions was supported by the data of Short et al. (8), who found that EBP50, which can associate with the COOH terminus of CFTR, failed to interact with a PDZ-binding peptide derived from the skeletal muscle voltage-gated Na\textsuperscript{+} channel. We have made a limited attempt to identify the basis of selectivity of EBP50-PDZ1 and E3KARP-PDZ2 for CFTR-Ct binding. Amino acid sequence homology of these PDZ domains suggested that Pro\textsuperscript{230} in E3KARP-PDZ2 is conserved in EBP50-PDZ1, however, mutation of Pro\textsuperscript{230} to either Gin or Ala did not produce a significant affinity change in the CFTR-Ct interaction assay.\textsuperscript{2} This indicates that differen-
tional PDZ domain selectivity may not be conferred by specific shared residues but may be determined by more global interactions (23, 24).

Our cell fractionation studies show that E3KARP is expressed predominantly in the membrane fractions of Calu-3 and T84 cells, whereas EBP50/NHERF is about equally distributed between membranes and cytosol (Fig. 5). Although there is likely to be ample amounts of EBP50/NHERF in the plasma membrane, the gross distribution of E3KARP in these cells is more appropriate for interactions with CFTR. Moreover, we found that CFTR and E3KARP were co-localized at the apical membrane domain of polarized Calu-3 cells (Fig. 8). It is possible that E3KARP and EBP50 may mediate interactions of different proteins with CFTR under specific regulatory conditions or perhaps when CFTR is present in different cellular compartments. However, given the co-localization of E3KARP with CFTR in Calu-3 cells, we speculate that E3KARP is more important physiologically for the regulation of CFTR functions at the apical membrane of airway cells.

Functional Consequences of E3KARP-CFTR Interactions—The interaction of E3KARP with CFTR and the coordinate association of ezrin with E3KARP may serve several potential functions. For example, PDZ domain proteins are recognized to act as scaffolds for organizing cellular signaling molecules in the vicinity of their substrates (25). This scaffolding complex can recruit protein kinases and phosphatases to organize physically signal transduction pathways at specific cellular locations. For example, the Drosophila protein, INAD, contains five PDZ domains. It is expressed in photoreceptor cells where, in addition to its interaction with Ca2+ channels, INAD associates with PKC and PLCβ via additional PDZ interactions to organize the phototransduction cascade. Mutations that disrupt INAD PDZ domains interfere with G protein-mediated phototransduction (26).

Likewise, the formation of the CFTR-E3KARP-ezrin complex appears to sequester PKA at the apical membrane in close proximity with CFTR, because ezrin can serve as an AKAP (13, 14). This complex allows PKA to be compartmentalized with its substrate, CFTR (27), and it may promote molecular specificity of PKA-mediated phosphorylation events. PKA displays a broad substrate specificity in vitro, and it has the potential to phosphorylate many targets within cells (28). Indeed, CFTR is apically polarized in most epithelia, but cAMP-mediated receptors, and thus the site of cAMP production, are generally localized to the basolateral membrane (29). It is thought that the specificity of substrate phosphorylation under such conditions is provided by a close association of kinase with its target (30). In a recent study, Weinman et al. (31) found that cAMP-mediated inhibition of NHE3 activity, as well as in vivo phosphorylation of the protein, required binding of both NHERF and ezrin. In addition, there are nine consensus dibasic PKA phosphorylation sites present in the R-domain of CFTR, yet only five of these appear to be used physiologically (3). In fact, one PKA site within the R-domain is inhibitory to Cl− channel gating. These properties imply that the phosphorylation of CFTR may be site-selective, and in addition to their accessibility, the physical position of PKA in relation to CFTR may confer phosphorylation site selectivity.

In addition to providing for compartmentalization of PKA in microdomains where CFTR resides, the association of CFTR with E3KARP and ezrin could potentiate CFTR Cl− currents by other mechanisms. First, the interaction with ezrin may facilitate the retention of CFTR at the apical membrane via interactions with the actin cytoskeleton, since ezrin is an actin-binding protein (32). Support for this concept is provided by the naturally occurring CFTR mutation, S1455X, which deletes part of the CFTR COOH terminus (33). This resulted in mis-localization of CFTR protein to the basolateral membranes of Madin-Darby canine kidney cells when this mutant was expressed exogenously (34). In addition, disruption of the actin cytoskeleton by cytochalasin D in cells expressing CFTR inhibited CFTR activity (35, 36). The stabilizing effect of PDZ protein interactions has been observed previously (37). Second, the formation of a regulatory complex involving PKA may augment its catalytic activity during cAMP stimulation. For example, HEK293 cells express low levels of endogenous AKAPs, and exogenous expression of AKAP75, to immobilize type II PKA in the cortical cytoskeleton, elicited 3-fold higher levels of catalytic subunit activity and proportionately increased the rate and magnitude of cAMP-dependent phosphorylation (38). Thus, the efficiency of PKA-dependent CFTR activation may be enhanced by formation of this regulatory complex.

In light of our pull-down assay showing that both PDZ domains are capable of binding the COOH terminus of CFTR, one possible role of E3KARP could be to link two CFTRs as a functional channel unit at the apical membrane of epithelial cells. Two different groups have proposed that CFTR functions as a dimer. By using freeze fracture electron microscopy, Eskandari et al. (39) found that Xenopus oocytes expressing CFTR exhibit particle patterns on their cell surface membranes that are consistent with the physical presence of two CFTR monomers (i.e. the cross-sectional packing area corresponded to 24 transmembrane helices). In addition, when studied in planar lipid bilayers, the single channel properties derived from expression in HEK293 cells of a tandem repeat of two CFTRs were similar to the single channel properties obtained from expression of CFTR monomers (40). Co-expression of CFTR with deletion mutants of either E3KARP or EBP50/NHERF could be used to test further the hypothesis that these PDZ domain proteins can mediate the formation of CFTR homodimers.

Finally, CFTR-PDZ domain associations may influence the regulation of membrane trafficking events. For example, the sorting of β2-adrenergic receptors between recycling endosomes and lysosomal compartments is controlled by their association with EBP50 (41). Disrupting this interaction causes selective mis-sorting of endocytosed β2-adrenergic receptors to lysosomes, and disrupting the actin cytoskeleton had the same effect. β2-Adrenergic receptors are linked to the actin cytoskeleton via EBP50-ezrin interactions because ezrin contains actin-binding sites at its COOH terminus (42). Accordingly, such interactions may also influence the endocytic sorting and recycling of CFTR, a process that is influenced by CFTR phosphorylation (43).

In summary, these results, together with our prior findings (14), indicate that a CFTR regulatory complex consisting of E3KARP, ezrin, and PKA is present at the apical membranes of human airway epithelial cells. This complex is likely to be important in determining the kinetics and magnitude of cAMP-stimulated CFTR Cl− channel currents, as well as the density of CFTR channels at the apical membrane of airway epithelial cells.

Acknowledgments—We thank Yuee Wang for technical assistance, Simon C. Watkins for assistance with image analysis, and Tony Hunter (Salk Institute) for the ezrin cDNA.

REFERENCES
1. Sheppard, D. N., and Welsh, M. J. (1999) Physiol. Rev. 79, S23–S45
2. Pilewski, J. M., and Frizzell, R. A. (1999) Physiol. Rev. 79, S215–S255
3. Gadsby, D. C., and Nairn, A. C. (1999) Physiol. Rev. 79, S77–S107
4. Naren, A. P., Nelson, D. J., Xie, W., Jovov, B., Pevsner, J., Bennett, M. K., Benor, D. J., Quick, M. W., and Kirk, K. L. (1997) Nature 390, 362–365
5. Peters, K. W., Qi, J., Watkins, S. C., and Frizzell, R. A. (1999) Am. J. Physiol. 277, C174–C180
6. Prince, L. S., Peter, K., Hatton, S. R., Zaliauskiene, L., Cotlin, L. F., Clancy, Salk Institute for the ezrin cDNA.
J. P., Marchase, R. B., and Collawn, J. F. (1999) *J. Biol. Chem.* **274**, 3602–3609
7. Weixel, K. M., and Bradbury, N. A. (2000) *J. Biol. Chem.* **275**, 3655–3660
8. Short, D. B., Trotter, K. W., Reczek, D., Kreda, S. M., Bretscher, A., Boucher, R. C., Stutts, M. J., and Milgram, S. L. (1998) *J. Biol. Chem.* **273**, 19797–19801
9. Wang, S., Raab, R. W., Schatz, P. J., Guggino, W. B., and Li, M. (1998) *FERS Lett.* **427**, 103–108
10. Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., and Lefkowitz, R. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8496–8501
11. Weinman, E. J., Steplock, D., Tate, K., Hall, R. A., Spurney, R. F., and Shenolikar, S. (1998) *J. Clin. Invest.* **101**, 2199–2206
12. Reczek, D., and Bretscher, A. (1998) *J. Biol. Chem.* **273**, 18452–18458
13. Dransfield, D. T., Bradford, A. J., Smith, J., Martin, M., Roy, C., Mangeat, P. H., and Goldenring, J. R. (1997) *EMBO J.* **16**, 35–43
14. Sun, F., Hug, M. J., Bradbury, N. A., and Frizzell, R. A. (2000) *J. Biol. Chem.* **275**, 14360–14366
15. Mohler, P. J., Kreda, S. M., Boucher, R. C., Sudol, M., Stutts, M. J., and Milgram, S. L. (1999) *J. Cell Biol.* **147**, 879–890
16. Yun, C. H., Oh, S., Zizak, M., Steplock, D., Tse, C. M., Weinman, E. J., and Donowitz, M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3010–3015
17. Yun, C. H., Lamprecht, G., Forster, D. V., and Sidor, A. (1998) *J. Biol. Chem.* **273**, 25856–25863
18. Lamprecht, G., Weinman, E. J., and Yun, C. H. C. (1998) *J. Biol. Chem.* **273**, 29972–29978
19. Leffing, J., Moyer, B. D., McCoy, D., and Stanton, B. A. (1998) *Am. J. Physiol.* 275, C913–C920
20. Haws, C., Finkbeiner, W. E., Widdicombe, J. H., and Wine, J. J. (1994) *Am. J. Physiol.* 266, L502–L512
21. Brant, S. R., Yun, C. H., Donowitz, M., and Sudol, M. (1995) *Am. J. Physiol.* 268, C198–C206
22. Kim, E., Cho, K. O., Rothschild, A., and Sheng, M. (1996) *Neuron* 17, 103–113
23. Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996) *Cell* 85, 1067–1076
24. Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chishiti, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Canliy, L. C. (1997) *Science* 275, 73–77
25. Fanning, A. S., and Andersen, J. M. (1999) *J. Clin. Invest.* 103, 767–772
26. Tsuoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Scollich, M., and Ziker, C. S. (1997) *Nature* 388, 243–249
27. Huang, P., Trotter, K., Boucher, R. C., Milgram, S. L., and Stutts, M. J. (2000) *Am. J. Physiol.* 278, C417–C422
28. Colledge, W., and Scott, J. D. (1999) *Science* 281, 126–128
29. Lamprecht, G., Weinman, E. J., and Yun, C.-H. (1998) *J. Biol. Chem.* 273, 29972–29978
30. Cantiello, H. F. (1996) *Exp. Physiol.* 81, 505–514
31. Prat, A. G., Xiao, Y. F., Ausiello, D. A., and Cantiello, H. F. (1995) *Am. J. Physiol.* 268, C152–C1561
32. Perego, C., Vanoni, C., Villa, A., Longhi, R., Kaech, S. M., Frohli, E., Hjalal, A., Kim, S. K., and Pietrini, G. (1999) *EMBO J.* 18, 2394–2399
33. Feliciello, A., Li, Y., Avvedimento, E. V., Gottesman, M. E., and Rubin, C. S. (1997) *Curr. Biol.* 7, 1011–1014
34. Eskandari, S., Wright, E. M., Kreman, M., Starace, D. M., and Zampighi, G. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 11235–11240
35. Zerhusen, B., Zhao, J., Xie, J., Davis, P. B., and Ma, J. (1999) *J. Biol. Chem.* 274, 7627–7630
36. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* 401, 286–290
37. Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Soffier, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., Grinstein, S., and Lefkowitz, R. J. (1998) *Nature* 392, 626–630
38.Bradbury, N. A., Jilling, T., Berta, G., Sorch, E. J., Bridges, R. J., and Kirk, K. L. (1992) *Science* 256, 530–532