An Apical PDZ Protein Anchors the Cystic Fibrosis Transmembrane Conductance Regulator to the Cytoskeleton*

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The function of the cystic fibrosis transmembrane conductance regulator (CFTR) as a Cl^- channel in the apical membrane of epithelial cells is extensively documented. However, less is known about the molecular determinants of CFTR residence in the apical membrane, basal regulation of its Cl^- channel activity, and its reported effects on the function of other transporters. These aspects of CFTR function likely require specific interactions between CFTR and unknown proteins in the apical compartment of epithelial cells. Here we report that CFTR interacts with the recently discovered protein, EBP50 (ERM-binding phosphoprotein 50). EBP50 is concentrated at the apical membrane in human airway epithelial cells, *in vivo*, and CFTR and EBP50 associate in *in vitro* binding assays. The CFTR-EBP50 interaction requires the COOH-terminal DTRL sequence of CFTR and utilizes either PDZ1 or PDZ2 of EBP50, although binding to PDZ1 is of greater affinity. Through formation of a complex, the interaction between CFTR and EBP50 may influence the stability and/or regulation of CFTR Cl^- channel function in the cell membrane and provides a potential mechanism through which CFTR can affect the activity of other apical membrane proteins.

Cystic fibrosis $(CF)^1$ is a lethal autosomal recessive disease characterized by defects in epithelial ion transport (1). CF is caused by mutation in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR), which functions as a cAMP-regulated Cl^- channel at the apical cell surface (1–3). The CF phenotype includes changes in cellular processes distinct from those involving Cl^- transport, including sodium hyperabsorption and abnormalities in the processing of mucins (4–6). The most common cause of CF are mutations that lead to the formation of an abnormally folded CFTR pro-

tein that does not reach the cell surface (2). Even wild type CFTR is inefficiently transported to the cell surface, with up to 70% of the newly synthesized proteins failing to achieve a stable conformation that escapes quality control mechanisms in the endoplasmic reticulum (2, 7, 8). Knowledge of the protein-protein interactions that are involved in CFTR-mediated regulation of other epithelial transport proteins, and the interactions that control the trafficking, localization, and regulation of CFTR, is incomplete. Recently, the amino terminus of CFTR was shown to interact with syntaxin 1, with implications both for insertion of CFTR into the plasma membrane and regulation of channel activity (9). Other interactions that stabilize CFTR or regulate its function remain to be identified.

Compartmentalization of CFTR in a multiprotein complex might regulate CFTR activity by stabilizing the protein at the cell surface or by increasing the efficiency by which kinases and phosphatases control the channel. The presence of such a complex may also explain how CFTR modulates the activity of other epithelial cell transport proteins. A common mechanism to establish multiprotein complexes is via protein-protein interactions with submembranous scaffolding proteins (10–12). Ion channels and transport proteins may associate via their COOH-terminal cytosolic tails with proteins that contain PDZ domains (13–16). PDZ domains (originally identified in postsynaptic density-95, discs large, and ZO-1) are found in a large number of multifunctional proteins, where they mediate protein-protein interactions at structures including the postsynaptic density in neurons and junctional complexes in epithelia (10–12, 17). There is a short consensus sequence at the COOH terminus of membrane proteins that is critical for the interaction with PDZ domains; one consensus sequence consists of the amino acids (D/E)(T/S)XV (10, 18).

The COOH-terminal cytosolic domain of CFTR is highly conserved across species and terminates with the amino acids DTRL (1). Therefore, we reasoned that CFTR may exist in a multiprotein complex at the apical plasma membrane via interaction with a PDZ-containing scaffolding protein. Although several PDZ-containing proteins are known to be expressed in epithelia, many, including ZO-1 and ZO-2, are largely restricted to junctional complexes (19, 20). Recently, a 50-kDa human protein containing two PDZ domains was identified and cloned based on its ability to associate with ezrin, a protein found in the apical domain of epithelial cells (21). This protein, ERM-binding phosphoprotein 50 (EBP50), is also localized at the apical surface and is expressed in a variety of epithelial tissues (21). Human EBP50, and its rabbit homologue NHE-RF (Na^+/H^+ exchange regulatory factor) share domain organization and sequence homology with E3KARP (NHE3 kinase A regulatory protein), and both proteins may associate with the Na^+-H^+ exchanger NHE3 to confer cAMP-mediated inhibition

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; EBP50, ERM-binding phosphoprotein 50; NHE-RF, Na^+/H^+ exchange regulatory factor; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; PCR, polymerase chain reaction.

of Na$^+$-H$^+$ exchange (22, 23). In addition, the COOH terminus of the β_2-adrenergic receptor has recently been shown to associate with the first PDZ domain of EBP50 (24). We determined the distribution of EBP50 in human airway epithelia and studied the biochemical interaction between EBP50 and CFTR. The identification of proteins that associate with CFTR, either directly or via scaffolding proteins, will facilitate our understanding of why absence of CFTR leads to severe abnormalities in epithelial cell function.

MATERIALS AND METHODS

Analysis of EBP50 Expression and Distribution—Polyclonal antiserum B60 directed against the COOH terminus of EBP50 was generated in rabbits as described (21). Sections from human bronchus were fixed in 4% paraformaldehyde and permeabilized as described (25). The sections were subsequently blocked in 20% normal goat serum in 50 mM sodium phosphate, pH 7.4, 150 mM NaCl (PBS) and incubated in antiserum B60 or pooled normal rabbit sera diluted 1:50 in PBS. Texas Red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was used as secondary antibody. Tissues were mounted in Vectashield containing 4,6-diamidino-2-phenylindole to label nuclei (Vector Laboratories, Burlingame, CA).

For immunoblot analysis, cultured cells were homogenized in 20 mM NaCl, 5 mM EDTA, 1 mM EGTA, 20 mM Hepes, pH 7.2 (Buffer A), 1% Triton X-100 + protease inhibitors (26), centrifuged 14,000 × *g* for 20 min, and the supernatant collected. To analyze the distribution of proteins in soluble and particulate fractions, cells were homogenized in the same buffer minus Triton X-100, homogenates were centrifuged at 413,000 × *g* for 20 min, and pellets were resuspended in Buffer A + 0.2% SDS. Protein concentrations of whole cell lysates or soluble and particulate fractions were determined using the BCA assay kit (Pierce). Samples (20 μg) were electrophoresed on SDS-PAGE gels and analyzed by Western blot analysis as described previously (26). Mouse anti-ezrin was obtained from Transduction Laboratories (Lexington, KY), and rabbit anti-CFTR R domain antiserum was described previously (27).

Plasmids for Bacterial Expression and Purification of Fusion Proteins—Full-length EBP50 (amino acids 1–358), PDZ1 (amino acids 1–97), and PDZ2 (amino acids 139–248) of EBP50 were expressed as GST fusion proteins from constructs created either by PCR amplification or subcloning of sequences from human EBP50 cDNA. PCR products were generated with unique restriction sites on their ends, and the fragments to be subcloned were directionally ligated into the polylinker of the appropriate pGEX vector (Amersham Pharmacia Biotech). All PCR products were verified by DNA sequencing and found to be free of mutations.

Full-length GST-EBP50 or fusion proteins containing domains of EBP50 were expressed in *Escherichia coli* DH5α by induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C. Bacterial lysates were prepared by sonication in ice-cold PBS in the presence of protease inhibitors; proteins were purified from the soluble fraction on glutathione-Sepharose 4B.

Gel Overlays—Five-hundred ng of GST-EBP50, GST-PDZ1, GST-PDZ2, or GST-β2-syntrophin PDZ domain were electrophoresed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were blocked in 10% non-fat dry milk in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4 + 0.05% Tween 20 (TTBS) and probed with 100 nM biotinylated CFTR peptide diluted in TTBS for 2 h at room temperature. After extensive washing in TTBS, the membranes were incubated with streptavidin-conjugated horseradish peroxidase diluted 1:10,000 in TTBS. Blots were developed using enhanced chemiluminescence (ECL).

Peptide or Fusion Protein Affinity Chromatography—Peptides corresponding to the COOH-terminal 22 residues of full-length human CFTR or mutant CFTR (CFTRmut), where the last four residues were replaced by glycines were synthesized at University of North Carolina Peptide Synthesis Facility. Peptides were coupled to an amino-terminal biotin via the four-residue spacer SGSG and purified by high pressure liquid chromatography. The skeletal muscle voltage-gated sodium channel peptide (SkM2; biotin-SPDRDRESIV) (16) was kindly provided by Dr. Stan Froehner, University of North Carolina.

Biotinylated wild type CFTR, CFTRmut, and SkM2 peptides (25 μg) were immobilized onto 25 μl of streptavidin-agarose beads (Sigma) in 50 mM Tris, pH 7.5, 1 mM EDTA, and 1 mM EGTA (TEE) overnight at 4 °C. The beads were washed in TEE + 1% Triton X-100 to remove unbound peptide. GST-EBP50 fusion protein (25 μg) was immobilized onto 25 μl of glutathione-agarose beads for 1 h at room temperature in PBS, and the beads were washed in PBS + 1% Triton X-100. Five-
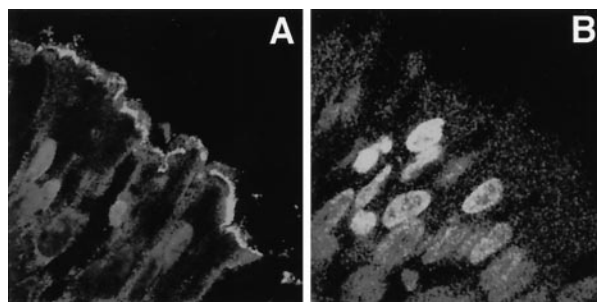

FIG. 1. **Confocal microscopy to determine the distribution of EBP50 in human bronchus.** Sections from human bronchi (6–10 μm thick) were prepared as described. The sections were first incubated with either an antiserum directed against EBP50 (*A*) or a pooled normal rabbit serum (*B*) and subsequently incubated with Texas Red-conjugated goat anti-rabbit IgG. Sections were analyzed using a Leica TCS 4D confocal microscope. Magnification = × 430.

hundred μg of CalU3 cell lysate in a total volume of 500 μl was added to the beads, fresh aliquots of protease inhibitor were added, and the beads were tumbled at 4 °C for 2 h. Samples were washed in TEE and bound proteins removed from the beads by boiling in sample buffer. In some experiments, unbound fractions were precipitated by addition of 1 ml of acetone overnight at 4 °C. Although CFTR was not stable when precipitated in acetone overnight, we observed CFTR proteolytic fragments with antisera to the R domain or COOH terminus (Not shown). Bound and unbound fractions were separated on SDS-PAGE gels and analyzed by immunoblotting with EBP50 or CFTR antisera.

To directly assess binding of CFTR peptides and full-length EBP50, biotinylated peptides were immobilized as described, and 100 nM GST-EBP50 was added to the beads in a total volume of 0.5 ml of TEE and incubated for 2 h at 4 °C. Bound and unbound fractions were analyzed as described above using mouse anti-GST (Santa Cruz Biotechnology, Santa Cruz, CA).

Surface Plasmon Resonance—The comparative binding of full-length EBP50 and EBP50 PDZ domains to CFTR peptides was measured using surface plasmon resonance (28–30). All experiments were performed on a BIAcore 2000 instrument at the Macromolecular Interactions Facility at the University of North Carolina. Biotinylated peptides were immobilized onto neutravidin-coated CM5 sensor chips (BIAcore, Piscataway, NJ) as described (16). After injection of 10 mM HCl to remove unbound peptide, final surface densities varied from 50–100 resonance units. Purified GST-EBP50 fusion proteins were injected onto the peptide surfaces at a flow rate of 20 μl/min.

RESULTS AND DISCUSSION

Because ezrin is concentrated at the apical membrane of many epithelia (25, 31), we first determined if EBP50 was similarly distributed in airway epithelium. Immunohistochemical analysis of sections from human bronchi using antisera directed against EBP50 demonstrated intense staining of the apical cell surface with little staining of cilia or internal structures (Fig. 1). This localization establishes that EBP50 is concentrated in airway epithelial cells in the apical compartment, where CFTR is targeted and functions as a Cl$^-$ channel.

Both ezrin and EBP50 are expressed in several epithelial cell lines derived from bronchus, colon, and kidney (Fig. 2*A*), but not in cells of neuroendocrine origin (not shown). Multiple EBP50 species are seen in all the cell lines shown, most likely representing differentially phosphorylated forms (21). Ezrin and EBP50 are binding partners (21, 32, 33), so these proteins could form part of a subapical membrane-anchoring complex specific to epithelia. To evaluate this possibility in airway epithelial cells, we analyzed the expression and distribution of CFTR, ezrin, and EBP50 in soluble and particulate fractions prepared from CalU3 cell lysates. CalU3 cells are a human airway epithelial cell line that expresses robust levels of CFTR. CFTR was present only in the particulate fraction, whereas EBP50 and ezrin were distributed equally in soluble and particulate fractions (Fig. 2*B*). Because ezrin and EBP50 do not have membrane-spanning domains, their presence in the par-
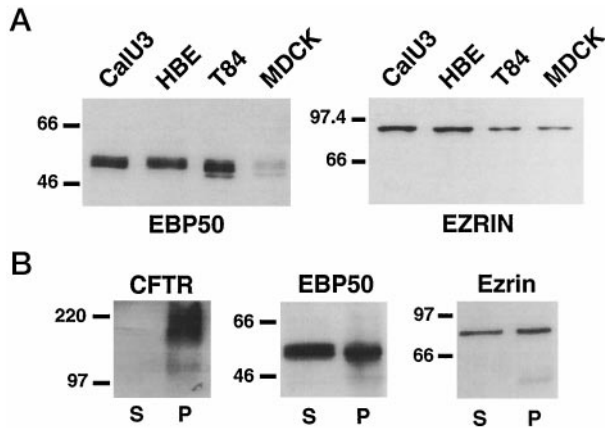
FIG. 2. **Distributions of EBP50 and ezrin in epithelial cell lines.** *A*, whole cell lysates (20 μg) prepared from epithelial cell lines were analyzed by immunoblotting with rabbit anti-EBP50 (1:10,000) or mouse anti-ezrin (1:1000) antisera. Proteins were visualized following incubation with horseradish peroxidase-conjugated secondary antisera and ECL reagent. *B*, CalU3 cell homogenates were separated into soluble and particulate fractions and analyzed by immunoblotting using rabbit anti-CFTR R domain (1:200), rabbit anti-EBP50 (1:5000), or mouse anti-ezrin (1:1000). Samples from two separate extractions were analyzed with similar results.

ticulate fraction is consistent with their cytoskeletal origin or association with membranes through an interaction with integral membrane proteins of epithelia.

To determine whether CFTR and EBP50 might interact, we immobilized GST or GST-EBP50 on glutathione-agarose beads and incubated these affinity resins with CalU3 cell lysates. Endogenous full-length CFTR was recovered bound to the GST-EBP50 beads but not to GST beads (Fig. 3*A*), indicating that EBP50 and CFTR are capable of interacting. When we performed similar assays in the presence of a peptide corresponding to the COOH-terminal 22 amino acids of CFTR, the association between GST-EBP50 and CFTR was significantly decreased (Fig. 3B). These results suggest that the COOH terminus of CFTR is involved in the interaction with EBP50. Because the CFTR peptide, in which the final four amino acid residues were replaced by glycines (CFTRmut), failed to block the association between GST-EBP50 and full-length CFTR (Fig. 3*B*), the last four residues of CFTR (DTRL) must play a critical role in the association.

To determine whether the COOH terminus of CFTR was sufficient to mediate the interaction with EBP50, we immobilized biotinylated peptides corresponding to the COOH-terminal 22 amino acids of CFTR (Fig. 3*B*) on streptavidin beads and incubated the immobilized peptide with CalU3 cell lysates. We found that EBP50 bound to the CFTR COOH-terminal peptide and was depleted from the lysate (Fig. 3*C*). In contrast, there was no association between EBP50 and the CFTRmut peptide (Fig. 3*C*). These data demonstrate that EBP50 and CFTR interact and further demonstrates that the COOH terminus of CFTR is sufficient to mediate the interaction. To test the specificity of the interaction between CFTR and EBP50, we performed assays using a peptide derived from the COOH terminus of the skeletal muscle voltage-gated sodium channel (SkM2) as an affinity ligand. It is known that the SkM2 peptide can precipitate syntrophins from lysates via a PDZ interaction (16, 34). SkM2 failed to bind EBP50 (Fig. 3*C*), demonstrating that the PDZ domains of EBP50 discriminate between the cytosolic tails of different proteins containing COOH-terminal PDZ-binding motifs.

The reciprocal affinity purifications demonstrate that EBP50 and CFTR proteins associate and that the association is mediated by the COOH terminus of CFTR. However, these results
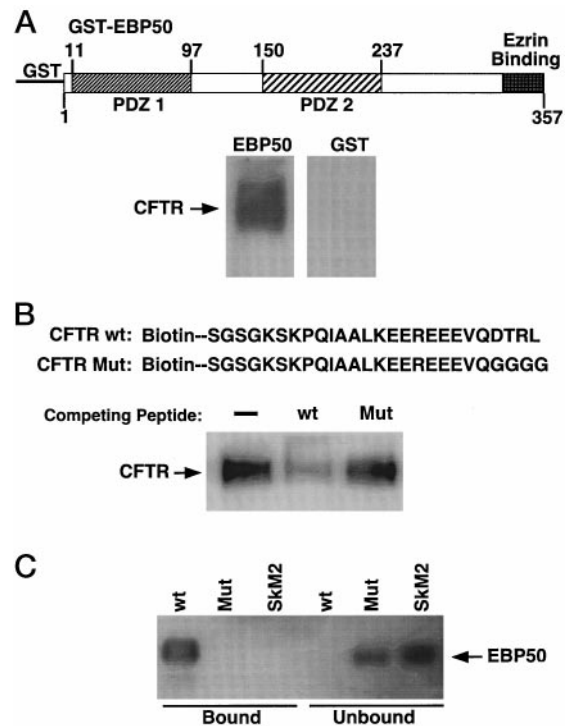

FIG. 3. **Affinity precipitation of EBP50 and CFTR from CalU3 cell lysates.** *A*, GST or GST-EBP50 were immobilized on glutathione-agarose beads and incubated with ~500 μg of CalU3 cell lysate for 2 h at 4 °C. The bound fraction was electrophoresed on 7% SDS-PAGE and immuoblotted with rabbit anti-CFTR R domain (1:200). A schematic of GST-EBP50 with PDZ1, PDZ2, and the COOH-terminal ezrin binding domain is shown. *B*, binding of CFTR to GST-EBP50 was assayed as described for *A* in the presence of 400 nM wild type CFTR or CFTRmut peptide. Binding of CFTR to GST-EBP50 in the presence of CFTR or CFTRmut peptides was determined by immunoblot analysis as described for *A*. The sequences of wild type CFTR and CFTRmut peptides are shown. *C*, CFTR, CFTRmut, and SkM2 peptides were immobilized on streptavidin-agarose and incubated with ~500 μg of CalU3 cell lysate for 2 h at 4 °C. The beads were extensively washed, and bound and unbound fractions were electrophoresed on 10% SDS-PAGE and immunoblotted with rabbit anti-EBP50 antisera (1:5000).

do not distinguish direct binding of CFTR and EBP50 from binding that occurs via an accessory protein. We used a combination of *in vitro* binding assays, gel overlays, and surface plasmon resonance to determine whether CFTR and EBP50 associate directly. EBP50 contains two PDZ domains and has an ezrin binding site at its extreme COOH terminus (Ref. 33; Fig. 3*A*). We immobilized the biotinylated wild type and CFTRmut peptides on streptavidin-agarose beads and incubated the beads with purified GST-EBP50. Wild type CFTR bound GST-EBP50, and, as we observed previously, the mutant CFTR peptide lacking the COOH-terminal DTRL did not (Fig. 4*A*). We also demonstrated a direct interaction between the COOH terminus of CFTR and full-length EBP50 in gel overlay assays (Fig. 4*B*). Furthermore, we observed that the COOH-terminal peptide could bind both PDZ1 and PDZ2 of EBP50 (Fig. 4*B*). Although the CFTR peptide binds PDZ1 and PDZ2 of EBP50 in overlay (Fig. 4*B*) and pull-down assays (Not shown), we did not observe binding of the CFTR peptide to the PDZ domain of β_2-syntrophin (Fig. 4*B*).

We performed surface plasmon resonance measurements to compare the relative strength of binding of the CFTR peptide with the EBP50 PDZ domains. We confirmed that the mutant CFTR peptide was unable to bind full-length EBP50, even when tested at very high concentrations (Fig. 4*C*), and found that PDZ1 bound the wild type CFTR peptide significantly better than PDZ2 (Fig. 4*D*). The apparent binding affinity for
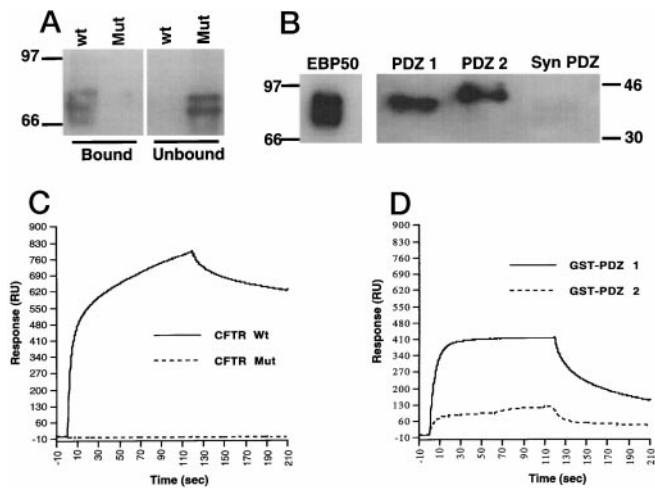
FIG. 4. *In vitro* **interactions between CFTR peptides and EBP50.** *A*, CFTR and CFTRmut peptides were immobilized on streptavidin-agarose and incubated with 100 nM GST-EBP50 for 2 h at 4 °C. Bound and unbound fractions were electrophoresed on 10% SDS-PAGE and GST-EBP50 was detected by immunoblotting using mouse anti-GST antisera (1:5000). *B*, 500 ng of each fusion protein was electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with 100 nM CFTR peptide and visualized by incubation with streptavidin-horseradish peroxidase and enhanced chemiluminescence. The experiment was performed three times with identical results. In one experiment, CFTR peptide failed to bind 500 ng of GST, and CFTRmut peptides failed to bind all samples. *C*, CFTR and CFTRmut peptides were immobilized on neutravidin-coated CM5 sensor chips, and 1.25 μg of GST-EBP50 was applied at a flow rate of 20 μl/min for 2 min. Relative binding is plotted in resonance units (*RU*) on the *y* axis and time in seconds is plotted on the *x* axis. *D*, CFTR peptide was immobilized on neutravidin-coated CM5 sensor chips, and GST-PDZ1 OR GST-PDZ2 was applied in increasing concentrations. The sensorgram shows relative binding in resonance units for 1.25 μg of fusion protein.

PDZ1 was ~40 nM, suggesting a high affinity interaction between the COOH terminus of CFTR and EBP50. Recently the rabbit homologue of EBP50 (NHE-RF) was shown to bind the COOH terminus of the β_2-adrenergic receptor via PDZ1(24). This raises the possibility that the tails of CFTR and the β_2-adrenergic receptor compete for binding to EBP50; however, it is not well established that the β_2-adrenergic receptor is expressed on apical membranes where CFTR resides.

Our data demonstrate that EBP50 in airway epithelial cells is concentrated at the apical membrane (Fig. 1), and EBP50 can associate directly with the cytoplasmic tail of the apical membrane protein, CFTR (Figs. 3 and 4). The association occurs because of the interaction of the terminal DTRL of the cytoplasmic carboxyl terminus of CFTR with the PDZ domains in EBP50. The PDZ domains of EBP50 do not recognize the COOH terminus of SkM2 (Fig. 3*A*) and the COOH terminus of CFTR will not interact with the PDZ domain of β2-syntrophin (Fig. 4*B*). These observations and the close cellular localization of CFTR and EBP50 argue that the interactions between EBP50 and CFTR are specific.

Together with previous data demonstrating that ezrin and EBP50 interact directly in cells (21), our experiments identify three members of a potential regulatory complex present at the apical membrane of airway epithelial cells (Fig. 5). Furthermore, because ezrin is a major actin binding protein in epithelia (25, 35, 36), our results identify EBP50 as an intermediate for linking an apical membrane protein to the cortical actin cytoskeleton. Such an interaction provides a mechanism through which modifications of cytoskeletal structure can be transmitted to CFTR (37–39). Moreover, the association of CFTR with a cytoskeletal complex could serve as an anchor that determines its specific location within microdomains of the
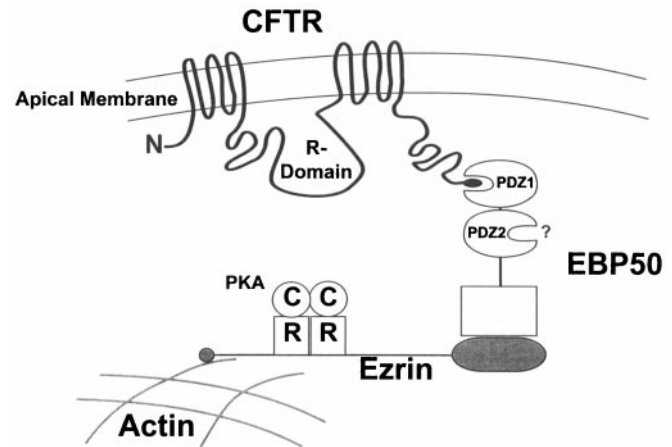

FIG. 5. **Hypothetical model of apical membrane CFTR, EBP50, and ezrin.** CFTR interacts with EBP50 by association with PDZ1; proteins that associate with PDZ2 with high affinity remain to be identified. The COOH terminus of EBP50 interacts with ezrin and ezrin associates with actin. Ezrin may serve to position PKA in close proximity to CFTR.

apical membrane (40) or its residence time at the cell surface. CFTR open probability is regulated by the competing actions of protein kinases and phosphatases, and ezrin was recently reported to function as an anchoring protein for PKA (41). Thus, binding to EBP50 may position the phosphorylation sites of CFTR within close proximity of PKA, and it would not be surprising if such a complex included other kinases or phosphatases that regulate CFTR (42–44).

Because EBP50 contains two PDZ domains (Fig. 3*A*) and PDZ1 binds CFTR with higher affinity (Fig. 4*D*), it is likely that additional apical proteins interact with PDZ2. Participation of CFTR and other epithelial ion transport proteins in a common complex in the subapical compartment could be the basis for reported functional observations that CFTR influences the activity of other epithelial ion transporters (45–49). EBP50 and E3KARP interact with NHE3 to confer cAMP-induced inhibition of NHE3 (22, 23). Although NHE3 is not expressed in lung (50), NHE3 and CFTR are co-expressed in intestinal epithelia. The interaction of CFTR and NHE3 with subapical scaffolding proteins may explain why CFTR is also required for cAMP-mediated inhibition of Na^+ absorption driven by Na^+/H^+ exchange in mouse intestine (49). The further characterization of complexes containing ion transport proteins, scaffolding proteins, and cytoskeletal elements may help explain molecular mechanisms that regulate epithelial ion transport.

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