Protein Kinase A Associates with Cystic Fibrosis Transmembrane Conductance Regulator via an Interaction with Ezrin*

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The cystic fibrosis transmembrane conductance regulator (CFTR) is an epithelial Cl\(^-\) channel whose activity is controlled by cAMP-dependent protein kinase (PKA)-mediated phosphorylation. We found that CFTR immunoprecipitates from Calu-3 airway cells contain endogenous PKA, which is capable of phosphorylating CFTR. This phosphorylation is stimulated by cAMP and inhibited by the PKA inhibitory peptide. The endogenous PKA that co-precipitates with CFTR could also phosphorylate the PKA substrate peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly (kemptide). Both the catalytic and type II regulatory subunits of PKA are identified by immunoblotting CFTR immunoprecipitates, demonstrating that the endogenous kinase associated with CFTR is PKA, type II (PKA II). Phosphorylation reactions mediated by PKA-associated PKA II are inhibited by Ht31 peptide but not by the control peptide Ht31P, indicating that a protein kinase A anchoring protein (AKAP) is responsible for the association between PKA and CFTR. Ezrin may function as this AKAP, since it is expressed in Calu-3 and T84 epithelia, ezrin binds RII in overlay assays, and RII is immunoprecipitated with ezrin from Calu-3 cells. Whole-cell patch clamp of Calu-3 cells shows that Ht31 peptide reduces cAMP-stimulated CFTR Cl\(^-\) current, but Ht31P does not. Taken together, these data demonstrate that PKA II is linked physically and functionally to CFTR by an AKAP interaction, and they suggest that ezrin serves as an AKAP for PKA-mediated phosphorylation of CFTR.

The cystic fibrosis transmembrane conductance regulator (CFTR)\(^1\) is the basis of the cAMP-activated anion conductance pathway at the apical membranes of epithelial cells (1). In secretory epithelia, CFTR is often the rate-determining step in salt and water transport, accounting for impairments in fluid secretion observed in patients having CFTR mutations. To date, more than 800 mutations in CFTR have been observed in patients with cystic fibrosis, but relatively few of these (~5%) are found in the regulatory domain of CFTR (CFTR data base, available on the World Wide Web), perhaps because of its functional importance. The key regulatory pathway determining CFTR activity involves elevation of cAMP and activation of protein kinase A (PKA) (2). Nine consensus sites for PKA phosphorylation lie in the central R domain region of CFTR. An important issue in the regulation of epithelial cell secretion has been the specificity of this process. The receptors for cAMP-mediated secretory agonists as well as the associated adenylate cyclase are localized to the basolateral membrane, yet the principal target of activated PKA, the CFTR, is apically localized.

In recent years, it has become apparent that the selective actions of signaling mediators that do not inherently possess substrate specificity (e.g. PKA) are conferred by the formation of regulatory complexes that provide privileged access of regulators to their substrates (3). Indeed, it is now appreciated that different PKA isofoms are compartmentalized in the soluble and particulate cell fractions and that the association of PKA with cytoskeletal or membrane structures is mediated primarily by a physical association between the type II regulatory subunits (RII subunits) of PKA and protein kinase A anchoring proteins (AKAPs). AKAPs are thought to sequester the regulatory and catalytic components of PKA in proximity to their substrates and thereby confer the needed specificity. Indeed, a model Cl\(^-\) secretory epithelium, T84, was found to express both RI and RII isofoms of PKA; and in these cells, about 5% of total PKA activity was due to RII that was localized on cellular structures (4). These findings raise the possibility that PKA may regulate CFTR via compartmental restrictions that are based on protein interactions and that this arrangement may lead to phosphorylation of CFTR at specific sites within the protein.

PDZ domain proteins are emerging as important organizing centers for regulatory complexes, and these scaffold-based regulatory proteins are often polarized to specific sites in polarized epithelial cells (5). For example, the Na\(^+/H\(^+\) exchange regulatory factor (NHERF, also termed ezrin-binding phosphoprotein 50 or EBP50) was identified initially from its ability to confer PKA-mediated inhibition of the apical Na\(^+/H\(^+\) exchanger in rabbit renal brush border membranes (6). The human homologue of NHERF, EBP50, binds to members of the ERM (ezrin-radixin-moesin) family of proteins (7). The C terminus of CFTR corresponds to a PDZ interaction motif (TRL), and it binds to the first PDZ domain of EBP50 with high affinity (8–10). It has been proposed that the EBP50-CFTR association, together with other proteins that are sequestered in a regulatory complex through these physical interactions, may provide the basis for functional interactions observed between CFTR and other ion channels (11). Recently, Mohler et al. (12) have provided evidence that a Yes-kinase-associated protein interacts with the second PDZ domain of EBP50. Ac-
coordinately, CFTR may bring other regulators, such as this Src family kinase, into its vicinity though these interactions.

The functional impact of CFTR-EBP50 interactions is not fully understood, but the interaction of this PDZ domain protein with ezrin is of interest concerning its possible role in the regulation of CFTR by PKA. The PKA-dependent regulation of NHE3 is mediated by EBP50/NHERF (13), which is a known ezrin-binding protein. In addition, protein overlay methods have implicated an interaction between ezrin and the regulatory subunit of PKA (14). Accordingly, ezrin’s interaction with EBP50 may localize PKA in close proximity to CFTR. The purpose of this study was to examine this hypothesis using both protein interaction assays and functional measurements of CFTR activity. Our findings show that CFTR is part of a regulatory complex in human airway cells and that this complex contains ezrin and both the catalytic and regulatory subunits of PKA. Disruption of these interactions blocks CFTR- and PKA-specific substrate phosphorylation reactions. Ezrin was found at the apical membrane domain of both airway and intestinal secretory epithelia, and it bound RII in protein overlay and co-immunoprecipitation experiments. Finally, in patch clamp experiments, the functional activation of CFTR by PKA could be disrupted by conditions that interfere with ezrin binding of RII. These findings provide physical and functional evidence that PKA regulation of CFTR is AKAP-mediated, and they suggest that ezrin is a CFTR-associated AKAP in secretory epithelial cells.

EXPERIMENTAL PROCEDURES

Materials—H331 and H331P peptides (15, 16) were obtained from Genemed Synthesis (San Francisco, CA). Protein A/G-agarose beads and molecular weight markers were obtained from Life Technologies, Inc. PKA catalytic subunit, CAMP, and cAMP-agarose were purchased from Sigma. Renaissance Chemiluminescence Reagent Plus and [γ-32P]ATP (3000 Ci/mmol) were obtained from NEN Life Science Products. PKA inhibitor peptide (residues 5–24) (PKI) and the SignalTR-GT37m-CAMP-dependent protein kinase assay system were purchased from Promega (Madison, WI). Protease inhibitor tablets and restriction endonucleases were from Roche Molecular Biochemicals. Phosphatase inhibitors were from Alomone (Jerusalem, Israel). Other reagent grade chemicals were obtained from Sigma.

Antibodies—Monoclonal anti-PKA catalytic subunit antibody was obtained from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against RIIα regulatory subunit (RIIα) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-CFTR antibodies were from Genzyme (Framingham, MA). Monoclonal anti-GST and anti-ezrin antibodies were obtained from Sigma.

Cell Culture—Calu-3 cells were grown in Dulbecco’s modified Eagles’s medium/Ham’s F-12 medium containing 15% fetal bovine serum. Cells were incubated in a humidified atmosphere containing 5% CO2 at 37 °C. T84 cells were grown under similar conditions, except that the medium contained 10% fetal bovine serum. For confocal microscopy, Calu-3 or T84 cells were seeded onto Costar Transwell cell culture inserts, and the culture media were changed every 2 days. 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 was performed after 14–21 days in culture.

Cell Fractionation—Confluent cell monolayers were scraped into buffer (10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, and protease inhibitors) and homogenized using a Dounce type homogenizer. Post-nuclear supernatants were obtained by centrifugation (14,000 x g for 1 min), and from this supernatant, cytosolic and membrane fractions were obtained by centrifugation at 100,000 x g for 60 min. Cytosolic proteins were concentrated by precipitation with 10% trichloroacetic acid and then resuspended in lysis buffer (50 mM HEPS (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol). Membrane fractions were resuspended in lysis buffer directly.

GST and GST-Ezrin Fusion Proteins—Full-length ezrin cDNA (a gift from Dr. T. Hunter, Salk Institute) was amplified by polymerase chain reaction using the following primers: 5'-GAATTCCGGAAACCAAAT-CAAGTGC and 5'-GATATCTTAAAGGGGCTGGAACTCAGTGC, which resulted in the generation of an EcoRI site at the N terminus and an EcoRV site at the C terminus of ezrin (sites indicated by underlining). The polymerase chain reaction product was cloned into pCr2.1 (Invitrogen). Fidelity of the polymerase chain reaction product was confirmed by DNA sequencing. Ezrin cDNA was released with EcoRI and EcoRV and then subcloned into pEzrin vector at EcoRI and SmaI sites. Expression and purification of GST-ezrin fusion protein or GST protein in bacteria followed the manufacturer’s instructions (Amersham Pharmacia Biotech) at EcoRI and SmaI sites. Expression and purification of GST-ezrin fusion protein or GST protein in bacteria followed the manufacturer’s instructions (Amersham Pharmacia Biotech).

Co-immunoprecipitation—In attempts to detect endogenous protein kinase activity associated with CFTR, precleared Calu-3 cell lysates were mixed with 1 μg of anti-CFTR R domain antibody or control antibody (anti-GST) for 1.5 h at 4 °C in lysis buffer. Twenty μl of washed protein G-Sepharose beads were added to each immunoprecipitation and incubated for 1 h at 4 °C with gentle rotation. Immunocomplexes with protein G-Sepharose beads were precipitated by centrifugation at 12,000 x g for 10 s and washed three times with 1 ml of lysis buffer and once with phosphorylation buffer (50 mM Tris HCl (pH 7.5), 10 mM MgCl2, 0.1 mg/ml bovine serum albumin). Where indicated, 4 μM H331 was added to CFTR immunoprecipitates to disrupt AKAP-RII subunit interactions (3). The control peptide, H331P, inserts a proline to disrupt the helical structure of H331; it was used at the same concentration. Samples were immediately subjected to phosphorylation reactions. For other co-immunoprecipitation experiments, Calu-3 cell lysates (150 μg) were mixed with experimental or control antibodies at 4 °C for 1.5 h in lysis buffer with gentle rotation. Twenty μl of washed protein A- or protein G-Sepharose beads were then added to each immunoprecipitate and incubated for 1 h at 4 °C. Immunocomplexes were pelleted by centrifugation at 12,000 x g for 10 s and washed four times with 1 ml of lysis buffer. Pellets were resuspended in Laemmli sample buffer and resolved by SDS-PAGE.

Phosphorylation of CFTR and Synthetic Peptide by Endogenous Protein Kinase—Phosphorylation of immunoprecipitated CFTR was performed as described previously (17). Briefly, immunoprecipitated CFTR was phosphorylated by endogenous kinase or by adding 5 units of PKA catalytic subunit in the presence of 0.15 μM [γ-32P]ATP (3000 Ci/mmol). Phosphorylation reactions were performed in the presence or absence of 10 μM cAMP and incubated at 37 °C in phosphorylation buffer for 10 min followed by two washes with lysis buffer. Phosphorylation reagents were removed by 7.5% SDS-PAGE and processed for autoradiography or phosphor imaging (Bio-Rad). Phosphorylation of biotinylated kemptide (amino acid sequence LRRASLG) was performed according to the manufacturer’s instructions (Promega). [32P] Incorporation was quantified by liquid scintillation counting.

Expression and Purification of RII—Expression, purification, and radiolabeling of RIIα were performed as described previously (18). Briefly, a plasmid containing the mouse RIIα cDNA (a gift of Dr. J. D. Scott, Vollum Institute) was transformed into Escherichia coli BL21 (DE3) competent cells (Novagen). Expression of RIIα 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 with protease inhibitors. Resuspended bacteria were lysed by nitrogen cavitation and then centrifuged at 10,000 x g for 15 min. The supernatant was mixed with 10 μg of ammonium sulfate at 4 °C for 15 min. Precipitated proteins were separated from soluble material by centrifugation at 10,000 x g for 15 min and then resuspended in PBS containing protease inhibitors. The resuspended material was mixed with 5 μl of cAMP-agarose for 16 h at 4 °C. Non-specific binding was removed by washing with high salt buffer. Bound RIIα was eluted with a solution containing 25 mM cAMP. Purified RIIα was radiolabeled by incubation with [γ-32P]ATP and the catalytic subunit of PKA in phosphorylation buffer for 1 h at 30 °C. Separation of labeled RIIα from free ATP was achieved using desalting columns (Pierce).

RII Overlay—10 μg of GST-ezrin fusion protein and GST protein were resolved by 7.5% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in blocking buffer (5% nonfat milk, 10 mM Tris (pH 8.0), 150 mM NaCl) containing 1% bovine serum albumin for 3 h at room temperature and then incubated for 4 h with 100,000 cpm/ml of [32P]-labeled RIIα in blocking buffer containing 0.1% bovine serum albumin. Membranes were washed with labeled RIIα and 4 μM of H331 or H331P peptide. Membrane was washed three times in TBST (10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20), and the label was visualized by autoradiography.

Immunoblot Analysis—Samples 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 TBS (TBST lacking Tween 20). Membranes were incubated 1 h at room tempera-
ture with the appropriate primary antibodies. The membranes 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. The blots were washed five times for 5 min each with TBST, and reactive bands were visualized by Renaissance Chemiluminescence (NEF Life Science Products). Samples were exposed to x-ray film (Eastman Kodak Co.).

Whole-cell Patch Clamp Recordings—Calu-3 cells were seeded onto glass coverslips and used 1–3 days after seeding. Coverslips were placed in a chamber that was perfused at a rate of 7–15 ml/min with a solution of the following composition 120 mM NaCl, 25 mM NaHCO3, 0.4 mM KH2PO4, 1.6 mM K2HPO4, 1 mM MgCl2, 1.5 mM CaCl2, 5 mM glucose. This solution was gassed with 5% CO2, 95% O2 and heated to 37 °C. The perfusion chamber was mounted on the stage of an inverted microscope (Nikon Diaphot). Patch pipettes were pulled from borosilicate glass tubing (Warner Instrument Corp., Hartford, CT) and filled with a solution composed of 95 mM potassium glutonate, 30 mM KCl, 1.2 mM Na2HPO4, 4.8 mM NaH2PO4, 1 mM MgCl2, 5 mM glucose, 0.5 mM EGTA, 1 mM ATP, and 0.1 mM GTP. Average pipette resistance was 2–4 megohms. Pipettes were mounted to the headstage of an EPC7 patch clamp amplifier (List Medical Instruments, Darmstadt, Germany) and advanced by a mechanical micromanipulator (Narishige, Japan). After establishing the whole-cell configuration, membrane voltage was measured in the current clamp mode of the amplifier. Then the cells were voltage-clamped to −40 mV, and the whole-cell current was recorded. To precisely measure membrane conductance, Gm, impedance analysis was performed. For this purpose, four sine waves (203.45, 406.9, 813.8, and 1627.6 Hz) were superimposed onto the clamp voltage. The resulting currents were fed into a four channel lock-in amplifier (Quad synchron detector; Physiologisches Institut, Freiburg, Germany), and the ing currents were fed into a four channel lock-in amplifier (Quad synchron detector; Physiologisches Institut, Freiburg, Germany), and the imaginary (i) and imaginary (j) parts of the currents were sampled with a computer’s hard disc. On-line analysis was performed using the program Bioicap (Physiologisches Institut, Freiburg). The use of four frequencies allowed us to closely monitor changes in four parameters: pipette capacitance, access conductance, membrane conductance, and membrane capacitance. Here, we report on changes in Gm.

Confocal Microscopy—Filter-grown Calu-3 or T84 cells were fixed in 2% paraformaldehyde in PBS for 10 min followed by a permeabilization with a mixture of 2% paraformaldehyde and 0.1% Triton X-100 in PBS for 10 min. The cells were then washed three times in PBS containing 0.5% bovine serum albumin and 0.15% glycine at pH 7.4 (buffer A). This was followed by a 30-min incubation with purified goat serum at 25 °C and three additional washes with buffer A. Cells were incubated for 1 h with a primary antibody (monoclonal IgG2 against ezrin) followed by secondary antibodies (Alexa 488 fluorochrome used for labeling). Image stacks were exported to ImageSpace (Molecular Dynamics, Inc.) for subsequent reconstruction and processing. Final presentation of images uses a pseudocolor representation encompassing the black and white intensity range 0–255 as illustrated with the final images.

RESULTS

PKA Co-immunoprecipitates with CFTR—CFTR was immunoprecipitated from Calu-3 cell lysates with an antibody against the CFTR regulatory domain (CFTR-RD). The immunoprecipitate was incubated under phosphorylation conditions with [γ-32P]ATP, which included the addition of phosphatase inhibitors (see “Experimental Procedures”), separated on SDS-PAGE, and visualized by autoradiography. In the presence of CAMP, a major diffuse band (characteristic of CFTR) with a molecular weight of ~180 kDa was phosphorylated by a protein kinase that was co-immunoprecipitated with CFTR (Fig. 1, lane 3). We examined the effect of CAMP on the endogenous kinase activity by incubating the CFTR immunoprecipitate with [γ-32P]ATP and 10 μM cAMP. The addition of cAMP promoted the phosphorylation of CFTR relative to control experiments performed in its absence (Fig. 1, compare lanes 3 and 4).

The increase in γ-32P incorporation into CFTR with the addition of cAMP was ~50% as determined by densitometry. As is routine procedure for CFTR identification (19), this band was strongly phosphorylated by the addition of the catalytic subunit of PKA to the immunoprecipitate (Fig. 1, lane 1). In contrast, Calu-3 cell immunoprecipitate generated using an irrelevant antibody (anti-GST monoclonal) did not reproduce this phosphoprotein signal (Fig. 1, lane 2). To confirm that the band phosphorylated by the endogenous kinase was CFTR, we also probed the immunoprecipitate with an anti-CFTR C terminus antibody; this immunoblot identified a similar 180-kDa diffuse band (data not shown).

Next, we used a PKA-specific substrate peptide and PKI to determine whether the endogenous kinase associated with CFTR is PKA. Two experiments were performed. First, we tested whether the endogenous kinase associated with CFTR is able to phosphorylate the PKA-specific substrate peptide, kemptide. As shown in Fig. 2A, the endogenous kinase precipitated by anti-CFTR-RD phosphorylated kemptide. This phosphorylation was also stimulated by cAMP (Fig. 2B). The PKA activity associated with the CFTR immunoprecipitate was about 4-fold higher than that of the immunoprecipitate obtained with a control antibody (Fig. 2A). Second, we tested the effect of PKI on the ability of the endogenous kinase to phosphorylate kemptide. As shown in Fig. 2A, the phosphorylation of kemptide by the endogenous kinase associated with CFTR was blocked by PKI. PKI-insensitive phosphorylation (~10% of total phosphorylation) may reflect background activity of contaminating kinases. Taken together, these results strongly suggest that the kinase activity associated with CFTR is PKA.

PKA Regulatory and Catalytic Subunits Co-immunoprecipitate with CFTR—To determine whether PKA is physically associated with CFTR, we performed co-immunoprecipitations in which a CFTR-RD antibody was mixed with Calu-3 cell lysates and then precipitated with protein G-agarose beads. After SDS-PAGE and transfer to PVDF membrane, this immunoprecipitate was probed with anti-PKA antibodies. Fig. 3A shows that RIIs of PKA was present in the CFTR immunoprecipitate. In contrast, Calu-3 cell lysates immunoprecipitated with an irrelevant antibody did not yield a positive signal on RII immunoblots (Fig. 3A). A similar experiment was done to probe for the catalytic subunit of PKA (PKAc) in CFTR immunoprecipitates. As shown in Fig. 3B, the PKAc subunit was also
membranes. 32P incorporation was quantified by liquid scintillation.

The phosphorylation reactions were spotted onto streptavidin matrix for PKA.

Anti-CFTR-RD or control (anti-GST) antibodies were used to precipitate with CFTR and that CFTR is a substrate for this kinase. The control antibody was an anti-GST monoclonal IgG. Fifteen µg of Calu-3 cell lysates were loaded as the positive control. The antibodies used for immunoprecipitation (IP) and immunoblot (IB) in B were mouse monoclonals, resulting in detection of the heavy and light antibody chains. The results are typical of three experiments. In this and subsequent figures, molecular masses are indicated, in kDa.

Cell fractionation was employed to investigate the distribution of CFTR, RIIα, and PKAc expression in Calu-3 and T84 epithelia. In these experiments, cells were homogenized and subjected to centrifugation at 14,000 × g for 1 min. The resultant postnuclear supernatant was further separated at 100,000 × g for 60 min. A membrane fraction was prepared by suspending the pellet in lysis buffer, while a cytosolic fraction was made by concentration of the supernatant (See "Experimental Procedures"). As expected, CFTR was identified predominantly in the membrane fraction of Calu-3 and T84 cells (Fig. 4). The CFTR expression level is much higher in Calu-3 cells than that in T84 cells (Fig. 4, compare upper and lower panels), consistent with previous biochemical observations (17). RIIα and PKAc were mainly present in the membrane fraction of Calu-3 and T84 cells, although these proteins could be also detected in the cytosolic fraction.

Endogenous PKA Activity Is Inhibited by Ht31 Peptide—These results provide evidence that PKAII co-immunoprecipitates with CFTR and that CFTR is a substrate for this kinase. Since the association of PKAII with subcellular structures is generally mediated by AKAP(s) (20), we reasoned that an AKAP might link PKAII to CFTR. To test this hypothesis, we used Ht31, an amphipathic peptide that corresponds to the RII binding motif of a human thyroid AKAP (15), to determine whether a similar RII binding motif is responsible for linking PKAII to CFTR. CFTR immunoprecipitation from Calu-3 cell lysates was performed in the presence or absence of Ht31. Fig. 5 shows that preincubation of the immunoprecipitate with Ht31 decreased the phosphorylation level of CFTR that was due to endogenous PKA activity. The control peptide, Ht31P, did not alter CFTR phosphorylation. These data suggest that one or more AKAPs mediate the observed association of protein kinase activity with CFTR.

Ezrin Is an A-kinase Anchoring Protein—Previous studies (14, 21) have suggested that ezrin, a cytoskeleton-associated protein with a molecular mass of about 80 kDa, is an RII-binding protein. To determine whether ezrin can function as an AKAP in Calu-3 cells, we performed RIIα overlay assays in which 10 µg of GST-ezrin fusion protein and GST protein alone were resolved by SDS-PAGE and transferred to PVDF membrane, and probed with 32P-labeled RIIα. Fig. 6A shows that RIIα binds GST-ezrin but not GST. This binding could be blocked by the addition of 4 µM Ht31 in the overlay (Fig. 6A, right panel).

To function as an AKAP, ezrin should also interact with RIIα
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in vivo. To assess this, we performed co-immunoprecipitation experiments using ezrin and RIIα antibodies. In Calu-3 cell lysates, however, we were unable to detect the regulatory subunit of PKA in an ezrin immunoprecipitate (data not shown). This lack of consistency between the RIIα overlay of ezrin and the RIIα co-precipitation with ezrin prompted us to examine other cell fractions. Using the cytosolic fraction, we could detect RIIα in the ezrin immunoprecipitate from Calu-3 cells. Fig. 6B shows that ezrin co-precipitated the regulatory subunit of PKA, while the control antibody did not. Similar results were obtained using cytosolic fractions from T84 cells (data not shown).

Ezrin Co-immunoprecipitates with CFTR—Next, we attempted to identify an interaction between CFTR and ezrin in vivo. The membrane fraction from Calu-3 cells was mixed with an anti-CFTR-RD antibody, precipitated with protein G-agarose beads, and then probed with an anti-ezrin antibody. Fig. 7 shows that ezrin was present in the CFTR immunoprecipitate. When the membrane fraction from Calu-3 cells was precipitated with beads alone (without CFTR antibody), no ezrin signal could be detected. These data indicate that ezrin interacts with CFTR in vivo.

CFTR Cl− Conductance Is Inhibited by Ht31 in Calu-3 Cells—To gain functional evidence for a role of AKAP(s) in the PKA-dependent regulation of CFTR Cl− channels in Calu-3 cells, we performed whole-cell patch clamp recordings. Membrane current (I) and Gm were measured as described under Experimental Procedures. Calu-3 cells grown on glass coverslips were subjected to conventional whole-cell voltage clamp using pipette solutions containing either 10 μM Ht31 or 10 μM Ht31P. To activate CFTR Cl− conductance pathways, Calu-3 cells were stimulated with a mixture containing 2 μM forskolin and 100 μM 8-chlorophenylthio-cAMP. Cells dialyzed with the standard pipette solution (no-peptide control) showed basal and stimulated Gm values of 7.1 ± 0.8 and 54 ± 12 nS (n = 51), respectively. Representative traces of membrane current and conductance during cAMP stimulation are illustrated in Fig. 8A. The cAMP-stimulated conductance increase could be blocked by the addition of 1 mM diethylstilbestrol or 100 μM diphenyl-2-carboxylate to the bath, as shown. Cells dialyzed with Ht31 showed a marked reduction in their cAMP response (basal Gm = 4.7 ± 0.8 nS; stimulated Gm = 12 ± 2.1 nS; n = 11; see Fig. 8B). In contrast, cells dialyzed with Ht31P exhibited a basal conductance during cAMP stimulation, which was not different from the no-peptide control (basal Gm = 9.2 ± 1.5 nS; stimulated Gm = 80 ± 29 nS; n = 13; see Fig. 8B). Thus, introduction 10 μM of Ht31 into Calu-3 cells attenuated the cAMP-activated membrane conductance. The reduction due to Ht31 was ∼80%. These results indicate that Ht31 disruption of a PKA-AKAP complex interferes with cAMP-mediated activation of the CFTR Cl− conductance. The finding that basal Gm results were similar in peptide-dialyzed and control cells (Fig. 8B) suggests that the inclusion of these peptides in the pipette solution per se does not have an effect on the membrane conductance properties of nonstimulated Calu-3 cells.

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Fig. 5. Effect of Ht31 peptide on CFTR phosphorylation by endogenous protein kinase. CFTR immunoprecipitates from Calu-3 cell lysates were obtained with anti-CFTR-RD (1 μg) and preincubated with either 4 μM Ht31 or 4 μM Ht31P. The subsequent phosphorylation reaction was performed with [γ-32P]ATP in the presence 10 μM cAMP. Proteins were separated on 7.5% SDS-PAGE, and 32P phosphorylation products were detected by autoradiography. A monoclonal anti-GST protein or GST protein were loaded on SDS-PAGE, transferred to PVDF membrane. The membrane was probed with a polyclonal RIIα antibody, and the signal was visualized by ECL using horseradish peroxidase-conjugated secondary antibodies. Negative control experiment. The results are typical of three experiments.

Fig. 6. Interaction of ezrin with RIIα detected in overlay assays and by co-immunoprecipitation. A. 10 μg of GST-ezrin fusion protein or GST protein were loaded on SDS-PAGE, transferred to PVDF membrane, and probed with 32P-labeled RIIαs in the presence or absence of 4 μM Ht31 as indicated. Signals were visualized by autoradiography. B, ezrin immunoprecipitates from the cytosolic fraction (~1 mg of protein) of Calu-3 cells were resolved on 10% SDS-PAGE and transferred to PVDF membrane. The membrane was probed with a polyclonal RIIα antibody, and the signal was visualized by ECL using horseradish peroxidase-conjugated secondary antibodies. Negative control was performed using monoclonal anti-GST. The results are typical of five (A) and two (B) experiments.

Fig. 7. Co-immunoprecipitation of ezrin with CFTR. Immunoprecipitates were obtained with anti-CFTR-RD from Calu-3 cell lysates, resolved on 7.5% SDS-PAGE, and transferred to PVDF membrane. The membrane was probed with an anti-ezrin antibody (also monoclonal, 1:1000 dilution), and the signal was visualized by ECL using horseradish peroxidase-conjugated secondary antibody. The negative control omitted anti-CFTR-RD. Fifteen μg of Calu-3 cell lysate was loaded as the positive control. Antibodies for both immunoprecipitation (IP) and Western blot were mouse monoclonals, resulting in detection of heavy and light chains. The band with molecular mass of ~100 kDa may represent non dissociated heavy chains. The results are typical of three experiments.
logical assays suggest that ezrin should be found in proximity to CFTR, which is localized at the apical membrane domain of polarized secretory epithelia. Because Calu-3 and T84 epithelia express endogenous CFTR, we examined the localization of ezrin in these cells. Using laser-scanning confocal immunofluorescence microscopy, ezrin was identified at the apical membrane domain of polarized Calu-3 and T84 epithelia. Fig. 9, A–C and D–F, shows a series of en face (xy plane) images of Calu-3 and T84 epithelia, respectively, beginning at the apical membrane and progressing toward the basal aspect of the cells. Fig. 9, G and H, shows reconstructed vertical (xz plane) sections of polarized Calu-3 and T84 epithelia, respectively. The finding that ezrin was present in the apical membrane domain of polarized Calu-3 and T84 epithelia is consistent with the observed physical and functional interactions between these proteins. We were unable to investigate the co-localization of ezrin and CFTR by double labeling, because both the anti-ezrin and anti-CFTR antibodies are mouse monoclonals.

DISCUSSION

The results of our biochemical studies demonstrate that endogenous PKA activity is associated with CFTR. Both the catalytic and the regulatory subunits of PKA were present in CFTR immunoprecipitates. Moreover, the PKA holoenzyme was functionally active in CFTR immunoprecipitates, since CFTR could be phosphorylated \textit{in vitro} by the addition of cAMP and ATP. This phosphorylation of CFTR was inhibited by PKI. These findings are consistent with previous observations, which demonstrate that PKA is the major kinase involved in the regulation of CFTR channel gating (22–25). The physical association of PKA with CFTR could be disrupted by the RII binding peptide, Ht31, suggesting that the PKA-CFTR interaction is mediated by an AKAP. The lack of complete Ht31 inhibition of CFTR phosphorylation mediated by the endogenous kinase activity might be due to kinases other than PKA (26, 27) that could be present in CFTR immunoprecipitates or to dilution of kinase activity after disruption of its association with CFTR. Three lines of evidence implicate ezrin as an AKAP linking PKA to CFTR: (i) RII\(\alpha\) binds ezrin in RII overlay assays \textit{in vitro} (Fig. 6A), (ii) ezrin co-immunoprecipitates RII\(\alpha\) from Calu-3 cell lysates (Fig. 6B), and (iii) ezrin co-precipitates with CFTR from Calu-3 cells (Fig. 7). The results of these protein interaction studies implicate a PKA-AKAP-CFTR complex in CFTR activation, and they suggest that ezrin is an AKAP that can link PKA to CFTR. Nevertheless, we cannot exclude the
Our functional data also implicate a PKA-AKAP-CFTR interaction in the cAMP-mediated regulation of CFTR activity. Ht31 inhibited ~80% of the cAMP-stimulated CFTR Cl− conductance increase observed during whole-cell current measurements (Fig. 8B). This indicates that PKA-AKAP-mediated phosphorylation of CFTR is the preferred pathway for CFTR activation in Calu-3 cells. A number of studies have shown the functional consequences of PKA anchoring, either by disrupting RII-AKAP interactions with Ht31 or by expression of compartment-specific AKAPs that redistribute PKA to defined subcellular sites (28). Many of these studies have focused on PKA-modulated ion channels. For example, introduction of Ht31 into cultured hippocampal neurons caused a time-dependent inhibition of cAMP-activated α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) kainate channel currents (29). Disruption of the RII-AKAP complex by Ht31 also inhibited L-type Ca2+ channel potentiation by cAMP in cardiac and skeletal muscle cells (30), while overexpression of AKAP15/18, which co-localizes with the Ca2+ channels in skeletal myocytes, stimulated cAMP-dependent channel activity (31). Finally, a cell membrane-permeable Ht31 (S-Ht31) has been shown to arrest sperm motility (32), which is a cAMP/PKA-dependent process. All of these lines of evidence suggest that AKAPs are functionally important in the organization of PKA-dependent signaling events. Similar to our findings with CFTR, disrupting this interaction impairs cAMP/PKA-mediated signaling processes.

The critical role played by AKAPs in cAMP-mediated signal transduction processes may be the control of their specificity. It is anticipated that there are 2000 different protein kinase genes (33). It has also been shown that PKA has many potential phosphorylation targets within cells (31). Therefore, it is important to understand the basis for substrate specificity in response to cAMP/PKA-dependent agonist stimulation. Compartmentalization of protein kinases with their substrates, in part through subcellular kinase targeting by AKAPs, is a mechanism that is thought to promote specificity of intracellular phosphorylation events (34). For example, yotiao, an AKAP associated with the N-methyl-d-aspartate receptors, tethers PKA at postynaptic sites and enhances the cAMP-dependent potentiation of N-methyl-d-aspartate channel currents (28). Our results show that ezrin is localized at the apical membranes of Calu-3 and T84 epithelia (Fig. 9), where it is positioned appropriately to regulate CFTR Cl− currents. Together with the results of our biochemical data implicating a physical interaction of ezrin with CFTR and RII, these findings suggest that ezrin can serve as an AKAP that links PKA to CFTR.

Our results demonstrating ezrin association with RIIα are consistent with previous findings (14, 21). Although RII was present in an ezrin immunoprecipitate from Calu-3 cytosol, we were unable to co-immunoprecipitate RII with ezrin from the membrane fraction. This may due to competition between ezrin and other RII-binding proteins in Calu-3 cells that would occur during cell disruption. Our unpublished data from RII overlay assays performed using Calu-3 cell lysates indicate that these cells express at least five distinct RII binding proteins. Other investigators have suggested that cells generally express 10–15 AKAPs that mediate RII associations. Previous work has suggested that ezrin is a relatively low affinity AKAP, with an $IC_{50} \approx 30 \mu M$ for RIIα (14). Therefore, high affinity AKAPs present in cell lysates may compete effectively with ezrin for RII binding when cells are disrupted and detergent-solubilized. We could detect RIIα in ezrin immunoprecipitates prepared from lysates of HEK293 cells transiently expressing ezrin (data not shown). HEK293 cells express relatively few endogenous AKAPs (35). In CFTR immunoprecipitates, we identified ezrin as a component of the regulatory complex (Fig. 7), but we were unable to consistently detect RII binding to an 80-kDa protein in overlay assays performed using the CFTR immunoprecipitates. This could be explained by the reported low affinity of ezrin for RII (14). In the experiment for Fig. 6A, we loaded 10 μg of GST-ezrin fusion protein for the RII overlay assays, a protein level that the immunoprecipitates cannot achieve. This raises the question of whether ezrin binds a significant amount of RII in vivo. Burton et al. (36) have recently reported that RIα binds to AKAPs with a 500-fold lower affinity than RIα, but this low affinity is apparently sufficient for PKA anchoring in vivo because no functional defects have been detected in RIα knockout mice. More importantly, using immunogold electron microscopy, Bradbury and colleagues (4) found that RIIs is concentrated at the plasma membranes of T84 cells. These findings suggest that ezrin binds RII and may therefore serve as the AKAP for CFTR in secretory cells. Nevertheless, its association with RII is difficult to demonstrate in cell membrane lysates after cell disruption, possibly because RII binding to ezrin is effectively competed by other RII-binding proteins under these conditions.

The probable site of PKA linkage to CFTR has been inferred in recent findings from several groups (8–10). These investigations have identified EBP50/NHERF as a protein that can interact with the C terminus of CFTR via PDZ domain interactions. Moreover, EBP50/NHERF is a known ezrin-binding protein (37). Accordingly, the C-terminal residues of CFTR would interact with EBP50/NHERF through PDZ domain binding, and ezrin would associate with the C terminus of EBP50/NHERF to form a regulatory complex. An interaction of ezrin with RII would then bring PKA into close proximity to CFTR. Thus, EBP50/NHERF could serve as a bridge for associating ezrin with CFTR. Our recent findings suggest that a related protein, E3KARP, can also serve this function (38).

Ezrin was initially identified as a structural component of microvilli (39) and a substrate for the epidermal growth factor receptor protein-tyrosine kinase (40). Prior studies show that ezrin plays multifunctional roles in different cells (41), including determination of cell shape, cell-cell adhesion, and transmembrane signal transduction. Ezrin is found at the apical membrane domain of many epithelia (42, 43). It is present in several epithelial cell lines derived from bronchus, colon, and kidney (8) that express CFTR endogenously. The well documented interaction of ezrin with F-actin has been particularly interesting, since endocytic and exocytic membrane trafficking is maintained by actin filaments and microtubules. Previous studies show cAMP-dependent endocytosis and exocytosis in CFTR-expressing cells (44), and others indicate insertion of CFTR into plasma membrane during cAMP stimulation (45). Identification of CFTR association with ezrin raises the possibility that ezrin’s association with the cytoskeleton may be involved in the regulation of apical CFTR trafficking. Another very interesting aspect of ezrin function is the maintenance of cell polarity. A recent study concluded that deletion of the C-terminal three amino acids of CFTR led to inappropriate targeting of CFTR to the lateral membranes of polarized Madin-Darby canine kidney cells (46). A possible explanation for this finding could be that an interaction between CFTR and ezrin is needed for CFTR stability at the apical membrane domain or for the membrane trafficking events that lead to CFTR retention in a stable apical compartment. The absence of such interactions may result in steady-state mislocalization of CFTR. It is apparent from this work that a CFTR-AKAP-PKA association can provide the needed specificity for phosphorylation.
tion dependent regulation of CFTR channel gating. In addition, this regulatory complex, including the proteins tethered to CFTR by additional PDZ domain interactions (12), provides the potential to explain CFTR’s functional interactions with other cellular processes, including the activity of other ion channels.

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