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The Cystic Fibrosis Transmembrane Conductance Regulator Is Regulated by a Direct Interaction with the Protein Phosphatase 2A*

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated chloride channel expressed at the apical surface of epithelia. Although the regulation of CFTR by protein kinases is well documented, channel deactivation by phosphatases is not well understood. We find that the serine/threonine phosphatase PP2A can physically associate with the CFTR COOH terminus. PP2A is a heterotrimeric phosphatase composed of a catalytic subunit and two divergent regulatory subunits (A and B). The cellular localization and substrate specificity of PP2A is determined by the unique combination of A and B regulatory subunits, which can give rise to at least 75 different enzymes. By mass spectrometry, we identified the exact PP2A regulatory subunits associated with CFTR as Aα and B′ϵ and find that the B′ϵ subunit binds CFTR directly. PP2A subunits localize to the apical surface of airway epithelia and PP2A phosphatase activity co-purifies with CFTR in Calu-3 cells. In functional assays, inhibitors of PP2A block rundown of basal CFTR currents and increase channel activity in excised patches of airway epithelia and in intact mouse jejunum. Moreover, PP2A inhibition in well differentiated human bronchial epithelial cells results in a CFTR-dependent increase in the airway surface liquid. Our data demonstrate that PP2A is a relevant CFTR phosphatase in epithelial tissues. Our results may help reconcile differences in phosphatase-mediated channel regulation observed for different tissues and cells. Furthermore, PP2A may be a clinically relevant drug target for CF, which should be considered in future studies.

Cystic fibrosis (CF)[2] is an autosomal lethal disease characterized by abnormal ion transport in epithelial tissues. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an apical membrane chloride channel. The regulation of CFTR by the cAMP-dependent protein kinase (PKA) and other protein kinases has been extensively documented. PKA can phosphorylate the CFTR regulatory domain (R domain) on at least 11 serine residues (1–3). *In vivo*, PKA phosphorylation increases CFTR open probability and the number of channels in the plasma membrane (4, 5). Work from our laboratory and others has demonstrated that PKA and other regulatory proteins are compartmentalized in close proximity to CFTR. The cellular machinery capable of generating cAMP, including the adenosine receptor and membrane-bound adenylate cyclase are present with CFTR in apical membrane patches (6). A kinase-anchoring proteins (AKAPs) target PKA to protein complexes with CFTR (7–9), and the disruption of the PKA/AKAP interaction abolishes the ability of PKA to activate CFTR in response to physiologic stimuli (9). In addition, the phosphodiesterase PDE4D is also present with CFTR in patch preparations and forms a cAMP diffusion barrier at the apical plasma membrane (10). Other CFTR regulatory molecules including protein kinase C and the AMP-activated protein kinase are found in protein complexes associated directly with CFTR (11, 12).

Less is known about the ability of serine/threonine phosphatases to regulate CFTR activity or how they are compartmentalized with CFTR. Work from many laboratories suggests that multiple phosphatases, including PP2A, PP2B, PP2C, and alkaline phosphatase, are involved in the deactivation of CFTR (13–17). *In vitro*, PP2A and PP2C are most effective in dephosphorylating purified CFTR and recombinant R domain. Furthermore, exogenous PP2A and PP2C inactivate CFTR in excised membrane patches (13, 18). In human sweat ducts, cardiac myocytes, 3T3 fibroblasts, and Hi-5 insect cells, inhibitors of PP2A increase CFTR channel activity (14, 16, 18, 19). Likewise, PP2B inhibitors stimulate CFTR in NIH 3T3 fibroblasts (17). However, the contribution of PP2A and PP2B to CFTR deactivation may vary in different cell types (15, 20). In baby hamster kidney cells, PP2C co-immunoprecipitates with exogenously expressed CFTR (20). Additionally, PP2C overexpression in Fischer rat thyroid cells decreases CFTR chloride conductance (15). However, the ability of endogenous PP2C to regulate CFTR in native epithelial tissues is unclear, since no PP2C inhibitors have been identified. To date, no single phosphatase has been demonstrated to be both necessary and sufficient to completely down-regulate CFTR channel activity, suggesting that CFTR is dephosphorylated by multiple phosphatases. Here, we present evidence for a direct interaction between CFTR and the serine/threonine phosphatase PP2A. This interaction involves the COOH terminus of CFTR and the PP2A B′ϵ regulatory subunit. PP2A localizes to the apical cell surface, where it negatively regulates CFTR channel activity in Calu-3 cells and intact mouse jejunum. Furthermore, inhibition of PP2A increases the airway surface liquid in primary human bronchial epithelial (HBE) cells by a mechanism requiring CFTR channel activity. We conclude that PP2A is indeed a relevant CFTR phosphatase in epithelial tissues.

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[2] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PKA, cAMP-dependent protein kinase; AKAP, A kinase-anchoring protein; HBE, human bronchial epithelial; MS, mass spectrometry; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; PCL, periciliary liquid.

EXPERIMENTAL PROCEDURES

Cell Culture and Immunofluorescence—Calu-3 cells were maintained as described previously (21). Human airway epithelial cells were obtained from freshly excised bronchial specimens from normal subjects by protease digestion, seeded directly as primary cultures on 12-mm Transwell Col membranes (T-Col; Costar) in modified BEGM media under ALI conditions, and studied when fully differentiated (3–5 weeks). Cultures with transepithelial resistances (R_t) > 300 ohms cm^2 were studied. Immunofluorescence and confocal microscopy were performed as described previously using anti-PP2A subunit antibodies (22). Monoclonal anti-catalytic subunit, monoclonal anti-A regulatory subunit, and polyclonal anti-B′ subunit were acquired from Upstate Biotechnology, Inc. (Lake Placid, NY).

Affinity Purification of CFTR-interacting Proteins—Peptides corresponding to residues 1375–1401, 1411–1441, 1451–1476, and 1471–1480 of human CFTR synthesized with an N-terminal biotin tag (Genemed Synthesis) were resuspended in 50 mM Tris-Cl (pH 7.4). 20 nmol of the peptides were immobilized on 100 μl of streptavidin-agarose (Sigma) and incubated with Calu-3 cell lysates. Lysates were prepared by incubating twenty 100-mm dishes of Calu-3 cells in binding buffer (50 mM Tris-Cl, 150 mM NaCl, 0.2% CHAPS, and protease (Roche Applied Science) and phosphatase (Sigma) inhibitor mixtures) at 4 °C for 1 h. Following ultracentrifugation, the soluble fraction was precleared over empty streptavidin beads and incubated with CFTR peptides conjugated to streptavidin beads for 1 h. The bound fractions were washed extensively in binding buffer, eluted with 5% formic acid, and lyophilized. Prior to MS analysis, the protein samples were reduced, alkylated, and digested with proteomics grade trypsin (Sigma). The peptides were analyzed by liquid chromatography-MS/MS on a Q-Tof *micro* (Waters, Manchester, UK). All data were acquired using Masslynx 4.0 software and than processed using Proteinlynx module. The processed data were searched against updated NCBInr and Sprot data bases using the Mascot search engine. Mascot probability-based Mowse individual ion scores of >46 were accepted as indicating identity or extensive homology ($p < 0.05$). The MS/MS spectrum scores between 20 and 45 were examined individually, with the acceptance criteria being that the parent and fragment ion masses were within the calibrated tolerance limits and that the spectrum contained an extended series of consecutive y- or b- ions. Small scale experiments analyzed by Western blot were performed using lysates from two 100-mm dishes of Calu-3 cells and 2 nmol of CFTR peptide.

In Vitro Binding Assays—Binding assays were performed as described previously, with several exceptions (21, 23). Briefly, the cDNA sequence of the human PP2A B′ε subunit was amplified by PCR and cloned into the BamHI and XhoI sites of pET-28C (Novagen, Madison, WI). Recombinant B′ε subunit was produced in either rabbit reticulocyte lysates (Promega, Madison, WI) or BL21 *Escherichia coli* (Stratagene, La Jolla, CA). For reticulocyte lysates, the PP2A B′ε subunit was purified using Ni^{2+}-nitrilotriacetic acid beads (Qiagen) in binding buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100). To remove any reticulocyte proteins that co-purify with the B′ε subunit, the beads were washed three times in binding buffer plus 2 M urea followed by three washes in binding buffer. The B′ε subunit was eluted with binding buffer plus 10 mM EDTA. Bacterially, expression of B′ε subunit was performed as previously described (24). Purified PP2A core dimer consisting of the catalytic and A regulatory subunit was obtained from Promega (Madison, WI).

Purified proteins were then incubated with indicated CFTR peptides immobilized to streptavidin beads in binding buffer plus 0.2% bovine serum albumin for 1 h at 4 °C. Following extensive washing, the bound proteins were eluted, resolved by SDS-PAGE, and analyzed by Western blot or phosphorimaging analysis.

Co-immunoprecipitation and Phosphatase Assays—Calu-3 cells were scraped into a hypotonic lysis buffer (150 mM Tris-Cl, 10 mM NaCl, and protease inhibitors) and physically disrupted by Dounce homogenization on ice. The lysates were centrifuged at 500 × *g* for 10 min to remove nuclei and unbroken cells. The supernatant was subsequently centrifuged at 100,000 × *g* for 1 h to pellet cell membranes. Membranes were resuspended in binding buffer for 1 h on ice. CFTR was immunoprecipitated using CFTR 596 antibodies (gift of Dr. J. Riordan, University of North Carolina, Chapel Hill, NC) or isotype-matched control antibodies covalently conjugated to Protein G dynabeads. Bound proteins were washed extensively and analyzed by Western blot using specific antibodies for PP2A subunits or for phosphatase activity using a PP2A immunoprecipitation phosphatase assay kit (Upstate Biotechnology).

CFTR Currents in Outside-out Membrane Patches of Calu-3 Cells—The procedures were essentially as described previously (6). Briefly, CFTR Cl^- channel activity was recorded at a membrane potential of 30 mV with a 6–8-megaohm resistance for an open pipette. Both the pipette and the bath solutions were the same as described previously (6). CFTR channel activity was recorded digitally (PClamp software) for 300 s following patch excision. PP2A inhibitors or protein kinase A inhibitor was included in the pipette solution as indicated.

CFTR Currents in Mouse Jejunum—Details of this approach have been described previously (25, 26). Briefly, sections of the midportion of mouse jejunum were studied under short circuit current (I_{sc}) conditions during a 90-min recording. A constant voltage pulse (1–5 mV, 1-s duration) was applied to the tissue every minute (Physiologic Instruments, San Diego, CA). Potential difference and resistance were calculated by Ohm's law from the changes in I_{sc} in response to the voltage pulse. Tissues were treated with 100 μM endothall or vehicle.

Confocal Microscopy Measurement of Periciliary Liquid (PCL)—The technique has been described in detail (27). Briefly, phosphate-buffered saline (20 μl) containing 2 mg/ml Texas Red-dextran (10 kDa; Molecular Probes, Inc., Eugene, OR) and benzamil (10^{-4} M) was added to cultured bronchial epithelium, and excess was aspirated to bring PCL height to ~7 μm. The CFTR-specific inhibitor $CFTR_{inh172}$ was also included where noted at a concentration of 10^{-5} M. To measure the average height of the PCL, five predetermined points (one central and four 2 mm from the edge of the culture) were XZ-scanned as previously described (27). For all studies, perfluorocarbon was added mucosally to prevent evaporation of the PCL, and the culture was placed on the stage of the confocal microscope over a serosal reservoir (80 μl of TES-buffered Ringer). Okadaic acid was added to the apical surface as a dry powder in perfluorocarbon (5 μg of okadaic acid/25 ml of perfluorocarbon/cm^2 of culture). Perfluorocarbon has no effect on PCL height or ion transport, as previously described (27).

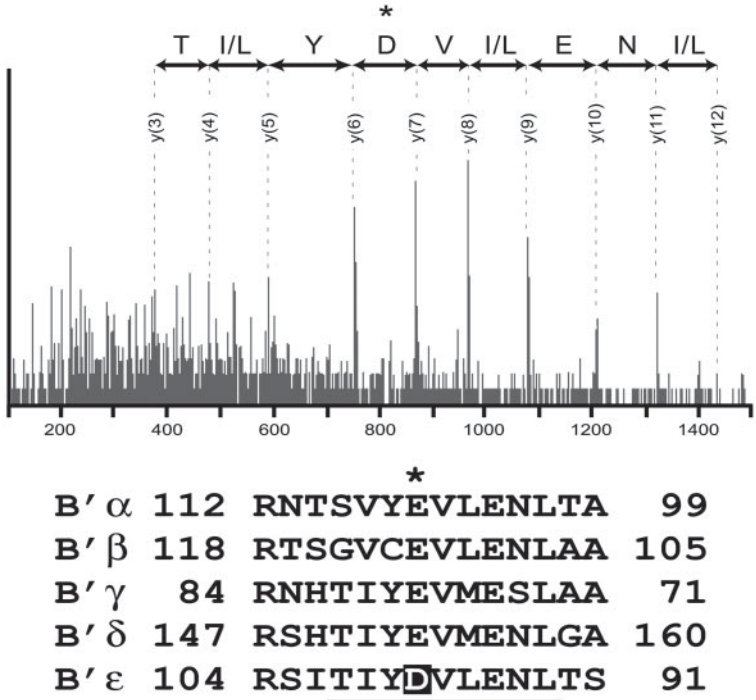
RESULTS

PP2A Physically Associates with the COOH Terminus of CFTR—The COOH terminus of CFTR mediates protein-protein interactions with PDZ proteins (7, 28–30), the adaptor protein AP-2 (31), and AMP kinase (12) to regulate cell surface stability, membrane trafficking, and channel activity. In the present study, we asked whether the highly conserved CFTR COOH terminus can interact with additional proteins that regulate channel function. We chose to focus on the last 25 amino acids of CFTR (encompassing residues 1451–1476), which precede, but do not include, the PDZ binding motif. Previous studies suggest that this region is important for CFTR trafficking and channel activity (32); however, no protein interactions have been reported. We immobilized bioti-

A

PP2A Subunit	Coverage	Mass	Sequence
Catalytic	3.6%	1339.66	YSFLQFDPAPR
Aa (PR65α)	7.1%	2193.16	IGPILDNSTLQSEVKPILEK
		1375.72	TDLVPAFQNLMK
		1108.53	LAGGDWFTSR
B'ε (PR61ε)	5.8%	1622.85	STLNELVDYITISR
		1522.77	FLESQEFQPSIAK

B



B′α	112	RNTSVYEVLENLTA	99
B′β	118	RTSGVCEVLENLAA	105
B′γ	84	RNHTIYEVMESLAA	71
B′δ	147	RSHTIYEVMENLGA	160
B′ε	104	RSITIYDVLENLTS	91

FIGURE 1. **MS analysis of PP2A subunits associated with the CFTR COOH terminus.** *A*, table of PP2A subunit peptides identified by MS/MS analysis of Calu-3 cell proteins associated with CFTR-(1451–1476). *B*, example of an MS/MS spectrum used to specifically identify the B′ϵ subunit. y ions and corresponding amino acids are labeled on the *spectrum*.

nylated CFTR-(1451–1476) peptides on streptavidin-agarose beads, incubated the peptides in cell lysates prepared from Calu-3 cells, and eluted the bound proteins with formic acid. Using nanoliquid chromatography MS/MS, we identified the serine/threonine protein phosphatase PP2A associated with CFTR-(1451–1476). PP2A dephosphorylates CFTR *in vitro* and decreases channel activity in multiple cell systems (14, 16, 18, 19). Recently, Vastiau *et al.* (33) also identified an interaction between the regulatory domain of CFTR and PP2A, suggesting that PP2A anchoring to CFTR may involve multiple contacts.

PP2A is a major cellular phosphatase that regulates many protein targets. PP2A functions as a heterotrimeric complex composed of a catalytic subunit and two regulatory subunits, A and B (34). The specificity of PP2A is determined by the unique combination of regulatory subunits associated with the catalytic subunit. The A regulatory subunit is encoded by one of two genes α and β, which are 86% identical. The A regulatory subunit is tightly associated with the catalytic subunit and functions as a scaffold to recruit the B regulatory subunit. The B regulatory subunit is highly divergent in comparison with the other PP2A subunits. The B regulatory subunit is divided into the B, B′, B″, and B‴ families, which are encoded by at least 14 different genes, some of which produce as many as five splice variants. The diversity of regulatory subunits gives rise to over 75 distinct PP2A enzymes. By mass spectrometry, we observe peptides from all three PP2A subunits (Fig. 1*A*). Importantly, the MS/MS spectra provide amino acid sequence information that allowed us to precisely identify the PP2A regulatory subunits associated with CFTR-(1451–1476) as Aα and B′ϵ (Fig. 1*B*).

By Western blot, we confirmed that the PP2A catalytic, A regulatory, and B′ regulatory subunits associate with CFTR-(1451–1476) but not other CFTR C-terminal peptides (Fig. 2*A*). Furthermore, these PP2A subunits also co-precipitate with endogenous CFTR from Calu-3 cell membranes (Fig. 2*B*), consistent with an interaction *in vivo*. The PP2A Bα subunit, which is structurally unrelated to the B′ family, does not co-purify with CFTR-(1451–1476) or co-immunoprecipitate with endogenous CFTR. In addition, we asked whether PP2A phosphatase activity purified with full-length CFTR. CFTR immunoprecipitates were assayed for PP2A using a colorimetric phosphatase assay. We find that a PP2A-like activity specifically co-precipitates with CFTR, but not an IgG control (Fig. 2*C*). Characteristic of PP2A, the phosphatase activ-

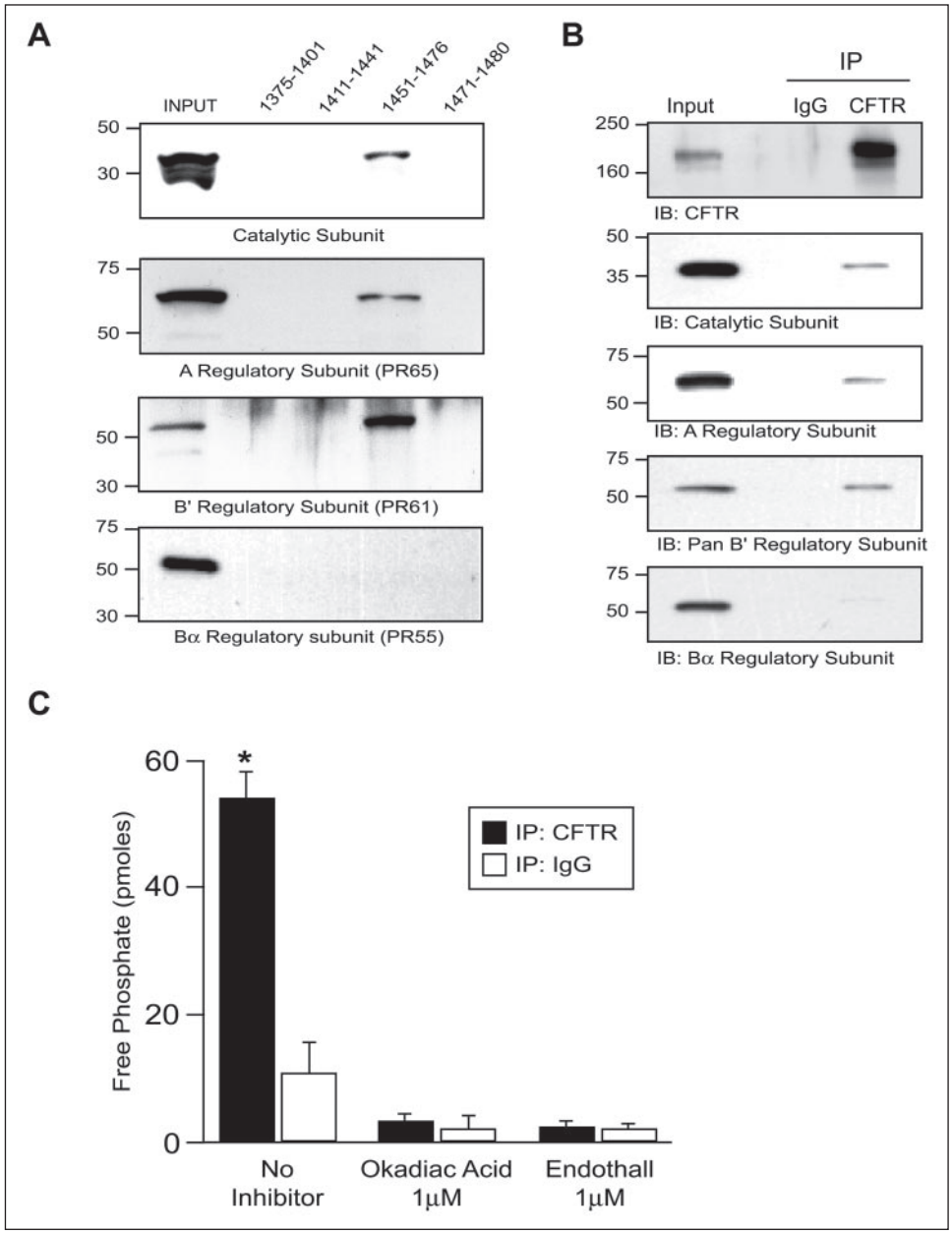


FIGURE 2. **The PP2A heterotrimer co-purifies with CFTR.** *A*, biotinylated CFTR peptides were immobilized on streptavidin beads and incubated in Calu-3 cell lysates. Bound proteins were resolved by SDS-PAGE and analyzed by Western blot using antibodies against the PP2A catalytic, A regulatory, B′ regulatory, and Bα regulatory subunits. *B* and *C*, Calu-3 cell lysates were incubated with either anti-CFTR monoclonal antibodies or isotype-matched mouse IgG covalently coupled to protein G dynabeads. The beads were extensively washed and analyzed by Western blot using specific PP2A subunit antibodies (*B*) or assayed for serine/threonine phosphatase activity using an Upstate Biotechnology phosphatase assay kit (*C*). To determine the contribution of PP2A to the total phosphatase activity, the PP2A inhibitors okadaic acid or endothall were added during the phosphatase assay ($n = 4$ for each condition). The data represent mean values ($n = 5$) ± S.E. *, $p < 0.0005$. *IP*, immunoprecipitation; IB, immunoblotting.

ity was inhibited by 1 μM okadaic acid or 1 μM endothall, potent inhibitors of PP2A (IC_{50} = 0.1 and 90 nM, respectively). Although okadaic acid and endothall can also inhibit PP1 (IC_{50} = 10 nM and 5 μM, respectively), previous studies have found no evidence to support the ability of PP1 to physically associate with CFTR, to dephosphorylate CFTR *in vitro*, or regulate channel activity (13, 18, 20). Although PP2C has been shown to be associated with CFTR in baby hamster kidney cells, the assay buffer does not contain Mg^{2+}, which is required for PP2C activity. Taken together, these data suggest that the phosphatase activity associated with CFTR is PP2A.

The PP2A B′ϵ Subunit Binds Directly to the COOH Terminus of CFTR—It is clear that multiple phosphatases can regulate CFTR channel activity. However, the degree to which PP2A contributes to this regulation varies in different functional assays and cell systems. Given the diversity of PP2A enzymes, we were intrigued by the possibility that the B regulatory subunit may, in part, account for these differences. Therefore, we asked whether the PP2A B′ϵ subunit could interact directly with CFTR. We find that PP2A B′ϵ subunit expressed and purified from reticulocyte lysates binds to CFTR-(1451–1476) but not other CFTR C-terminal peptides (Fig. 3*A*). NHERF-1, previously shown to interact with the extreme COOH terminus of CFTR, is enriched by the CFTR-(1471–1480) peptide, demonstrating the specificity of this assay (Fig. 3*A*). Although we do not find the catalytic or A subunit associated with the purified B′ϵ subunit from reticulocyte lysates (data not shown), we cannot rule out the possibility that these subunits are present at low levels and may be influencing binding to CFTR-(1451–1476). Using bacterially expressed, purified B′ϵ subunit, we observe direct and dose-dependent binding to CFTR-(1451–1476) (Fig. 3*B*). Furthermore, we also find that the core PP2A dimer, composed of the catalytic and A regulatory subunit, do not bind to CFTR-(1451–1476) unless the B′ϵ subunit is present (Fig. 3*C*). Whereas it is not clear whether the B′ϵ subunit alone is necessary and sufficient for targeting PP2A to CFTR, these data suggest that the B′ϵ subunit may play a critical role in the binding specificity.

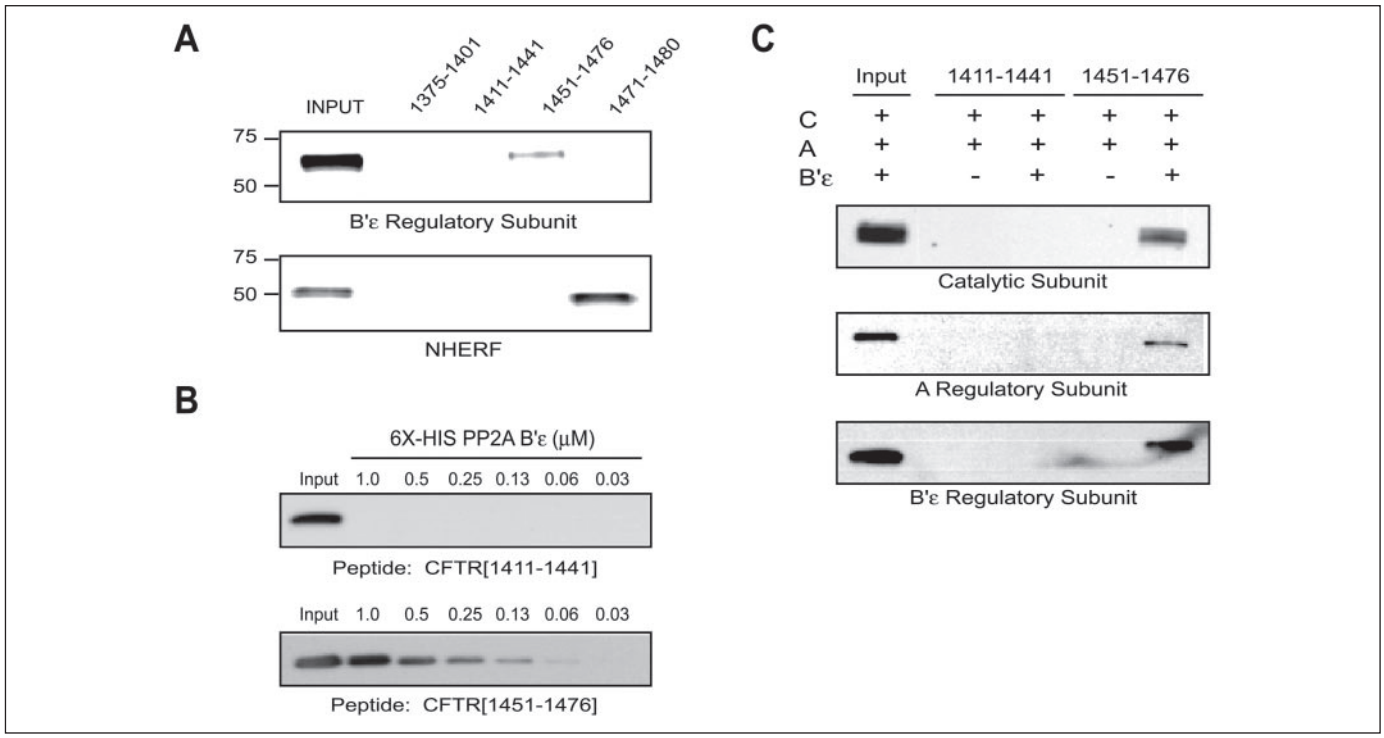


FIGURE 3. **The B′ϵ subunit directly interacts with the CFTR-(1451–1476).** *A*, the indicated CFTR COOH-terminal peptides were immobilized and incubated with radiolabeled B′ϵ subunit or NHERF-1. The bound fraction was separated by SDS-PAGE and analyzed by phosphorimaging analysis. *B*, 2 μM CFTR-(1411–1441) or CFTR-(1451–1476) was immbolized and incubated with bacterially expressed B′ϵ subunit at the indicated concentrations. Bound proteins were washed and analyzed by Western blot using a pan-B′ antibody. *C*, immobilized CFTR-(1411–1441) or CFTR-(1451–1476) was incubated with purified PP2A core dimer (0.05 units) with or without B′ϵ (1 μg). Samples were analyzed by Western blot using PP2A subunit antibodies.

PP2A Localizes to the Apical Cell Surface of Airway Epithelia—If PP2A is a physiologically relevant CFTR phosphatase, we reasoned that its subcellular localization should partially overlap with CFTR. Given the large number of PP2A substrates, we expect the ubiquitously expressed catalytic and tightly coupled A regulatory subunits to have a broad cellular distribution. However, specific B regulatory subunits, which target the PP2A enzyme to distinct subcellular compartments, should have a more restricted localization. Using a pan-B′ antibody, which recognizes all five gene products (α, β, γ, δ, and ϵ), we examined the localization of PP2A in the human airway. The B′ subunits exhibit a broad subcellular distribution in the human airway (Fig. 4). We find that B′ staining strongly localizes to the apical membrane of ciliated cells in superficial epithelia and gland ducts, consistent with the localization of CFTR in these tissues. Although we cannot unambiguously attribute the apical localization to the B′ϵ subunit, it is likely that this staining reflects the distribution of one or a combination of the cytosolic B′ subunits, B′α, B′β, B′δ, and B′ϵ (35). In addition, we observe staining of perinuclear membranes and nuclear speckles, which is consistent with the observation that the B′γ and B′δ localize to the nucleus (35, 36).

As we expected, the PP2A catalytic and A regulatory subunits are more broadly localized in airway epithelia (data not shown) but clearly localized to the apical cell surface of ciliated cells. The antibodies directed against the catalytic and A regulatory subunits detect both gene products for each subunit. Thus, these antibodies label every PP2A molecule that we expect to give a broad distribution in all cell types. Whereas the pan-B′ antibody detects five distinct gene products, this still only represents one of four families of the highly divergent PP2A B regulatory subunit. Accordingly, the staining pattern for the B′ subunit exhibits a more restricted distribution, including the apical cell surface. These localization data support our biochemical evidence for an *in vivo* interaction between CFTR and PP2A. Furthermore, the PP2A subunits are concentrated at the apical cell surface, suggesting a potential role in regulating the channel activity of CFTR at the plasma membrane.

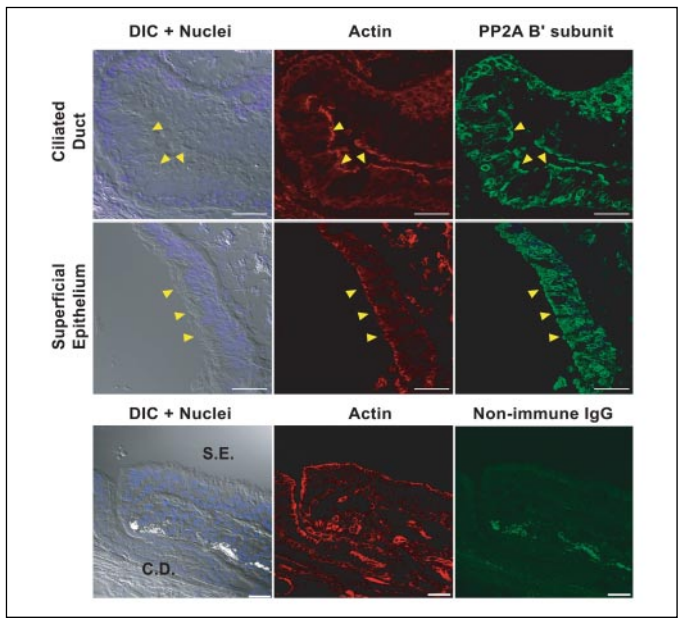


FIGURE 4. **The PP2A B′ regulatory subunit localizes to the apical compartment of ciliated airway cells.** Shown are representative images of the PP2A B′ regulatory subunit localization in gland ducts and superficial epithelium of the human airway. Confocal images were acquired in four channels with independent laser sources: *left*, differential interference contrast (*DIC*) and nuclear staining (4′,6-diamidino-2-phenylindole; *blue*); *center*, actin cytoskeleton (rhodamine phalloidin; *red*); *right*, PP2A B′ subunit family (*green*). The *arrowheads* indicate regions of apical staining. The *bottom panel* shows IgG controls on sections of superficial epithelium (*S.E.*) and ciliated ducts (*C.D.*). *Scale bars*, 40 μm.

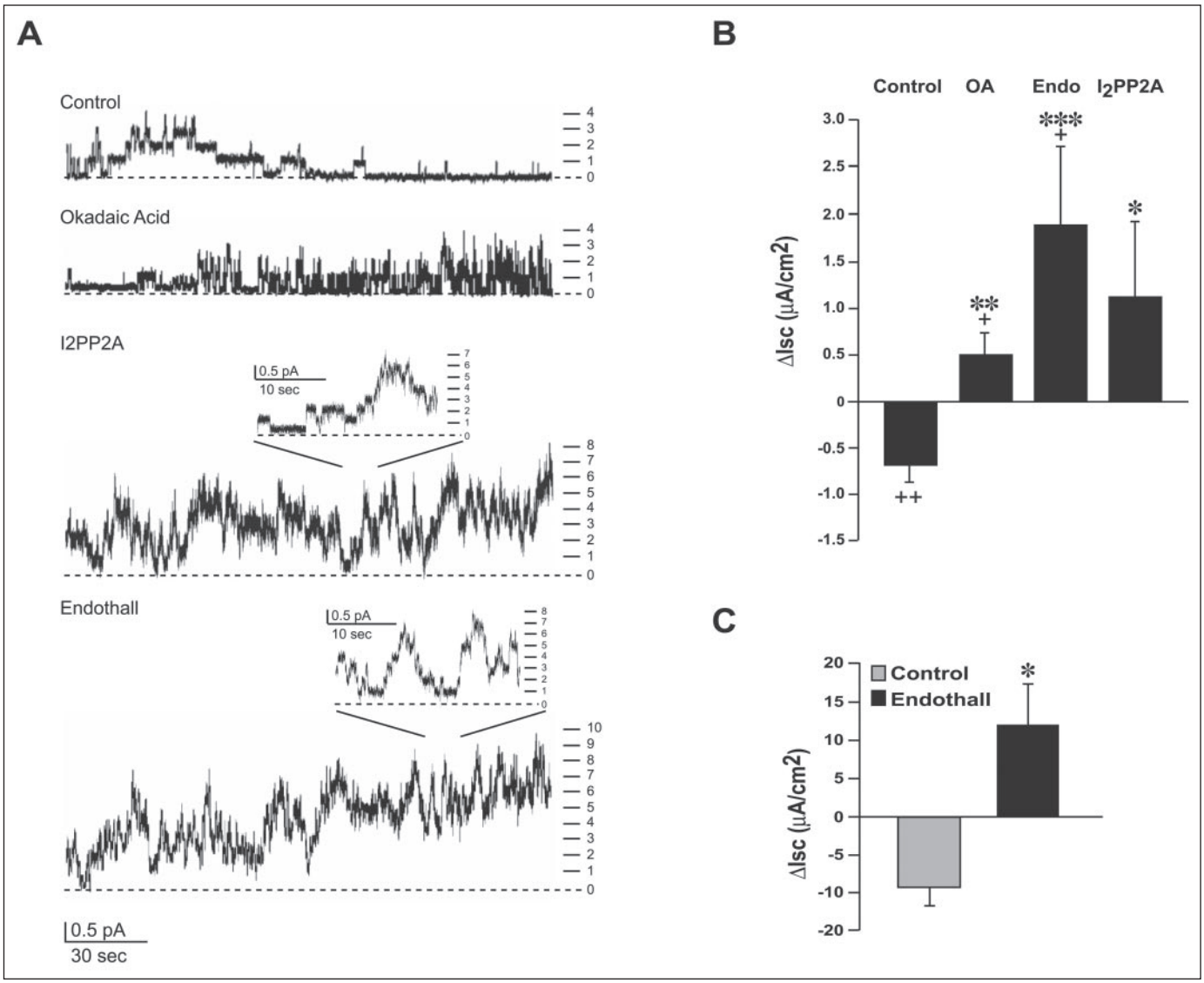


FIGURE 5. **PP2A inhibitors functionally regulate CFTR.** *A* and *B*, basal CFTR currents measured in apical patches were generated from Calu-3 cells in the outside-out configuration. Patches were recorded under control conditions as outlined under "Experimental Procedures." Where indicated, patches were prepared in the presence of okadaic acid (*OA*; 3 nM), endothall (1 μM), and I2PP2A (1.7 μM). *A*, representative traces for each condition are shown. For endothall and I2PP2A, traces were expanded on the *x* axis to clearly show discrete single channel events. *B*, summary of the patch clamp data in Calu-3 cells. The ΔI_{sc} values for each treatment are compared with $\Delta I_{sc} = 0$ (*t* test, $p < 0.05$; +, $p < 0.05$; ++, $p < 0.005$) and compared with ΔI_{sc} of control samples (*t* test, $p < 0.017$; Bonferroni's *post hoc* relative to control; *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$). *C*, CFTR currents were measured in intact mouse jejunum in Ussing chambers as described previously. Tissues were treated with endothall (100 μM) or vehicle for the duration of the recording. ΔI_{sc} is represented as the change in CFTR chloride currents at time 0 *versus* 90 min. The data represent mean values ($n = 4$) ± S.E. (*, $p < 0.005$).

PP2A Inhibition Prevents CFTR Rundown in Excised Patches—Our previous work and the results of others are consistent with the membrane localization of the cAMP signaling machinery that regulates CFTR. In excised membrane patches, endogenous PKA activity activates CFTR when exogenous cAMP is added in excess or when membrane-associated adenylate cyclase is activated by receptors (6, 9). In patch preparations, CFTR channel activity reflects the phosphorylation status of the channel, which is regulated by a balance between PKA and endogenous phosphatases (6, 18, 37). Consequently, CFTR channel gating is increased under conditions in which PKA activity is elevated, such as increased cAMP or the inhibition of phosphodiesterases (1, 10). We hypothesize that "rundown" of CFTR activity following excision results from a shift in the balance between endogenous PKA and phosphatase activities. Based on our biochemical analyses demonstrating a physical association between CFTR and PP2A, we tested this hypothesis by examining CFTR rundown in excised patches under control conditions and in the presence of PP2A inhibitors. We compared the average pipette current during the first 30 s of outside out recordings with the average pipette current measured from 270 to 300 s, as performed in previous studies (38). Under control conditions, the pipette solution contained ATP and GTP, conditions that allow CFTR gating to reflect the activity of endogenous PKA (6). Furthermore, current flow through the patch is mediated by Cl^- traversing CFTR, since no channel events are observed, and pipette current is near zero with protein kinase A inhibitor (100 nM) in the pipette (0.10 ± 0.10 at 0–30 s and 0.00 ± 0.00 at 270–300 s, $n = 5$). Under these conditions, CFTR activity declines variably over 5 min ($\Delta I = -0.66 \pm 0.21$, $n = 18$) (Fig. 5, *A* and *B*). However, the presence of the PP2A inhibitors okadaic acid or endothall in the pipette prevented channel rundown ($\Delta I = 0.51 \pm 0.24$ and $\Delta I =$ 01.81 ± 0.88, respectively, $n = 10$ for each treatment) (Fig. 5, *A* and *B*). Although endothall and okadaic acid inhibit PP2A most potently, they can also inhibit other phosphatases such as PP1, PP4, and PP5 (39, 40).

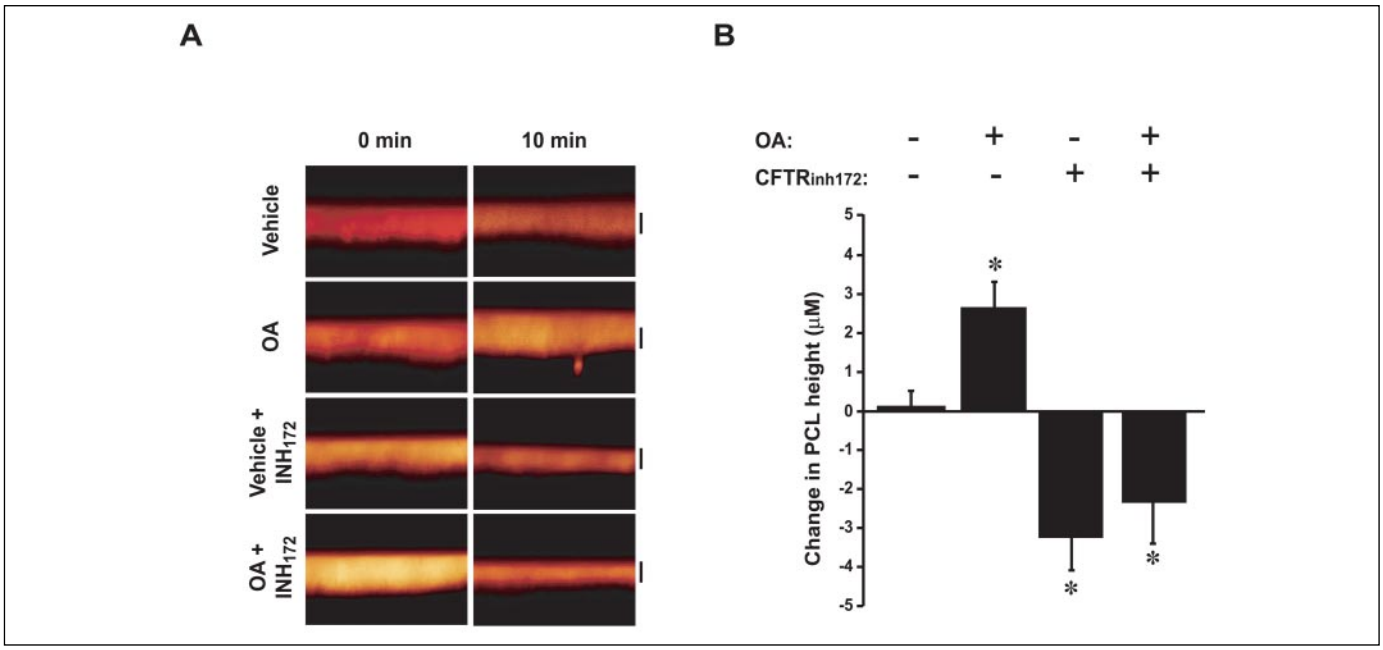


FIGURE 6. **PP2A inhibition increases PCL height in primary human airway cultures.** *A*, PCL height was assessed in primary cultures of human bronchial epithelia cells treated with vehicle (phosphate-buffered saline) or okadaic acid (*OA*; 5 μg/25 ml of perfluorocarbon/cm^2 of culture) in the presence or absence of CFTR inhibitor 172 (10^{-5} M) for 10 min. The PCL was labeled with Texas Red dextran, and the height was measured by confocal microscopy. Representative images taken at time 0 or 10 min for each condition are shown. *Scale bar*, 7 μm. *B*, summary of confocal data represented as percentage change in PCL height at time 0 *versus* 10 min of the indicated treatments. The data represent mean values ($n = 6$ for all conditions with the exception of $n = 7$ for okadaic acid) $\pm$ S.E. (Treatments were compared with controls by Mann-Whitney *U* test; *, $p < 0.02$.)

Therefore, we also tested the specific inhibitor I_2PP2A, which does not inhibit PP1, PP2B, or PP2C (41, 42). Similar to endothall and okadaic acid, I_2PP2A (1.7 μM) also prevented CFTR channel rundown ($\Delta I = 1.13 \pm 0.85$, $n = 8$) (Fig. 5, *A* and *B*).

PP2A Inhibitors Prevent CFTR Rundown in an Intact Epithelium—Consistent with our observation that CFTR and PP2A physically interact, the effect of PP2A inhibitors on CFTR in excised patches suggests that PP2A is present in the patches and is, therefore, compartmentalized in close proximity to CFTR. Furthermore, the inhibitor data demonstrate that endogenous PP2A can regulate CFTR channel activity. These results strongly suggest that PP2A functions as a relevant CFTR phosphatase in epithelial tissues. We also tested whether PP2A inhibitors altered basal CFTR activity in an intact epithelium. We measured basal short circuit chloride currents in freshly excised sections of mouse jejunum in Ussing chamber experiments. Previous studies demonstrated that CFTR constitutes the major chloride channel in these preparations, since jejunum from $CFTR^{-/-}$ mice do not exhibit significant chloride conductance (43). We find that over the course of control experiments, basal CFTR activity declined over time ($\Delta I_{sc} = -9.2 \pm 2.6$, $n = 4$) (Fig. 5*C*). In agreement with our patch clamp data, we find that the PP2A inhibitor endothall (100 μM) prevented rundown of CFTR activity, resulting in increased chloride conductance during the course of the 90-min recording ($\Delta I_{sc} = 12.4 \pm 4.9$, $n = 4$).

Inhibition of PP2A Increases the Airway Surface Liquid Height—Airway epithelial cells regulate the composition of the thin layer of PCL critical for innate host defense. In CF lung disease, PCL volume is depleted, reflecting an imbalance in solute transport across the epithelium (44–47). Ultimately, PCL depletion results in defective mucociliary clearance, which supports bacterial colonization and exposes individuals to life-threatening infections (27, 44–48). Previous studies have demonstrated that CFTR mediates the majority of PCL secretion and plays a fundamental role in maintaining basal PCL volume (36, 49). Therefore, we asked whether inhibition of PP2A would increase CFTR activity and thereby increase PCL levels in primary HBE cultures. The PCL was labeled with florescent dextran as previously described (27). PCL height was measured immediately before and 10 min after the indicated treatments. Vehicle treatment did not significantly change PCL height (Fig. 6). However, cultures treated with okadaic acid exhibited an ~30% increase in PCL height (ΔPCL $= +2.61 \pm 0.7$ μm, $n = 6$) (Fig. 6). Because PP2A inhibition is likely to influence the activities of many cellular proteins, we asked whether CFTR was required for the increase in PCL. Consistent with previous data demonstrating the importance of CFTR in PCL secretion, we find that the CFTR inhibitor, $CFTR_{inh172}$, causes PCL height to decrease by ~30% (ΔPCL $= -3.21 \pm 0.9$ μm) (50). Furthermore, in the presence of $CFTR_{inh172}$, okadaic acid was similar to the $CFTR_{inh172}$ alone (ΔPCL $= -2.3 \pm 1.1$ μm). Thus, the inhibition of PP2A with okadaic acid increases PCL height by a mechanism that requires CFTR. These results are consistent with our biochemical and electrophysiological studies demonstrating that CFTR is physically and functionally associated with PP2A in epithelial tissues.

DISCUSSION

The compartmentalization of signaling machinery with downstream effectors ensures the specificity and fidelity of signal transduction in response to extracellular stimuli. Previously, we demonstrated that CFTR is localized in close proximity with regulatory proteins *in vivo*, including the adenosine receptor, adenylate cyclases, PKA, and phosphodiesterases (6, 9, 10). In addition, many signaling proteins that regulate CFTR activity, including PKA (7–9), protein kinase C (11), PP2C (20), and AMP kinase (12), have been shown to be organized in multiprotein complexes directly associated with CFTR. The data in the present study add to this model by demonstrating that the phosphatase PP2A is both physically and functionally linked to CFTR.

The regulation of CFTR by phosphatases has been extensively studied. Whereas it is clear from the work of many laboratories that multiple phosphatases can affect CFTR channel activity, the specificity of this regulation is poorly understood. We identified PP2A in a biochemical screen for proteins that associate with residues 1451–1476 of the CFTR

COOH terminus. The power of our approach is illustrated by the fact that we unambiguously identified the exact PP2A regulatory subunits associated with CFTR by mass spectrometry as Aα and B$'\epsilon$. To date, at least 21 distinct PP2A regulatory subunits have been identified (2 A regulatory and 19 B regulatory subunits) (for a review, see Ref. 34). Importantly, it is the unique combination of regulatory subunits that governs the enzymatic activity, substrate specificity, and localization of PP2A enzymes (35, 51). Thus, the identification of the exact subunits associated with CFTR provides insight into the specificity of CFTR channel regulation by phosphatases.

Using purified proteins, we find that the divergent B$'\epsilon$ subunit interacts directly with CFTR-(1451–1476) and is required for the binding of the catalytic and A regulatory subunits. These data suggest that the B$'\epsilon$ subunit may target the PP2A enzyme to CFTR. It is intriguing to speculate that the expression of the B$'\epsilon$ subunit may account for the differences in the ability of PP2A to regulate CFTR channel activity in different cell types and tissues. Many of the CFTR phosphatase studies have utilized heterologous expression systems such as Chinese hamster ovary cells (13), baby hamster kidney cells (20), and Hi-5 insect cells (19), which may or may not express relevant CFTR phosphatases and subunits (13, 19, 20). We are unable to ask whether the PP2A B$'\epsilon$ subunit is expressed in these cell types by reverse transcription-PCR, since there is insufficient sequence information available at this time. However, the expression of specific PP2A subunits in these cells should be an important consideration for future experiments. Recently, Vastiau *et al.* (33) reported an interaction between CFTR and PP2A, which involves the direct binding of the ubiquitously expressed PP2A Aα regulatory subunit to the CFTR regulatory domain. To demonstrate the specificity of the CFTR/Aα interaction, Vastiau *et al.* (33) showed that the overexpressed heat repeats 1–10 cause a decrease in CFTR rundown, presumably due to the disruption of the Aα/CFTR interaction. However, the PP2A B subunits also bind to heat repeats 1–10 of the A subunit and would have been dislodged from the phosphatase complex (52, 53). Thus, in these assays, the observed decrease in CFTR rundown could be attributed to the disruption of the interaction between CFTR and both the PP2A A and B regulatory subunits. Nonetheless, the anchoring of PP2A to the CFTR chloride channel may involve multiple sites of interaction mediated by the PP2A Aα and B$'\epsilon$ subunits.

Previous studies have demonstrated a clear role for PP2A in the deactivation or rundown of CFTR in native sweat ducts, cardiac myocytes, baby hamster kidney cells, 3T3 fibroblasts, and Hi-5 cells (14, 16, 18, 19). However, the importance of PP2A in CFTR regulation in epithelial tissues seems variable. Thus, we chose to study the physical and functional association of CFTR and PP2A in airway epithelia. Indeed, we found that CFTR and PP2A interacted in Calu-3 cells as the PP2A catalytic, A regulatory, and B′ regulatory subunits specifically co-precipitate with endogenous CFTR (Fig. 2*B*). Additionally, we examined the localization of PP2A subunits in the human airway. If, in fact, PP2A regulates CFTR channel activity in epithelial tissues, we would expect the phosphatase to be present at the apical cell surface, consistent with the localization of CFTR. The ubiquitously expressed PP2A catalytic subunit and A regulatory subunit had a diffuse staining pattern, which clearly included the apical membrane of the superficial epithelium (data not shown). Alternatively, staining with a pan-B′ regulatory subunit antibody showed a more restricted distribution, including strong staining at the apical plasma membrane (Fig. 4). Thus, the PP2A subunits associated with CFTR are present at the apical cell surface where CFTR functions. Since the available B′ antibodies detect all family members, we cannot rule out the possibility that other B′ subunits other than B$'\epsilon$ may also associate with CFTR or localize to the apical surface of airway epithelia. Dozier *et al.* (54) identified an interaction between Chk2 and the PP2A B$'\gamma$ subunit and found that the other highly similar B′ family members, B$'\alpha$, B$'\beta$, B$'\delta$, and B$'\epsilon$, were also able to bind to Chk2 *in vitro*. Consequently, other PP2A B′ regulatory subunits may interact with CFTR. However, we did not find an association between CFTR and a structurally unrelated B regulatory family member, Bα (PR55a) by pull-down (Fig. 2*A*) or immunoprecipitation (Fig. 2*B*). Thus, the PP2A molecules associated with CFTR at least appear to be specific for the B′ family.

We found that CFTR and PP2A are functionally associated in airway and intestinal epithelial cells. In the presence of PP2A inhibitors, CFTR rundown was blocked in outside-out membrane patches from Calu-3 cells and intact mouse jejunum (Fig. 5). Consistent with our biochemical observations, the presence of PP2A in the membrane patches containing CFTR strongly suggests that these proteins are closely compartmentalized. Furthermore, our data suggest that PP2A is a relevant CFTR phosphatase in epithelial tissues, in agreement with Vastiau *et al.* (33), who found that the addition of okadaic acid in Caco-2 epithelial cells delayed channel rundown following forskolin treatment. In T84 and airway epithelial cells, PP2A inhibitors did not block the rundown of CFTR following maximal activation by forskolin (15, 20). A major difference between our data and data of other studies is that we chose to measure the effect of PP2A inhibitors on basal CFTR currents as opposed to CFTR currents following maximal activation with forskolin or PKA. It is possible that endogenous PP2A plays a role in regulating basal CFTR currents but that other phosphatases contribute to the deactivation of hyperphosphorylated CFTR. Future studies are needed to address the relative contribution of different phosphatases to the regulation of CFTR channel activity in epithelial tissues.

In the airways, the mucus layer provides a defensive barrier that traps and clears bacteria and noxious particles from the airways (44–47). The underlying PCL provides a low viscosity fluid that facilitates ciliary beating and mucus clearance (12, 55, 56). Based on inhibitor studies, CFTR has been demonstrated to play a central role in the maintenance of basal PCL height (36, 49). Furthermore, the loss of CFTR function results in decreased PCL height and increased viscosity. If, in fact, PP2A is a relevant CFTR phosphatase in airway epithelia, we reasoned that the inhibition of PP2A should increase CFTR chloride conductance and ultimately increase PCL height in primary HBE cultures. We find that the inhibition of PP2A by the addition of okadaic acid to primary HBE cultures produces a 30% increase in PCL height, consistent with an increase in CFTR activity (Fig. 6). Additionally, when CFTR channel activity is blocked by $CFTR_{inh172}$, okadaic acid does not have a significant effect of the PCL. Thus, okadaic acid treatment increases PCL height through a CFTR-dependent mechanism, consistent with our hypothesis that CFTR is compartmentalized with and regulated by PP2A in epithelial tissues.

In addition to regulating channel activity by dephosphorylating CFTR, PP2A may also be important for mediating other signaling and trafficking events for CFTR. For example, AMP-activated protein kinase, which directly binds to CFTR and negatively regulates channel activity, can also directly interact with PP2A via the A regulatory subunit (12, 56). Moreover, the direct interaction between CFTR and AMP-activated protein kinase has been mapped to CFTR residues 1420–1457, adjacent to the residues where we observe PP2A binding (residues 1451–1476) (12). It is interesting to speculate that PP2A and AMP-activated protein kinase may function to stabilize each other's interactions with CFTR or may compete for binding to the CFTR COOH terminus.

In summary, we establish that CFTR is physically and functionally associated with PP2A. Our data demonstrate that PP2A directly inter-

acts with CFTR via the B′ϵ regulatory subunit. Furthermore, we provide the first evidence that PP2A regulates CFTR in airway epithelia, the major tissue affected in CF. Finally, we demonstrate that the inhibition of PP2A in well differentiated HBE cultures increases PCL height. Because decreased PCL underlies the basic defect in airway clearance observed in CF patients, our data suggest that PP2A may be a useful therapeutic target for CF.

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