Protein Kinase CK2, Cystic Fibrosis Transmembrane Conductance Regulator, and the ΔF508 Mutation

F508 DELETION DISRUPTS A KINASE-BINDING SITE***

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Deletion of phenylalanine 508 (ΔF508) from the first nucleotide-binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR) is the most common mutation in cystic fibrosis. The F508 region lies within a surface-exposed loop that has not been assigned any interaction with associated proteins. Here we demonstrate that the pleiotropic protein kinase CK2 that controls protein trafficking, cell proliferation, and development binds wild-type CFTR near F508 and phosphorylates NBD1 at Ser-511 in vivo and that mutation of Ser-511 disrupts CFTR channel gating. Importantly, the interaction of CK2 with NBD1 is selectively abrogated by the ΔF508 mutation without disrupting four established CFTR-associated kinases and two phosphatases. Loss of CK2 association is functionally corroborated by the insensitivity of ΔF508-CFTR to CK2 inhibition, the absence of CK2 activity in ΔF508 CFTR-expressing cell membranes, and inhibition of CFTR channel activity by a peptide that mimics the F508 region of CFTR (but not the equivalent ΔF508 peptide). Disruption of this CK2-CFTR association is the first described ΔF508-dependent protein-protein interaction that provides a new molecular paradigm in the most frequent form of cystic fibrosis.

Cystic fibrosis (CF) is a common autosomal recessive multisystem disease resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). The molecular mechanisms that control CFTR function are complex and incompletely understood. CFTR belongs to the ATP-binding cassette family of transmembrane pumps involved in ATP-driven substrate transport. Uniquely among ATP-binding cassette proteins, CFTR is an ion channel, but from the transport characteristics of other chloride channels it remains unclear why CFTR requires two ATP-binding domains if passive chloride transport is its sole function. Deletion of F508 (ΔF508) from the first nucleotide-binding domain (NBD1) of CFTR is by far the commonest pathogenic mutation (80–90% of alleles) and induces a multisystem disease. The resultant clinical features are difficult to reconcile with defects in ion transport alone (4–7) because ΔF508-CFTR perturbs inflammation, cell metabolism and multiple ion-channel physiology. ΔF508 attenuates CFTR biosynthesis (8), cell surface expression (9), and channel gating (10), but it remains unknown how loss of F508 leads to defective gating because the F508 residue lies remote from both the channel pore and the site of ATP binding on NBD1 (11–13). Neither is it established how ΔF508 CFTR alters the function of unrelated proteins, including other epithelial ion channels (14). CFTR is part of a macromolecular complex in the apical membrane of epithelia (2) comprising (among others) a number of protein kinases (see below), syntaxins, ezrin-binding phosphoprotein 50 (15), and CAP70 (16). Provided ATP is available for NBD binding, CFTR channel function is activated by protein kinases such as PKA and PKC (17) but is inhibited by AMP-activated kinase (AMPK) (18, 19). CFTR also binds nucleoside diphosphate kinase (NDPK), which regulates AMPK without the need for AMP (20–22). We observed that the crystal structure for the NBD1 domain shows that F508 is located on the surface of NBD1 where it is accessible for protein-protein interactions (12, 13, 23). However, no such molecular interactions have yet been assigned to the F508 residue or its adjacent region in wild-type CFTR, and this report investigates whether regulatory proteins interact with this region in a ΔF508-dependent manner. We hypothesized that an explanation for the multisystem nature of CF may reside in differences between proteins bound to wild-type and ΔF508 CFTR.
The amino acid sequence of CFTR adjacent to F508 (boldface) contains a consensus sequence (KENIIFGVSYDEYR) for phosphorylation by protein kinase CK2 (formerly known as casein kinase II) (24), with a potential target serine located at serine 511 (italic underline). This protein kinase has many unusual features and a diverse range of targets making it an attractive candidate for study in CF cells (25–27). For example, CK2 inhibits NDPK, and NDPK not only binds CFTR but also controls AMPK (20–22).

CK2 is a heterotetramer containing two 47-kDa α subunits (catalytic) and two 26-kDa β subunits (regulatory) that targets over 300 proteins linking its promiscuous activity to essential cellular functions (27). CK2 can also use either ATP or GTP as the phosphate donor for kinase activity toward multiple substrates thus adding to the complexity (27). Unlike most protein kinases, CK2 is often described as constitutively active, but CK2 function may nevertheless be regulated, first by restricting its subcellular localization and second by modification of the regulatory interactions between the α and β subunits. Specific inhibitors are correspondingly either directed toward the nucleotide-binding α subunit (exploiting its unique structure) or to the site of polyamine binding on the acidic groove in the regulatory β subunit. We investigated whether CK2 interacts functionally with the F508 region of CFTR and whether deletion of F508 disrupts binding of this signaling molecule using immunofluorescence, inhibitor studies, electrophysiology, and site-directed mutagenesis of Ser-511 in CFTR.

EXPERIMENTAL PROCEDURES

Cell Culture—16HBE14o− and CFBE41o− cells were cultured to confluence in medium M199 containing 10% fetal calf serum, 2 mM l-glutamine, 5% antibiotics, and 5% Fungizone. Calu-3, Hep-G2, TB4, and BHK cells were cultured in Dulbecco’s modified Eagle’s medium with the same supplements.

Ussing Chambers—After mounting Calu-3 epithelia (Rt > 200 Ω; where Rt is transepithelial resistance) in Ussing chambers, basolateral membranes were permeabilized with amphotericin B (10 μM) and an outwardly directed Cl− gradient imposed as detailed previously (28). Cl− current was stimulated with 10 μM forskolin, and 4,6,7-tetramethylbenzotriazole (TBB) or diphenylamine-2-carboxylate (DPC) was added as indicated.

Single Channel Patch Clamp Studies—CFTR Cl− channels were recorded in either cell-attached or excised inside-out membrane patches. For cell-attached recordings, the pipette solution contained the following: 140 mM N-methyl-d-glucamine (NMDG), 3 mM MgCl₂, 10 mM TES, and 1 mM cesium EGTA, pH 7.3, with HCl ([Cl−], 147 mM). The bath solution contained the following: 137 mM NaCl, 4 mM KCl, 3 mM MgCl₂, and 10 mM TES, pH 7.3, with NaOH ([Cl−], 147 mM) and was maintained at 37 °C; pipette potential was 50 mV. For excised recordings, the pipette (extracellular) solution contained the following: 140 mM NMDG, 140 mM aspartic acid, 5 mM CaCl₂, 2 mM MgSO₄, and 10 mM TES, pH 7.3, with Tris ([Cl−], 10 mM). The bath (intracellular) solution contained the following: 140 mM NMDG, 3 mM MgCl₂, 1 mM cesium EGTA, and 10 mM TES, pH 7, with HCl ([Cl−], 147 mM; [Ca²⁺]ᵢ = 10⁻⁸ M), and was maintained at 37 °C; voltage was −50 mV. In cell-attached recordings, CFTR Cl− channels were activated with forskolin (20 μM) and in excised inside-out membrane patches by PKA (75 nM) and ATP (1 mM). CFTR Cl− channels were filtered at 500 Hz, digitized at 5 kHz, and digitally filtered at 100 Hz. To measure i, Gaussian curves were fit to current amplitude histograms. To measure Pᵢ, we created lists of open and closed times and calculated Pᵢ as described (29). The number of channels in a membrane patch was determined from the maximum number of simultaneous channel openings observed during an experiment (29).

Xenopus Oocyte Studies—Oocytes were isolated and microinjected as described (30). In brief, cRNAs for CFTR, cDNAs encoding human wt-CFTR or ΔF508-CFTR were linearized in pbLuescript or pTLN with NotI and in vitro transcribed using T7, T3, or SP6 promoter and polymerase (Promega). After isolation from adult Xenopus laevis frogs (Horst Kähler, Hamburg, Germany), oocytes were dispersed and defolliculated with collagenase (45 min, type A; Roche Applied Science). Subsequently, oocytes were rinsed and stored at 18 °C in ND96 buffer as follows: 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, sodium pyruvate 2.5, pH 7.55, supplemented with theophylline (0.5 mM) and gentamicin (5 mg/liter).

Two-electrode Voltage-clamp—Oocytes were injected with cRNA (1–10 ng) in 47 nl of double-distilled water (Nanoliter Injector WPI, Germany). Water-injected oocytes served as controls. 2–4 days after injection, oocytes were impaled with two electrodes (Harvard Apparatus), which had resistances of <1 megohms when filled with 2.7 mol/liter KCl. Using two bath electrodes and a virtual ground head stage, the voltage drop across Rᵢ was effectively zero. Membrane currents were measured by voltage-clamping the oocytes (Warner oocyte clamp amplifier OC725C) in intervals from −90 to +30 mV, in steps of 10 mV, each at 1 s. Conductances were calculated using Ohm’s law. Oocytes were perfused continuously with physiological solutions at a rate of 5–10 ml/min. All experiments were conducted at room temperature (22 °C).

For the poly(E:Y) peptide, 43 nl of poly(E:Y) peptide (Sigma) was injected into CFTR-expressing oocytes reaching a final concentration of 10–20 μM. Currents were assessed 3–24 h after injection.

For the KENIIF/KENII peptide, peptides were injected into CFTR-expressing oocytes to a final concentration of 100 μM. Currents were assessed 20 h after injection.

Immunofluorescence—Nasal ciliated epithelial cells harvested from the inferior turbinate of patients undergoing unrelated surgery (approved by local ethical committee) were maintained in cell culture medium M199 prior to fixation in 4% paraformaldehyde. Cells were permeabilized using 1% Triton X-100, washed three times in PBS, and then blocked in 1% glycine for 15 min, followed by 5% donkey serum for 15 min. Pelleted cells were resuspended in PBS containing primary antibodies (goat anti-CK2α (Santa Cruz Biotechnology) and mouse anti-CFTR NB1 (Neomarkers) at a 1:100 dilution) and incubated at room temperature with shaking, overnight. After three washes in PBS, pelleted cells were resuspended in PBS containing fluorescein isothiocyanate-labeled anti-goat and rhodamine-labeled anti-mouse IgG secondary antibodies (1:100; Jackson ImmunoResearch). After a 2-h incubation, with
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shaking, the cells were washed five times in PBS and resuspended in 15 μl of anti-fade mountant (6% n-propyl gallate in 70% glycerol, 100 mM Tris/HCl, pH 7.4) for mounting on glass slides. Coverslips were sealed with nail varnish for image capture using a Zeiss 510 laser scanning confocal microscope.

Immunoblotting—Immunoblotting was carried out as described previously (21). Briefly, blotted membranes were blocked for 30 min in TBS/Tween with 5% milk powder; and anti-NBD1 antibody (Neomarkers) was applied for 1.5 h followed by four 15-min washes, and then horseradish peroxidase-labeled anti-mouse secondary antibody was applied at a 1:5000 dilution for 45 min followed by four 15-min washes. Bound horseradish peroxidase was visualized using a chemiluminescence system and exposure to x-ray film.

Kinase Assays—5 μg of protein (or 50 μg in 10 × samples) were assayed for CK2 activity (27). Briefly, [γ-32P]ATP or [γ-32P]GTP was used as phosphate donor to detect endogenous CK2 activity directed toward the specific target peptide RRRADDSDDDDD (gift of F. Meggio, S. Sarno, and L. Pinna). 10 μM TBB specified the activity.

Cloning of NBD1 from CFTR—To clone an NBD1 construct (amino acids 351–665 of human CFTR), we used full-length cfr cDNA (gift; C. Boyd, Edinburgh, Scotland) as template and designed the following primers (forward, 5'-ctcgagatgtggtatgacctcttgga-3'; reverse, 5'-tcagtagcactagatcagccggcg-3') incorporating Xhol and NotI restriction sites 5’ and 3’, respectively. PCR was performed with these primers and template (92 °C for 1 min, 38 °C for 2.5 min, and 46 °C for 3.5 min, for 35 cycles); the product (~950-base fragment) was gel-purified and inserted into a standard ligation reaction with XhoI/NotI-digested plasmid vector; the product (70% glycerol, 100 mM Tris/HCl, pH 7.4) for mounting on glass slides. The product was gel-purified and subsequently ligated into our expression vector. The mutant was then transformed into a competent JM109 E. coli cell line for expression. Amplification and protein purification as described for the NBD1 wild-type fragment.

In Vivo Phosphorylation of CFTR—Hep-G2 cells were transfected with vectors encoding either NBD1 alone or full-length CFTR. 48 h after transfection, some plates were treated with TBB (10 μM) to specify CK2 phosphorylation, and all cultures were maintained for 12 h in the presence of cell-permeable inhibitors of PKA (100 μM), PKC (10 μM), and calmodulin-dependent protein kinase II (1 μM; all from Calbiochem) to reduce background phosphorylation by other kinases. Cells were lysed into RIPA buffer containing phosphatase inhibitors, and CFTR was purified and enriched by immunoprecipitation. Transfected Hep-G2 cells showed consistent levels of CFTR expression with all mutants and transcripts (Fig. 4C, lower blots).

Co-immunoprecipitation of CK2 with CFTR Mutants—BHK cells were transfected with vectors encoding wild-type, ΔF508, S511A, or S511D CFTR. 48 h after transfection, all cultures were maintained for 12 h in the presence of cell-permeable inhibitors of PKA (100 μM), PKC (10 μM), and calmodulin-dependent protein kinase II (1 μM; all from Calbiochem) to reduce background phosphorylation by other kinases. Cells were lysed into RIPA buffer containing phosphatase inhibitors, and CFTR was purified and enriched by immunoprecipitation. Transfected Hep-G2 cells showed consistent levels of CFTR expression with all mutants and transcripts (Fig. 4C, lower blots).

RESULTS

CK2 and CFTR in a Differentiated Human Epithelium—Using immunofluorescence and confocal microscopy, we tested whether the subcellular localization of CK2 coincides with the known apical distribution of CFTR in biopsies of normal and homozygous ΔF508 CF ciliated human nasal epithelial cells. Fig. 1 demonstrates that CK2α is not only enriched at the apical membrane but also colocalizes with CFTR in wild-type but not ΔF508 CF epithelial cells (CFTR antibody specificity is shown in supplemental Fig. S1). Levels of CFTR protein are reported to be reduced when F508 is deleted (31); thus, the absence of CFTR staining at the apical membrane of the CF cell is not surprising. However, the enrichment of CK2α at the apical membrane in wild-type cells and the lack of CK2 where CFTR levels

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are depleted are novel results and suggest that CK2 localization is somehow dependent on CFTR.

CAMP-dependent Chloride Transport Is Dependent on CK2—CFTR regulates CAMP-dependent fluid and electrolyte transport across epithelia (3). To investigate CK2-dependent regulation of a classical CFTR function, we tested the effects of pharmacological CK2 inhibition on forskolin-stimulated, apical membrane Cl\(^-\) currents (I\(^{Cl}\) (apical)) with the Ussing chamber technique (28). Fig. 2A demonstrates that preincubation of Calu-3 epithelia (a robust model for CFTR-mediated anion transport reviewed in 32) with the CK2-selective inhibitor TBB (33) decreased I\(^{Cl}\) (apical) with half-maximal inhibition at \(15 \mu M\) that causes potent inhibition of CK2 activity in mammalian cells (33). I\(^{Cl}\) (apical) was similarly abrogated in 16HBE144–bronchial epithelia (32) (HBE; supplemental Fig. S2A). TBB targets the ATP-binding site on CK2α and is without effect on 30 other protein kinases, including PKA, PKC, and AMPK (33). Nevertheless, we determined whether this agent might interact with NBD1 and found that TBB (50 nm to 1 mM) did not compete with ATP for NBD1 binding (supplemental Fig. S2B). To understand better how TBB inhibits CFTR-mediated Cl\(^-\) transport, we studied the effects of TBB on the single-channel activity of CFTR using the patch clamp technique. Fig. 2B, panel a, demonstrates that addition of TBB (10 \(\mu M\)) to the solution bathing an intact cell caused a prompt inhibition of channel activity in a cell-attached membrane patch (compare expanded trace 1 with expanded traces 2 and 3 in Fig. 2B, panels a and d). TBB mediated its effects in two ways. First, the drug caused a dramatic time-dependent prolongation of the closed time interval between bursts of channel openings and, hence, decreased open probability (\(P_o\)) to zero (Fig. 2B, panel e, histogram, left). Second, the drug caused a small decrease in current flow through individual channels (Fig. 2B, panel f, histogram, right). Interestingly, Fig. 2B (panels b–f, middle traces and histograms) shows that when TBB (10 \(\mu M\)) is directly added to the intracellular solution using an excised inside-out membrane patch, the drug has little effect on channel gating and hence \(P_o\), but it still reduced current flow through individual channels. Two conclusions can be drawn from these data. First, the small effect of TBB on CFTR-mediated current flow most likely represents the direct interaction of TBB with CFTR. Second, the effect of TBB on CFTR channel gating that is observed in intact cells, but lost on patch excision (summary histogram for \(P_o\), suggests that TBB exerts its effect via a CFTR-interacting protein. Given that TBB is a selective inhibitor of CK2, the simplest interpretation of the data is that CK2 regulates CFTR channel gating in intact cells.

Wild-type (but Not ΔF508) CFTR—Next, we confirmed that a structurally unrelated peptide inhibitor of CK2 (poly(E:Y) peptide 4:1) that targets the CK2 β subunit also inhibited forskolin-stimulated CFTR current. First, we used the Xenopus oocyte expression system to test both wild-type and ΔF508 CFTR for their sensitivity to CK2 inhibition. We either incubated oocytes with TBB or injected them with poly(E:Y), exploiting the near identity between human and Xenopus CK2 (94% identity α subunit; 98% β subunit). Dramatic inhibition of CAMP-stimulated CFTR-mediated Cl\(^-\) current was observed with either a CK2-specific concentration of TBB (1 \(\mu M\)) or poly(E:Y) (10 \(\mu M\)) (Fig. 3A), further suggesting that CK2 is a novel CFTR regulator. To confirm that TBB acts specifically on CK2 activity, we co-transfected oocytes with wild-type CFTR and a TBB-insensitive mutant form of CK2 (gift of L. A. Pinna). TBB had a greatly reduced effect on CFTR current in the presence of this mutant CK2 (supplemental Fig. S5). Consistent with our hypothesis that CK2 would not bind when F508 was absent, we found that the corresponding ΔF508-CFTR Cl\(^-\) current was insensitive to either CK2 inhibitor (Fig. 3B). Because inhibition of CK2 reduces wild-type CFTR Cl\(^-\) current, but is without effect on ΔF508 CFTR, this strengthens the evidence that ΔF508 CFTR lacks CK2.

CK2 Associates with Wild-type but Not ΔF508 CFTR—Despite consistent results with two unrelated pharmacological CK2 inhibitors, our hypothesis that CK2 regulates CFTR required confirmation by independent strategies. However, this was not a trivial matter because CK2 is an essential protein, and short interfering RNA or dominant negative approaches do not adequately inhibit CK2 function.\(^5\) We predicted that a peptide corresponding to the wild-type sequence of CFTR around the F508 site should sequester CK2 away from CFTR. We gen-

\(^5\) L. Pinna, unpublished data and personal communication.
erated wild-type and ΔF508-CFTR peptides, which both contain the putative CK2 consensus sequence around serine 511 and determined their effects on CFTR activity and CK2 binding. Fig. 2C shows that wild-type (but not ΔF508) CFTR peptide inhibits forskolin-stimulated CFTR current when injected into CFTR-expressing oocytes. Next, we made detergent extracts of airway epithelial membranes and overlaid them on peptide dot blots followed by detection with anti-CK2α antibody, which demonstrated that CK2 only binds the peptide sequence corresponding to wild-type CFTR (KENIIF; Fig. 4A). No interaction was detected with either the ΔF508-CFTR peptide (KENII) or a control peptide from another region of CFTR (unrelated peptide). No peptide-bound PKC was detected; this is a control, CFTR-directed kinase (34) (Fig. 4A).

To test the association of CK2 with CFTR, we isolated membranes from wild-type HBE (32) cells and apically enriched sheep tracheal epithelium (35). Fig. 4B demonstrates that CFTR and CK2 co-immunoprecipitate under stringent conditions from epithelial membranes in different species. We tested the ΔF508 dependence of the CFTR-CK2 interaction using a CF bronchial cell line homozygous for the ΔF508 mutation (CFBE410−/−; CFBE) (32). Because low levels of ΔF508 CFTR protein are present in membranes from CFBE cells (~10% of wild-type CFTR levels; Fig. 4C, left panel), we compensated by using a 10-fold excess of membrane protein (10× CFBE). CK2α was not detected in 10× CFBE membranes (Fig. 4C, right panel), confirming the absence of CK2 immunofluorescence in native ΔF508-CFTR cells (Fig. 1). The selectivity of the defect toward CK2α was demonstrated by the observations that the regulatory half of CF2 (CK2β), PKA holoenzyme, and PKC were unaffected by the loss of F508 and co-immunoprecipitated with CFTR from 10× CFBE in quantities comparable with HBE membranes (Fig. 4, C and D). Similarly, equivalent amounts of PP2Bα and PP2C, two protein phosphatases that dephosphorylate CFTR (34) and two other CFTR-bound kinases (NDPK-A and AMPPKα), were detected in both HBE and 10× CFBE (supplemental Fig. S3A and kinase data not shown). Finally, CK1, another protein kinase requiring Glu/Asp amino acid residues near its target serine, was not detected in either cell membrane preparation (supplemental Fig. S3B). Thus, the data demonstrate the F508 dependence of the CK2-CFTR interaction and its selectivity toward the catalytically active domain of CK2. The retention of CK2β with ΔF508 CFTR is consistent with our previous finding that CK2α can translocate between substrates independent of CK2β (20).
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CK2 Phosphorylates Wild-type but Not ΔF508 CFTR In Vitro—Next, we examined the potential signaling consequences. Overlying recombinant CK2 holoenzyme onto peptide dot blots in the presence of [γ-32P]ATP (or [γ-32P]GTP; data not shown), phosphorylated only the wild-type KENIIF peptide, despite the CK2 consensus sequence remaining intact in the ΔF508-CFTR peptide (Fig. 5A; phosphorylation was enhanced 8-fold by the CK2 activator, polylysine (27)). Consistent with these data, the phosphorylation of immobilized wild-type CFTR peptide was abolished by the presence of free wild-type CFTR peptide but not ΔF508 peptide (10 μM) in solution (Fig. 5A, inset). The total absence of CK2α protein from CFBE membranes observed in Fig. 3C was confirmed by testing for CK2 activity (27). CK2 activity was detected in HBE but not 10× CFBE membranes, whereas similar cytosolic activity was present in both cell lines (Fig. 5B). The F508 dependence of CK2 phosphorylation was further demonstrated by the failure of CK2 to phosphorylate purified recombinant ΔF508-CFTR NBD1 protein, whereas wild-type CFTR NBD1 was phosphorylated as expected with both ATP and GTP (Fig. 5C). Both proteins contain a PKA target at Ser-660 (36), and PKA phosphorylated the wild-type and ΔF508 proteins equivalently (data not shown). Thus, F508 is critical for CK2 to bind CFTR and phosphorylate Ser-511, and this differential phosphorylation of wild-type and ΔF508 NBD1 domains is specific to CK2.

CK2 Targets CFTR at Ser-511 in Vitro—To test whether Ser-511 is phosphorylated by CK2 in wild-type CFTR, we mutated Ser-511 in recombinant NBD1 (Fig. 5D). Phosphorylation assays using NBD1/NBD1-S511A protein demonstrated that PKA-dependent phosphorylation remained intact. In contrast, CK2 failed to phosphorylate the S511A mutant (Fig. 5D, open columns). TBB (10 μM) abolished the CK2-dependent phosphorylation of
wild-type CFTR NBD1 (Fig. 5D, filled columns), whereas PKA-dependent phosphorylation was unaffected, confirming the specificity of this CK2 inhibitor (33). Conversely, a PKA inhibitor was without effect on CK2-dependent phosphorylation but abolished PKA-dependent phosphorylation (Fig. 5D). The incorporation of $^{32}$P into wild-type NBD1 was similar with both kinases, consistent with NBD1 containing one target serine for each. Moreover, the phosphorylation of wild-type NBD1 with both kinases was additive, indicating that CK2 and PKA target distinct sites (Fig. 5D, last filled column). These results suggest that neither ΔF508-NBD1 nor S511A-NBD1 domains interact with CK2 in vitro; we therefore investigated whether this defect is mirrored in full-length CFTR.

Wild-type, but Not ΔF508 or S511A CFTR, Induces CK2 Membrane Localization—Having shown that CK2 membrane localization is dependent on the presence of wild-type CFTR in bronchial cell lines, we investigated whether this phenomenon could be reproduced by expression of CFTR in a CFTR-naive cell line. We tested CK2 binding to CFTR by overexpressing full-length wild-type and mutant CFTRs in BHK cells. ΔF508 CFTR was detectable in membrane fractions from transfected BHK cells (Fig. 6A), despite its reported trafficking defect, but this has been shown to vary with cell type for unknown reasons (37). We immunoprecipitated CFTR using an excess of membrane protein isolated from cells transfected with ΔF508 and Ser-511 mutants and probed for CK2α. No CK2 protein was found associated with S511A or ΔF508 mutant CFTR, whereas wild-type, S511D, and the ΔF508-S511D double mutant CFTR all bound CK2 (Fig. 6A). The specificity of CK2 association was confirmed by assay for membrane-bound kinase activity. CK2 activity was present in wild-type, S511D, and ΔF508-S511D-CFTR-transfected membranes but was absent from those of S511A and ΔF508 (Fig. 6B). Thus, the S511D mutation rescues the CFTR-CK2 association abolished by ΔF508.

FIGURE 5. CK2 phosphorylates wild-type but not ΔF508-CFTR and targets Ser-511 in vitro. A, quantification of recombinant CK2-dependent (New England Biolabs) KENIIF/KENII/NBD2 CFTR peptide phosphorylation $^\pm$ polysine (CK2 activator-poly-K) (24) with [$^{32}$P]ATP. n = 3 ± range; inset, KENIIF-specific inhibition of phosphorylation of dot blot-immobilized peptides by competing free peptide (10 μM). B, CK2 activity is absent from CFBE membranes; assay for CK2 activity in HBE and CFBE membranes and cytosol. 5 μg of protein (or 50 μg in 10 x samples) from the indicated sources were assayed for CK2 activity (27). Either [$^{32}$P]ATP (filled bars) or [$^{32}$P]GTP (open bars) was used as phosphate donor to detect CK2 phosphorylation directed toward the specific target peptide (gift of O. Marin, F. Meggio, S. Sarno, and L. Pinna) RRADDSDDDDD (n = 3, ± range). C, differential phosphorylation of wild-type (NBD1) and ΔF508-NBD1 protein by CK2 (New England Biolabs) from [γ-$^{32}$P]ATP/GTP; stimulation with polylysine (polyK) and inhibition with 10 μM TBB, (n = 3 ± range). 7-Fold higher phosphorylation occurs with wild-type NBD1 compared with ΔF508-NBD1, irrespective of polylysine. D, CK2-dependent but not PKA-dependent phosphorylation is abolished in S511A-NBD1; CK2 and PKA phosphorlate wild-type NBD1 additively at distinct sites, and their respective inhibitors are specific (n = 3 ± range). All data are in the presence of polysine, and constructs were sequence-verified. Recombinant pol(His)-tagged proteins were purified to homogeneity as described in supplemental Fig. 54.

CFTR was detectable in membrane fractions from transfected BHK cells (Fig. 6A), despite its reported trafficking defect, but this has been shown to vary with cell type for unknown reasons (37). We immunoprecipitated CFTR using an excess of membrane protein isolated from cells transfected with ΔF508 and Ser-511 mutants and probed for CK2α. No CK2 protein was found associated with S511A or ΔF508 mutant CFTR, whereas wild-type, S511D, and the ΔF508-S511D double mutant CFTR all bound CK2 (Fig. 6A). The specificity of CK2 association was confirmed by assay for membrane-bound kinase activity. CK2 activity was present in wild-type, S511D, and ΔF508-S511D-CFTR-transfected membranes but was absent from those of S511A and ΔF508 (Fig. 6B). Thus, the S511D mutation rescues the CFTR-CK2 association abolished by ΔF508.

CK2 Phosphorylates Wild-type but Not ΔF508 CFTR in Vivo—We investigated whether Ser-511 could be phosphorylated by endogenous CK2 in intact cells using transfection of CFTR-naive cells and detection with an anti-phosphoserine antibody. Hep-G2 (human liver) cells were transfected with either wild-type or S511D CFTR (eliminating the putative CK2 target serine at Ser-511) using vectors encoding either NBD1 alone or full-length CFTR. Quantification of their phosphoserine content (Fig. 6C) demonstrates that NBD1 is constitutively phosphorylated at Ser-511 in Hep-G2 cells because the phosphoserine signal is reduced to the same degree with either CK2 inhibition or mutation of Ser-511 in NBD1 (left histogram). This identical reduction also indicates that Ser-511 is the sole CK2 target serine in NBD1 under these conditions. In cells expressing full-length CFTR, either adding TBB or transfecting S511D CFTR reduces the phosphoserine content; however, the effect of CK2 inhibition is more pronounced than Ser-511 mutation (Fig. 6C, right histogram). This indicates that CK2-dependent target serines are likely to exist in other domains of CFTR. The combined data confirm that CFTR can be phosphorylated by CK2 at Ser-511 in vivo.

Mutation of Ser-511 Inhibits CFTR Channel Activity—The role of Ser-511 mutation in CFTR function was investigated with the patch clamp technique. Fig. 7A demonstrates that mutation of Ser-511 in wild-type CFTR perturbs channel gating. S511D (ΔF508) and S511A decreased the P<sub>o</sub> of CFTR 3-fold, without altering current flow through individual chan-
Protein Kinase CK2, CFTR, and the ΔF508 Mutation

of Ser-511 disrupts channel gating while making Ser-511-mutated CFTR insensitive to CK2 inhibition. The consistency of our results using CFTR-transfected cells, oocyte expression, cultured epithelial cells and native tissue argues that the CK2-CFTR interaction is a general feature of CFTR-expressing cells. Surprisingly, when F508 is deleted, the ΔF508-CFTR-expressing cell membrane is devoid of CK2α protein and CK2 kinase activity, suggesting that a normal function of CFTR is to either maintain the CK2α content of the apical membrane or to provide a F508-dependent anchoring protein for CK2α. Given the plethora of targets for CK2, further work will be needed to determine whether the absence of CK2 staining at the apical membrane of ΔF508 CFTR-expressing cells is a direct or indirect phenomenon. We also show that Ser-511, the sole CK2 target in NBD1, can be phosphorylated by CK2 in vivo and that mutation of Ser-511 disrupts channel gating while rendering the Ser-511-mutant insensitive to CK2 inhibition.

We find that two CK2 inhibitors targeting different subunits of the CK2 heterotetramer are without effect on ΔF508-CFTR channel activity and yet potently inhibit the wild-type channel. These data are consistent with our finding that ΔF508 CFTR lacks CK2α. Because CK2 has over 300 in vivo targets (26, 27), dominant negative, inhibition, or knockdown studies inevitably impact on multiple cellular pathways making interpretation of the results problematic. In CK2 studies, it is therefore necessary to apply site-directed mutagenesis of the putative target. We complemented this approach with experiments using specific, competitive peptides to disrupt the local association of CK2 and CFTR. We note that site-directed mutation of the proposed CK2 target at Ser-511 significantly reduced the Pd, compared with wild-type CFTR. The Pd observed in S511A/S511D was similar to that of ΔF508 CFTR when it is induced to traffic to the membrane by a low temperature correction. Although this suggests that the phosphorylation status of Ser-511 is critical for channel gating, Ser to Asp mutation is not sufficient in this respect to mimic phosphorylation and restore the Pd to that of wild type. Failure to restore channel function is not mirrored by the successful reconstitution of the CFTR-CK2α interaction, where a negative charge at Ser-511 appears sufficient to restore CK2 binding. Thus we find that S511A and ΔF508 share an inability to bind CK2, but S511D restores CK2 binding with or without F508. Others have reported the functional limitation of using a single negatively charged carboxylate substitution in place of the physiologically reversible, double-negatively charged phosphate moiety when studying ion channels (38). Alternatively, our data may be interpreted as a need for reversible modification of Ser-511 for the complex gating cycle of CFTR to proceed (2, 39). We have found that the KENIIF (wild-type) peptide inhibits CFTR channel function in oocytes. We interpret these data to indicate that KENIIF can sequester CK2 away from that of F508 CFTR-expressing cells is a direct or indirect phenomenon. We also show that Ser-511, the sole CK2 target in NBD1, can be phosphorylated by CK2 in vivo and that mutation of Ser-511 disrupts channel gating while rendering the Ser-511-mutant insensitive to CK2 inhibition.

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DISCUSSION

Our studies have focused on identification of a pathogenic molecular mechanism in most cases of cystic fibrosis. This mechanism results in a disrupted interaction between CFTR and CK2; to our knowledge, this is the first report of such a pathogenic mechanism. Here we demonstrate that CK2 is a novel NBD1-directed CFTR kinase, and we establish that deletion of F508 selectively disrupts the binding of the α catalytic domain of this kinase when compared first to its own regulatory domain CK2β and second to six known CFTR-associated proteins. We also show that Ser-511, our proposed CK2 target in NBD1, can be phosphorylated by CK2 in vivo and that mutation

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FIGURE 6. CK2 association with CFTR is abrogated by ΔF508 or S511A mutation; CK2 phosphorylates CFTR at Ser-511 in vivo. A, full-length human CFTR with mutations as indicated was expressed in BHK cells. Membranes were isolated and immunoprecipitated with a limiting quantity of anti-CFTR antibody (supplemental Fig. S1). Precipitates were probed for CK2 and CK2α. B, membranes were assayed for CK2 kinase activity using recombinant NBD1 as phosphate acceptor substrate (n = 3 ± S.E.). C, the NBD1 domain of CFTR (left) or full-length CFTR (right) containing the indicated mutations were transiently expressed in Hep-G2 cells. Cells were treated with TBB (10 μM, 12 h) as indicated. CFTR was immunoprecipitated from lysed cells, and samples were probed with anti-phosphoserine (top) and anti-CFTR (bottom) antibodies. Histograms show quantification of CFTR phosphorylation under indicated conditions; n = 3 ± range.
transfected with these Ser-511 mutants. Thus, Ser-511 may be involved in trafficking, and this is currently under investigation.

What might be the consequences of ΔF508-induced CK2α mislocalization away from its natural apical membrane targets? Some of the cellular processes reported to be defective in CF are dependent upon CK2. Polyamines, such as spermidine, which are stimulators of CK2 activity, are increased in CF (41–43), consistent with a compensatory reaction to the absence of CK2. The residence time for ΔF508-CFTR at the apical membrane is reduced because of abnormal endocytic cycling (44), and coating and uncoating of such vesicles is a CK2-dependent process (45). CFTR maturation is dependent on its association with calnexin in the endoplasmic reticulum (46); this chaperone has a reported CK2 phosphorylation target (47). The CF antigen, which is overexpressed in both CF patients and model systems, is a regulator of CK2 kinase activity (48, 49). Finally, our earlier studies demonstrated defective function of NDPK-A in CF cells (50), and NDPK is an in vivo CK2 target (51) that we have recently shown to not only regulate AMPK α1 but also to bind to CFTR (21, 52). Thus, some of the reported targets of CK2 (27, 53) include proteins controlling unexplained aspects of CF disease (50, 54). Overall, our proposed role for CK2 is consistent with published data on the cellular biological consequences of CFTR mutation, but further work will be needed to determine whether the fracture of the CK2 holoenzyme and loss of CK2 catalytic domain in ΔF508-apositive membranes might selectively disrupt membrane-associated pathways dependent on CK2 activity.

One approach to CF therapy aims to rescue defective processing and trafficking of ΔF508-CFTR (55). Our data suggest that CK2-dependent regulation of ΔF508-CFTR might still be defective under conditions where ΔF508-CFTR has been induced to traffic, consistent with published data showing that restoration of trafficking fails to fully restore CFTR-mediated Cl− current (10, 40). Although ΔF508 is responsible for the majority of CF pathology, further work will be needed to determine which other pathogenic mutations in CFTR act by a CK2-related mechanism. Outside the CF field, understanding the CK2-CFTR interaction might generate new drugs for the treatment of secretory diarrheas because inhibitors of CFTR, selected for their impact on ion transport, have been proposed as treatments for cholera (56).

The data in this paper suggest not only that CK2 is a novel regulator of CFTR function but also that ΔF508-CFTR lacks the catalytic domain of CK2. We present a radically different explanation for the most common CF defect that links the deletion of F508 with defective binding of a pleiotropic kinase.

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Protein Kinase CK2, Cystic Fibrosis Transmembrane Conductance Regulator, and the ΔF508 Mutation: F508 DELETION DISRUPTS A KINASE-BINDING SITE
Kate J. Treharne, Russell M. Crawford, Zhe Xu, Jeng-Haur Chen, O. Giles Best, Eva A. Schulte, Dieter C. Gruenert, Stuart M. Wilson, David N. Sheppard, Karl Kunzelmann and Anil Mehta

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Synaptic vesicle protein 2 binds adenine nucleotides.

Jia Yao and Sandra M. Bajjalieh

On Page 20633, an incorrect image was used for Fig. 8B. The correct image is shown below.

Protein kinase CK2, cystic fibrosis transmembrane conductance regulator, and the ΔF508 mutation. F508 deletion disrupts a kinase-binding site.

Kate J. Treharne, Russell M. Crawford, Zhe Xu, Jeng-Haur Chen, O. Giles Best, Eva A. Schulte, Dieter C. Gruenert, Stuart M. Wilson, David N. Sheppard, Kar Kunzelmann, and Anil Mehta

This article has been withdrawn by the authors. Further experiments are under way to resolve difficulties with this study arising from cloning errors.