Biochemical Basis of the Interaction between Cystic Fibrosis Transmembrane Conductance Regulator and Immunoglobulin-like Repeats of Filamin

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Mutations in the chloride channel cystic fibrosis transmembrane regulator (CFTR) cause cystic fibrosis, a genetic disorder characterized by defects in CFTR biosynthesis, localization to the cell surface, or activation by regulatory factors. It was discovered recently that surface localization of CFTR is stabilized by an interaction between the CFTR N terminus and the multidomain cytoskeletal protein filamin. The details of the CFTR-filamin interaction, however, are unclear. Using x-ray crystallography, we show how the CFTR N terminus binds to immunoglobulin-like repeat 21 of filamin A (FlnA-Ig21). CFTR binds to β-strands C and D of FlnA-Ig21 using backbone-backbone hydrogen bonds, a linchpin serine residue, and hydrophobic side-chain packing. We use NMR to determine that the CFTR N terminus also binds to several other immunoglobulin-like repeats from filamin A in vitro. Our structural data explain why the cystic fibrosis-causing S13F mutation disrupts CFTR-filamin interaction. We show that FlnA-Ig repeats transfected into cultured Calu-3 cells disrupt CFTR-filamin interaction and reduce surface levels of CFTR. Our findings suggest that filamin A stabilizes surface CFTR by anchoring it to the actin cytoskeleton through interactions with multiple filamin Ig repeats. Such an interaction mode may allow filamins to cluster multiple CFTR molecules and to promote colocalization of CFTR and other filamin-binding proteins in the apical plasma membrane of epithelial cells.

Cystic fibrosis (CF) is a genetic disorder caused by mutations in an apical chloride channel, cystic fibrosis transmembrane regulator (CFTR). This disorder is characterized by high sweat chloride concentration, pulmonary disease with high production of dehydrated viscous secretions, and pancreatic insufficiency (1). CF affects all exocrine epithelia, with morbidity and mortality primarily caused by bacterial infection and inflammation in the lung. CF affects ~30,000 individuals in North America, of whom about 70% carry one copy of the mutation ΔF508, the most common of >1,000 CF-associated mutations. ΔF508 is a folding mutation that leads to rapid degradation at the endoplasmic reticulum. The small fraction of ΔF508-CFTR that is not degraded is characterized by inefficient trafficking to the apical plasma membrane and reduced residency in the plasma membrane (2, 3).

Although the levels of ΔF508-CFTR in the apical plasma membrane are low, ΔF508-CFTR retains partial function as a cAMP-activated chloride channel (4, 5). This justifies therapeutic approaches to promote delivery of ΔF508-CFTR and other functionally impaired CFTR mutants to the plasma membrane. A detailed understanding of factors that stabilize and regulate CFTR at the plasma membrane is important for the development of new therapies to correct CF-causing defects in vivo.

CFTR is regulated by intracellular cAMP levels and phosphorylated at multiple sites by cAMP-activated protein kinase, which modulates CFTR trafficking (6) and activity (7, 8). We and others have identified and characterized additional regulatory proteins that interact with cytoplasmic domains of CFTR. For example, the PDZ-containing adaptor molecule NHERF1/EBP50 binds to the C terminus of CFTR, where it interacts with microtubules and with receptor for activated C kinase-1 (RACK1) (9). By interacting with ERM (ezrin-radixin-moesin) domain actin-binding proteins such as ezrin, NHERF1 also connects CFTR to the cortical actin cytoskeleton (10, 11). CFTR associates with SNARE proteins (syntaxin 1A, SNAP23) and endocytic adaptors such as AP2, undergoing clathrin-me-
Filamin Ig Repeat Interactions with CFTR

EXPERIMENTAL PROCEDURES

Materials—Anti-human CFTR (C terminus-specific) monoclonal antibody was obtained from R&D Systems. Anti-FlnA antibody was from Millipore. Anti-NHERF1 was from ABCAM, and anti-RACK1 monoclonal antibody was from Transduction Laboratories. Horseradish peroxidase-coupled secondary antibodies were purchased from Santa Cruz Biotechnology. BioPORTER protein delivery system was obtained from Gene Therapy Systems, Inc. An enhanced chemiluminescence reagent was purchased from Denville. All other chemicals were reagent grade.

Protein and Peptide Expression and Purification—Human FlnA-Ig17 (residues 1861–1950), FlnA-Ig19 (residues 2045–2140), FlnA-Ig21 (residues 2236–2329), FlnA-Ig23 (residues 2424–2516), and FlnA-Ig10 (residues 1158–1252) were cloned into pGST (28) for expression as GST fusions. GST-Ig repeats were expressed in Rosetta2(DE3) cells (EMD Biosciences) and purified by glutathione-affinity column chromatography in buffers containing 150 mM NaCl, 50 mM sodium phosphate, pH 8.0. The GST tag was removed by overnight cleavage with tobacco etch virus protease followed by additional glutathione-Sepharose and gel filtration chromatography. 15N-Labeled FlnA-Ig repeats were expressed as GST fusions in minimal medium supplemented with 1 g/liter [15N]NH4Cl (Isotec), cleaved, and purified in the same manner as the unlabeled repeats.

CFTR N-terminal peptides were synthesized in the Molecular Biotechnology Core, Lerner Research Institute (Cleveland Clinic). The peptides are acetylated at their N termini and amidated at their C termini and were purified by high-performance liquid chromatography.

Crystal Structure Determination of FlnA-Ig21-CFTR4–22 Complex—Crystallization was carried out using 1 mM FlnA-Ig21 in 20 mM Tris, pH 7.4, 50 mM NaCl, mixed with 3 mM CFTR4–22 peptide (SPEKASVVKLEFKSWTRP) in water. The complex crystallized at 20 °C in hanging drops against 60% Tacso (29). The unit cell parameters are a = 74.15 Å, b = 74.15 Å, c = 289.06 Å, as confirmed by molecular replacement in Phaser (30). The FlnA-Ig21 monomer structure and a polyalanine model of the integrin β7 C terminus (Protein Data Bank (PDB) code 2BRQ) (Ref. 23) were used as search models. Refmac (31) and CNS (32) were used for refinement, and model rebuilding was carried out in COOT (33) to generate a final model with Rwork and Rfree of 26.3% and 29.8%, respectively. The anisotropy of the diffraction data contributed to a high average B factor. More than 97% of residues are in the most favored or additionally allowed regions of Ramachandran space, whereas no resi-

Mediated endocytosis (6, 12–14). A balance between cytoskeletal tethering and capture by the endocytic machinery may be crucial to maintain a sufficient population of CFTR at the cell surface.

Recently, Thelin et al. (15) identified the dimeric cytoskeletal adaptors filamin A and filamin B (FlnA and FlnB) as new and important binding partners of CFTR. FlnA and FlnB, which have high sequence similarity to each other, are homodimeric rod-like proteins that cross-link actin filaments at high-angle orientations (16). The filamins confer mechanical strength as well as flexibility and reversible deformability to cellular actin networks under mechanical stress (17). Filamins, however, also bind to cytosolic effectors and to membrane proteins such as integrin β subunits, the platelet adhesion receptor GPIβα, HCN1 pacemaker channels, calcitonin receptor, glutamate receptor type 7, D2/D3 dopamine receptors, CD4 receptor, Ror2 receptor tyrosine kinase, µ-opioid receptor, and others (for reviews, see Refs. 18, 19). Filamins tether such proteins to the membrane-proximal actin cytoskeleton and regulate their surface localization and dynamics. Filamins may also mediate direct signaling between these proteins and the cytoskeleton (20).

The filamins exhibit common structural and functional properties (for review, see Ref. 19). Filamins contain N-terminal globular actin-binding domains consisting of two calponin homology domains. These are followed by two extended rod domains connected by a hinge. The rod domains, respectively, consist of 15 and 8 immunoglobulin-like repeats, termed Ig1–Ig23. At the C terminus, a second hinge connects the second rod domain to a final repeat, Ig24, which is the dimerization element of the protein (21). Those Ig repeats that have been structurally characterized have 7-stranded β-sandwich folds (21, 22). Selected Ig-like repeats, primarily the odd-numbered repeats in the second rod domain, bind a diverse array of linear motifs from the cytoplasmic portions of the integral membrane protein-binding partners of filamins (23–26). Interestingly, some binding partners such as integrin β7 can bind to more than one of the Ig-like repeats in vitro (23, 27).

A CF-associated mutation, S13F, disrupts the interaction between the N terminus of CFTR and FlnA or FlnB (15). Disruption of the CFTR-filamin interaction results in greatly reduced CFTR surface levels due to rapid endocytosis. Unlike wild-type CFTR, the internalized S13F CFTR is targeted preferentially to lysosomes rather than being recycled to the plasma membrane (15). The CFTR-filamin interaction is thus crucial to maintain sufficient plasma membrane levels of CFTR, but the details of the interaction are unclear. It is also unclear how filamins fit into the diverse network of proteins that associate with CFTR and regulate its trafficking and activity at the plasma membrane. In this article, we present the crystal structure of the CFTR N terminus with immunoglobulin-like repeat 21 of filamin A (FlnA-Ig21). We also characterize the binding of the CFTR N terminus to other repeats in the C-terminal rod domain of filamin A using NMR. Our results explain why the S13F mutation disrupts the interaction between CFTR and filamins. In addition, we show that FlnA-Ig21 acts in a dominant negative fashion in cultured epithelial cells, disrupting the CFTR-filamin interaction and resulting in loss of surface CFTR. Our studies present the molecular details of the CFTR-filamin interaction and emphasize that coupling of CFTR to the actin cytoskeleton through filamin is crucial for the regulation of surface CFTR levels in epithelial tissues.
Filamin Ig Repeat Interactions with CFTR

19N-Ig repeats were titrated with CFTR7–20/F16E (EKASVVSKEFLESWT) and CFTR7–20/S13F/F16E (EKASVVFKEFLESWT) at ratios ranging from 1:0 up to 1:100 (FilA:peptide). These spectra exhibited much less line broadening and clearly defined chemical shift perturbations. Normalized chemical shift perturbations (Δδ) (39) were calculated and plotted with Octave (40) according to the equation Δδ = (Δ1H_1)/2 + (Δ15N_2)/2, where Δδ is the maximum change in chemical shift at saturation and [L] is the CFTR peptide concentration (41, 42). Binding constants are summarized in Table 2.

### Computational Modeling of Ig Repeat–CFTR Complexes—
CFTR4–22 peptide was docked to FilA-Ig repeats 17, 19, and 23 using ambiguous intermolecular distance restraints, derived from 1H/15N chemical shift perturbations (CSPs). Restraints were input into a combined rigid body/torsion angle dynamics simulated annealing protocol in Xplor-NIH (43). Docking calculations utilized molecule A from the crystal structure of FilA-Ig17 with GPIIbpeptide (PDB code 2BP1) (24), the NMR structure of FilA-Ig19 (PDB code 2K7Q) (37), and the NMR structure of FilA-Ig23 (PDB code 2K3T) (38). 1H/15N CSP maps were converted into ambiguous distance restraints. An additional distance restraint between the CFTR_S13 hydroxyl oxygen and the appropriate FilA-Ig repeat backbone amide was also introduced. For each FilA-Ig repeat–CFTR peptide complex, 100 structures were generated; the solution with the lowest total energy and no ambiguous distance restraint violations greater than 0.5 Å was selected as the best structure.

We built a homology model for FilA-Ig10 repeat 10 using the I-TASSER server (44), based on the FilB-Ig10 NMR structure (PDB code 2DIA). The I-TASSER C-score for the FilA-Ig10 homology model was 1.15, indicating a high likelihood that the model accurately predicts the FilA-Ig10 structure. As expected, the FilA-Ig10 homology model is similar in structure to FilB-Ig10 and other FilA-Ig repeats. A more complete description of the molecular modeling procedures is provided in the supplemental Methods.

### Cell Culture and Isolation—
Calu-3 cells were grown in cell culture on 100-mm² tissue culture plastic or on 0.4-μm pore

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### Table 1

| Data collection | ALS 4.2.2 |
|-----------------|-----------|
| Wavelength (Å)  | 1.0       |
| Space group     | P6₁,22    |
| Cell dimensions | a, b, c (Å) 74.15, 74.15, 289.06 |
| Resolution range (Å) 42.97–2.80 (2.90–2.80) |
| R<sub>free</sub> (%) | 7.4 (47.2) |
| I/erl           | 13.8 (3.3) |
| Completeness (%) | 96.6 (98.5) |
| Redundancy      | 8.53 (10.41) |

### Table 2

| Ig repeat                  | K<sub>d</sub> (μM) | Hydrogen bond donor residue* | Hydrogen bond length* |
|---------------------------|-------------------|-------------------------------|----------------------|
| FilA-Ig21                 | 90 ± 9            | Ala<sup>2261</sup>            | 1.81                 |
| FilA-Ig17                 | 315 ± 22          | Ala<sup>2088</sup>            | 2.09 ± 0.05          |
| FilA-Ig19                 | 135 ± 8           | Val<sup>2060</sup>            | 1.96 ± 0.02          |
| FilA-Ig23                 | 127 ± 9           | Val<sup>2052</sup>            | 1.99 ± 0.01          |

* Values in parentheses correspond to the highest resolution shell of reflections.
Transwell-Clear polyester filter inserts (Corning Costar) with a growth area of 4.4 cm² for biotinylation experiments. For immunofluorescence, cells were seeded at a density of 0.2 × 10⁶ cells/filter with a growth area of 1.0 cm². Minimum Eagle’s culture medium was supplemented with 2.4 mg of l-glutamine and 10% fetal bovine serum. Cell cultures were grown at 37 °C under 5% CO₂, humidified air. Culture medium was changed at 48-h intervals until the desired confluence was reached, as assessed by microscopic examination.

**BioPORTER Delivery of Proteins**—We used BioPORTER protein delivery system to deliver FlnA repeats into Calu-3 cells, as described previously (45). BioPORTER reagent was dissolved in methanol, aliquoted in 10-μl portions, and dried under an N₂ stream. Dried reagent was reconstituted in 50 μl of Hanks’ balanced salt solution with 10 mM HEPES, pH 7.5 (HPSS) per filter insert containing an aliquot of protein in phosphate-buffered saline (PBS). The total volume of the peptide-BioPORTER complex per filter insert was taken to 500 μl for 24-mm filter inserts or 300 μl for 12-mm filter inserts, using HPSS. Apical surfaces of cells were incubated with the protein-BioPORTER complex or BioPORTER reagent alone for 2.5 h at 35 °C. The apical solution was replaced with HPSS and the incubation continued for 2 h at 35 °C.

**Cell Surface Biotinylation**—Polarized Calu-3 cell monolayers were grown on 24-mm-diameter Transwell permeable supports. Cell surface proteins were biotinylated using EZ-Link sultosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS; Pierce) (46). Cells were cooled to 4 °C, washed in PBS, pH 8.2, supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂, and incubated twice with 1 mg sulfo-NHS-SS-biotin/ml for 30 min at 4 °C. Nonreacted sulfo-NHS-SS-biotin was quenched by washing cells with PBS, pH 8.2, with 100 mM glycine, 0.1 mM CaCl₂, and 1 mM MgCl₂. Cells were harvested in 4% paraformaldehyde for 15 min at room temperature, fixed cells were washed with PBS, pH 8.2, with 0.1 mM CaCl₂ and 1 mM MgCl₂, and incubated with 1 mg sulfo-NHS-SS-biotin/ml for 12-mm filter inserts, using HPSS. Apical surfaces of cells were incubated with the protein-BioPORTER complex or BioPORTER reagent alone for 2.5 h at 35 °C. The apical solution was replaced with HPSS and the incubation continued for 2 h at 35 °C.

Immunofluorescence Microscopy—Calu-3 cell monolayers at 50% confluence were dual-labeled for proteins of interest as described previously (9). Briefly, cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, washed three times with PBS, and permeabilized with 0.2% Triton X-100 in 10% normal goat serum in PBS. Fixed cells were stained for 1 h at room temperature with primary antibodies (Molecular Probes) and washed three times with PBS. Fluorophore-tagged secondary antibody was applied at a 1:200 or 1:500 dilution for 1 h at room temperature. After four additional PBS washes, the polyester filters were cut from their support inserts and mounted in Vectashield mounting medium (Vector Laboratories) on glass microscope slides.

Confocal images were collected with a Zeiss LSM 510 Meta microscope (Carl Zeiss Microimaging) with a Plan Apo 100×, 1.4 NA objective. Optical sections of 1024 × 1024 pixels were collected, and z-sectioning was carried out at 0.41-μm intervals. Images were preprocessed by background correction in ImageJ (47), thresholded, and analyzed for colocalization using the Colocalization Threshold plugin (48). Image brightness and contrast were further adjusted in Photoshop (Adobe Systems) for improved reproduction.

Data Analysis—Data are representative of at least three or more experiments, unless otherwise stated. Treatment effects were evaluated using a two-sided Student’s t test for unpaired samples and, where appropriate, one-way analysis of variance with Tukey-Kramer multiple comparisons test as a post test, using InStat 3.0 software (GraphPad).

RESULTS

**Structural Basis of the CFTR Interaction with Filamin Repeat Ig21**—It was initially unclear which of the many Ig domains of the filamins bind to the CFTR N terminus. We noted, however, that there is limited sequence similarity between the CFTR N-terminal sequence and filamin-binding motifs of the cytoplasmic tails of β-integrins and of the cytosolic protein migfilin (Fig. 1A). Several β-integrins and migfilin bind to FlnA-Ig21 (23, 25). We therefore screened a number of CFTR N-terminal peptides for binding to 15N-labeled FlnA-Ig21 using HSQC NMR titrations. Addition of the peptide CFTR₄₋₂₂ (SPELEKASSVSKLFFSWTAP) induced extensive line broadening and chemical shift perturbations in the HSQC spectrum of 15N-labeled FlnA-Ig21 (data not shown), indicating that CFTR₄₋₂₂ binds to the Ig21 repeat.
Filamin Ig Repeat Interactions with CFTR

FIGURE 2. Details of binding interfaces between FlnA Ig21 strands C/D with the CFTR4–22 peptide. Putative hydrogen bonds are shown as dotted lines, and water molecules are shown as red spheres. A, interactions between CFTR4–22 (white carbons) and Ig21 molecule A (yellow carbons). B, interactions between CFTR4–22 (white carbons) and Ig21 molecule B (pink carbons).

We therefore cocryrstallized the FlnA-Ig21-CFTR4–22 complex and solved its structure to 2.8 Å resolution (Table 1). The asymmetric unit of the crystal contains an unexpected arrangement of two FlnA-Ig21 molecules and a single CFTR4–22 peptide. The CFTR4–22 peptide forms a β-strand along the entire length of one of the Ig21 molecules. The C-terminal part of the peptide also forms a partial β-strand with the other Ig21 molecule (Fig. 1B and supplemental Fig. 1). The Ig21 repeats interact with different (though overlapping) portions of the CFTR4–22 peptide. CFTR residues Val11–Trp19 interact with one of the FlnA-Ig21 molecules (Ig21A), whereas Phe16–Pro22 interact with the other (Ig21B). The N-terminal residues of the peptide (Pro5–Ser10) are well ordered in the crystal but interact with hydrophobic groups from CFTR (Val11, Leu15, Phe17, Trp19) and some of which have counterparts in other FlnA-Ig21 complexes. A linchpin serine residue (Ser13) interacts with the main-chain carbonyl of S2279. The main-chain amide of Ig21-A2281 and a water molecule bridges the Ser13 side-chain and the main-chain carbonyl of S2279. The CFTR4–22 interaction is shown in greater detail in Fig. 2, and water molecules are shown as red spheres. A, interactions between CFTR4–22 (white carbons) and Ig21 molecule A (yellow carbons). B, interactions between CFTR4–22 (white carbons) and Ig21 molecule B (pink carbons).

The Ig21 molecules display the characteristic structure of filamin repeats, with two β-sheets consisting of strands ABDE and CFGF, respectively (Fig. 1B). The CFTR4–22 peptide forms antiparallel β-strand interactions with strand C and makes hydrophobic side-chain interactions with strand D of each Ig21 molecule. The Ig21A-CFTR4–22 and Ig21B-CFTR4–22 complexes bury 640 Å² and 450 Å² of accessible surface area, respectively. The overall binding mode resembles that found in other structures of Ig-repeat-peptide complexes, such as FlnA-Ig21-integrin-β7 tail (23), FlnA-Ig21-integrin-β2 tail (49), FlnA-Ig21-megilin or FlnC-Ig21-megilin (25, 26), and FlnA-Ig17-GplBα tail (24). The Ig21A-CFTR4–22 and Ig21B-CFTR4–22 interactions are shown in greater detail in Fig. 2, A and B, respectively.

The CFTR4–22-Ig21A interaction has several key features, some of which have counterparts in other FlnA-Ig21-ligand complexes. A linchpin serine residue (Ser13) interacts with the main-chain amide of Ig21-A2281 and a water molecule bridges the Ser13 side-chain and the main-chain carbonyl of S2279. The linchpin serine is conserved in all of the filamin-binding sequences listed in Fig. 1A. On either side of Ser13, alternating hydrophobic residues from CFTR (Val11, Leu15, Phe17, Trp19) interact with hydrophobic groups from β-strand D of Ig21A. Importantly, our crystal structure readily explains the basis of the defect in the CF-causing mutation S13F, which was previously shown to eliminate binding between CFTR and filamins (15). A bulky residue (such as phenylalanine) at the Ser13 position cannot accommodate the hydrogen bonds mediated by the serine side chain. More importantly, phenylalanine and other bulky side chains clash directly with the surface of FlnA-Ig21, specifically with the loop connecting β-strands C and D or with strand D itself. As shown in supplemental Fig. 2, one of the most common rotamers of phenylalanine induce such clashes.

The complex between CFTR4–22 and the second Ig21 repeat (Ig21B) superficially resembles the Ig21A-CFTR4–22 complex, but the peptide is displaced by several positions (Fig. 2B). Ser13 in the Ig21B-CFTR4–22 complex occupies a similar position as Ser13 in the Ig21A-CFTR4–22 complex. Ser13 is also flanked by hydrophobic moieties (Phe16, Pro22). Significantly, however, Ig21B makes no contacts with Ser13. Because the S13F mutation eliminates CFTR-filamin interactions in vivo (15), we conclude that the contacts between CFTR4–22 and Ig21B are a crystallographic artifact and do not represent a physiologically relevant interaction (see below).

The CFTR4–22 peptide exhibited nonspecific aggregation behavior, making it difficult to map its specific binding site onto FlnA-Ig21 by NMR methods or to measure its affinity for the Ig repeat. We therefore carried out HSQC NMR titrations with shortened CFTR peptides with F16E or F17E mutations, which were more soluble and did not aggregate. The peptide CFTR7–20-F16E (EKASVVSKEFSWT) perturbed chemical shifts that map to β-strands C and D of FlnA-Ig21, in agreement with the crystal structure (Fig. 3A). In contrast, addition of CFTR7–20-S13F/F16E had no effect (Fig. 3A; supplemental Fig. 3). The CFTR7–20-F17E and CFTR7–20-S13F/F17E peptides behaved similarly to their F16E counterparts (data not shown).

If the Ig21B-CFTR4–22 interaction mode observed in our crystal structure is also present in solution, this interaction should be insensitive to the S13F mutation. We observe, however, that the S13F mutation eliminates all binding of CFTR N-terminal peptides to Ig21 in solution. The HSQC NMR titration data therefore support the validity of the Ig21A-CFTR4–22 interaction and support the conclusion that the Ig21B-CFTR4–22 interaction is a crystallographic artifact.

CFTR Interactions with Other Filamin Ig Repeats—Some membrane protein partners of FlnA may bind to more than one Ig-like repeat of FlnA. For example, the integrin-β7 tail binds to both FlnA-Ig21 and, more weakly, to FlnA-Ig19 (23). We investigated whether the CFTR N terminus binds in vitro to FlnA-Ig17, Ig19, and Ig23, which have high sequence similarity to Ig21. We also tested FlnA-Ig10, in which β-strands C and D are more poorly conserved than in the former repeats (supplemental Fig. 4). The CFTR7–20-F16E peptide perturbs the HSQC spectra of 15N-FlnA-Ig17, Ig19, and Ig23 (Fig. 3B–D and supplemental Fig. 5). The chemical shifts of these repeats were previously assigned (24, 37, 38). HSQC NMR titration data show that CFTR7–20-F16E binds to the C and D strands of all these Ig repeats. In contrast, the corresponding S13F peptide does not bind to any of these repeats (supplemental Fig. 5).
Filamin Ig Repeat Interactions with CFTR

These data strongly suggest that the CFTR binding modes for these repeats are similar to that for Ig21.

We titrated the chemical shift perturbations of the Ig repeats to measure their affinities for the CFTR-N terminal peptide (Table 2 and supplemental Fig. 6). Although all of the affinities are modest, we found that Ig21, Ig19, and Ig23 had relatively similar affinities, whereas Ig17 bound more weakly. All of the binding curves fit well to a binding equation that assumes a 1:1 stoichiometry rather than a 1:2 stoichiometry, further supporting the notion that the Ig21B–CFTR interaction in our crystal structure is an artifact.

We used homology modeling and computational docking to examine putative binding modes between the CFTR N terminal and Ig17, Ig19, and Ig23 (Fig. 4, A–C). In all three cases, the peptide can accommodate most of the interactions found in the Ig21A–CFTR crystal structure. Interestingly, the apparent hydrogen bond distance between the Ser13 side-chain hydroxyl and the corresponding backbone amide group increases with decreasing CFTR peptide affinity of these Ig repeats (Table 2). In the Ig17 complex, the β-strand-like hydrogen bonding geometry and hydrophobic side-chain packing are suboptimal near the C terminus of the peptide, compared with the Ig21A–CFTR complex. This may account for the weaker in vitro affinity of the CFTR N-terminal peptide for Ig17 compared with Ig21.

Unlike the other repeats that were tested, Ig10 does not bind to CFTR–F16E (supplemental Figs. 6 and 7). We generated a homology model for FlnA–Ig10 to understand why this repeat does not bind to the CFTR N terminus (Fig. 5, D and E). Unlike the FlnA–Ig21 CD loop, the FlnA–Ig10 CD loop contains a 3-residue insertion, whereas a canonical proline is shifted by 2 positions toward β-strand D. This results in steric clashes between the FlnA–Ig10 CD loop and the CFTR N-terminal peptide. Additional clashes between CFTR and FlnA–Ig10 β-strand C result in poor packing. We were unable to model the CFTR peptide in a conformation that simultaneously relieves these clashes while maintaining a hydrogen bond between Ig10 and the Ser13 side chain. The CD loop of filamin Ig repeats may act as an important selectivity determinant for ligand binding.

Interaction of FlnA-Ig Repeats with Full-length CFTR—We investigated whether isolated FlnA-Ig repeats interact with native CFTR in a cellular context. We first determined whether single Ig repeats could compete with native FlnA for native full-length CFTR. We preincubated Calu-3 cell lysates with increasing amounts of Ig21, then immunoprecipitated FlnA and probed immune complexes for CFTR. Preincubation with Ig21 reduced coimmunoprecipitation of CFTR and native FlnA (Fig. 5A), showing that Ig21 competes with native FlnA for binding to CFTR. In contrast, Ig10 had no effect on the coimmunoprecipitation of native FlnA and CFTR, even at much higher concentrations than Ig21 (Fig. 5A).

CFTR N-terminal peptides transfected into CFTR-expressing cells reduce surface expression of CFTR by displacing CFTR from native filamins (15). We hypothesized that CFTR-binding Ig repeats could displace native filamins from native CFTR and would thus reduce surface levels of CFTR. We used the BioPORTER protein delivery system to deliver recombinant Ig repeats into Calu-3 cells (45). We quantified surface expression of CFTR by biotinylation of the apical plasma membrane followed by streptavidin bead pulldown and immunoblot analysis (46). BioPORTER reagent alone did not affect surface expression of CFTR (Fig. 5B), whereas FlnA-Ig21 decreased surface expression of CFTR in a dose-dependent manner (Fig. 5, C and D). Loss of surface CFTR is accompanied by a proportional loss in CFTR-associated FlnA and RACK1, a signaling protein that promotes CFTR expression at the apical surface (45). The results demonstrate that intracellular FlnA-Ig21 can act in a dominant negative fashion, reducing surface levels of CFTR by displacing native FlnA. These results reemphasize the importance of the CFTR-FlnA interaction for the maintenance of CFTR surface expression (15).
We investigated whether the other Ig repeats that bound CFTR N-terminal peptides in vitro had effects similar to those of Ig21 when delivered into Calu-3 cells (Fig. 5E). As expected, BioPORTER delivery of Ig10 had no effect on surface CFTR levels. Ig17, which binds more weakly to the CFTR N terminus than Ig21, also had no significant effects on surface CFTR. Ig19 also reduced surface CFTR. Surprisingly, Ig23 had little effect, even though Ig23 binds to CFTR N-terminal peptides in vitro. These results show that FlnA-Ig19 and Ig21 can effectively compete with CFTR-FlnA interactions in a native cellular context. This suggests that, at the very least, native CFTR/FlnA complexes involve interactions involving Ig19 and Ig21.

Using confocal microscopy, we examined the effect of Ig21 delivery on the localization of CFTR and native FlnA in Calu-3 cells. FlnA colocalizes extensively with cortical actin in these and other cell types and is effectively a membrane marker (data not shown). In untreated Calu-3 monolayers, we observed extensive colocalization of FlnA and CFTR at apical and lateral membranes (Fig. 6A). Limited punctate staining of CFTR in the cytoplasm may correspond to biosynthetic intermediates or endocytosed CFTR (50). Upon treatment with BioPORTER-delivered Ig21, FlnA-CFTR colocalization at apical and lateral membranes is decreased, accompanied by a small increase in cytosolic punctate CFTR staining (Fig. 6B). Total CFTR immunofluorescence is also reduced, likely due to lysosomal degradation of internalized CFTR (15). These data show that BioPORTER-delivered Ig21, by decoupling CFTR from filamin, promotes CFTR internalization from the plasma membrane.

**DISCUSSION**

A number of scaffolding and adaptor proteins are implicated in the regulation of expression and activity of CFTR at the cell surface (51, 52). Filamins apparently stabilize CFTR at the surface by linking the CFTR N terminus to the membrane-proximal actin cytoskeleton (15). Potentially, filamins also antagonize the recruitment of CFTR by the endocytic machinery. S13F-CFTR, which is unable to bind to filamins, exhibits reduced surface levels compared with wild-type CFTR. In addition, CFTR surface levels are suppressed in cells transfected with peptides that mimic the CFTR N terminus and ostensibly displace native CFTR from filamins (15). We now identify immunoglobulin-like repeats that bind to the CFTR N terminus and elucidate the structural basis of this interaction.

The relationship between the CFTR N terminus and previously characterized filamin-binding motifs is ambiguous at first glance (Fig. 1A). Our structural data demarcate the filamin-binding site of CFTR between residues Ser<sup>15</sup> and Trp<sup>19</sup>. The binding mode of this sequence to FlnA-Ig21 resembles that of
Filamin Ig Repeat Interactions with CFTR

FIGURE 5. Inhibition of the endogenous CFTR-FlnA interaction and suppression of surface CFTR levels by recombinant Ig repeats. A, immunoprecipitation of endogenous FlnA from Calu-3 cell lysates preincubated with of FlnA-Ig21 (upper panel) or FlnA-Ig10 (lower panel) for 30 min at 30 °C. Exposed bands were quantitated by densitometry, and the ratio of CFTR to FlnA was calculated from densitometric readings. FlnA-Ig21 results are representative of two experiments in which each condition was replicated twice. For FlnA-Ig10, results from two replicates are shown. Amount and approximate concentration of Ig repeat are shown above each lane (0.5-ml assay volume). B, Calu-3 cells incubated with BioPORTER reagent alone (--) or with BioPORTER-FlnA-Ig21 (+), as described under “Experimental Procedures.” Cells were biotinylated on the apical surface immediately after incubation with the BioPORTER reagent (0 time) or 2 h after incubation (2 h). Biotinylated proteins (Bead) and a 50-μg aliquot of supernatant from the streptavidin pulldown (Super) were immunoblotted for CFTR and an actin control. No actin was detected with biotinylated proteins, indicating that the cells retained their integrity. Typical results from one of five experiments are shown. C, dose-dependent loss of surface CFTR upon BioPORTER delivery of increasing amounts of FlnA-Ig21. FlnA and the CFTR-associated scaffold protein RACK1 were detected in reprobes of immunoblots of biotinylated proteins. Typical results from one of six experiments are illustrated. 0- indicates an experiment carried out without added BioPORTER. D, quantitation of surface CFTR from Ig21-treated Calu-3 cells; the data show means ± S.E. (error bars) for 5–11 separate experiments. E, effects on surface CFTR levels caused by BioPORTER delivery of 10 μg of selected Ig repeats (0.5-ml reactions). Plasma membrane CFTR was quantitated by surface biotinylation and streptavidin pulldown. Results shown are derived from three to six experiments for each repeat; standard errors are shown. Asterisks indicate that Ig19 and Ig21 suppression of surface CFTR levels is significant (*, p < 0.05) or highly significant (**, p < 0.01) compared with BioPORTER alone or compared with Ig10.

Conservation between these sequences and accumulating structural data emphasizes the importance of a conserved serine (Ser13 in CFTR) flanked by alternating hydrophobic residues. These side chains make hydrophobic contacts with strand D of Ig21, whereas the backbone of the CFTR N terminus forms an antiparallel β-strand with strand C of Ig21. The linchpin Ser13 residue intimately contacts the surface of FlnA-Ig21 and makes several hydrogen bonds. Our NMR titration data and computational modeling suggest that other FlnA-lg repeats that bind to the CFTR N terminus in vitro adopt a similar binding mode (Figs. 3 and 4). In addition, these repeats are highly similar in sequence to their counterparts in FlnB (68–84% identity; 91–100% similar). Thus, CFTR likely interacts with FlnB in a similar manner as with FlnA.

Substitution of Ser13 with phenylalanine disrupts the interaction between CFTR N-terminal peptides and all of the filamin Ig-like repeats that we tested because of steric clashes with the CD loop of the Ig repeats (supplemental Fig. 2). The S13F mutation is one of several mutations in the CFTR N terminus that are listed in the Cystic Fibrosis Mutation Database. Two other mutations in this region, P5L and W19C, are thought to cause CF primarily because they lead to biosynthetic defects (53). In titrations of 15N-FlnA-Ig21 with CFTR N-terminal peptides containing these mutations, the P5L interacted robustly with the Ig repeat whereas W19C interacted more weakly (data not shown). Unlike the S13F mutation, the CF-causing defect in these CFTR mutants is probably not primarily due to disruption of filamin-CFTR interactions.

The basis of specificity and selectivity of filamin-ligand interactions remains an area of extensive investigation and great interest. This is due both to the diversity of known filamin ligands and to the sheer number of filamin Ig repeats that potentially act as ligand binding sites. The relatively high degree of structural and sequence conservation among Ig repeats further complicates attempts to elucidate selectivity and specificity. A consensus is emerging that specific ligands likely interact with more than one Ig repeat and vice versa. Ig21, for example, interacts in a similar manner with integrin β-tails and in migfilin. Conservation between these sequences and accumulating structural data emphasizes the importance of a conserved serine (Ser13 in CFTR) flanked by alternating hydrophobic residues. These side chains make hydrophobic contacts with strand D of Ig21, whereas the backbone of the CFTR N terminus forms an antiparallel β-strand with strand C of Ig21. The linchpin Ser13 residue intimately contacts the surface of FlnA-Ig21 and makes several hydrogen bonds. Our NMR titration data and computational modeling suggest that other FlnA-lg repeats that bind to the CFTR N terminus in vitro adopt a similar binding mode (Figs. 3 and 4). In addition, these repeats are highly similar in sequence to their counterparts in FlnB (68–84% identity; 91–100% similar). Thus, CFTR likely interacts with FlnB in a similar manner as with FlnA.
Filamin Ig Repeat Interactions with CFTR

These criteria are fulfilled in other structurally characterized Ig repeat-ligand complexes and likely comprise the minimal requirements for micromolar or higher affinity interactions between Ig repeats and their partners. We limited our investigation to those Ig repeats in the second rod-like segment of FilA which were previously shown to interact with membrane proteins. Sequence analysis of other Ig repeats suggests that most of these are unlikely to bind to the CFTR N terminus. Our conclusion is based primarily on the sequence differences between their C and D strands and those of Ig21 (data not shown). In particular, the CD loops of these repeats differ in length and in predicted conformation from those of the Ig repeats tested in our work.

Although the ligand-binding criteria described above are fulfilled by the Ig21-CFTR interaction, the in vitro affinity is relatively modest compared with affinities measured for other Ig repeat-ligand interactions (23–26, 54). We investigated whether the relevant Ig repeats could bind to the CFTR N terminus in epithelial cells by testing their potential to act as competitive inhibitors of the interaction between CFTR and native filamins. The most strongly interacting repeat, Ig21, competed robustly with the CFTR-filamin interaction in Calu-3 cell lysates (Fig. 5). Significantly, BioPORTER-delivered Ig21 caused dislocation of CFTR from the apical plasma membrane as gauged both qualitatively by confocal microscopy and quantitatively by surface biotinylation assays (Figs. 5 and 6). Ig19 had a similar effect. The inability of Ig17 to compete with the native CFTR-filamin interactions is likely due to its lower intrinsic affinity for the CFTR N terminus. It is unclear, however, why Ig23 did not significantly suppress surface CFTR levels.

Interestingly, Ig21 bound to native CFTR and competed with full-length filamin at concentrations significantly below the in vitro affinity of Ig21 for CFTR N-terminal peptides. Instead, these concentrations fall into the range of effective affinities described in the accompanying paper by Playford et al. (56). Playford and co-workers measured affinities of individual Ig repeats by pulling down EGFIP-Ig repeat fusions from lysates using CFTR N-terminal peptides conjugated to agarose beads. Each bead likely displays multiple CFTR peptides. In addition, the slight tendency of EGFPI to oligomerize may allow avidity effects to come into play. The protocol of Playford et al. likely simulates an encounter between dimeric filamin (with multiple ligand-binding Ig repeats) and a cluster or array of CFTR molecules. Similarly, the BioPORTER-delivered Ig repeats likely encounter clusters or assemblies of CFTR in the cellular context. Filamins have the capacity to promote CFTR clustering through multivalent binding (see discussion below). There is also evidence that CFTR forms dimers or oligomers in the plasma membrane through direct lateral interactions or through linking proteins other than filamins (13, 55, 57; for review, see 52). The micromolar affinities measured in our solution NMR experiments thus probably represent an effective upper (weaker) limit, whereas the effective affinities in cells are stronger due to avidity effects.

How does CFTR interact with filamins in a cellular context, and what role does this interaction play within the greater collective of proteins associated with functional CFTR at the apical plasma membrane? Our findings suggest that CFTR and FilA in the CD loop is important. Structural changes in this loop, such as those caused by the introduction of a proline in the CD loop of Ig10 (Fig. 4), inhibit binding. Notably, Ig10 also does not bind the filamin-interacting site of migfilin (54), which contains a similar linchpin serine (Fig. 1A). Second, the portions of β-strands C and D near the C terminus of the ligand must be close enough to each other to allow for proper packing against hydrophobic side chains of the ligand while also accommodating backbone-backbone hydrogen bonds between strand C and the ligand. Satisfying these requirements may conflict with the maintenance of proper bonding geometry near the linchpin serine. Finally, the ligand must be able to adopt a conformation that accommodates 8–10 β-strand-like hydrogen bonds with strand C without substantial steric clashes.

FIGURE 6. Immunofluorescence labeling of endogenous FilA and CFTR in untreated and Ig21-treated Calu-3 cells. CFTR was immunolabeled with mouse anti-CFTR and Alexa Fluor 488-labeled secondary antibody (cyan). FilA was immunolabeled with mouse anti-human filamin antibody and Alexa Fluor 568-labeled secondary antibody (magenta). A, localization of CFTR and FilA in untreated Calu-3 samples. Both CFTR and FilA are primarily at the plasma membrane. Top row, apical XY slice from the Z series showing colocalization (white) of the proteins. Middle row, XY slice in the middle of the Z series, showing colocalization of the proteins at lateral membranes. Bottom row, transverse (YZ) slice of the Z series. B, CFTR and FilA immunofluorescence in Calu-3 cells treated with BioPORTER-Ig21. Apical, middle, and transverse sections similar to those in A are shown.
interact through the Ig21 or Ig19 repeats at the very least. Playford et al. (56) identify Ig23 as another major CFTR binding site. They also show that deletion of all the odd-numbered repeats in the second rod domain of filamin as well as repeats 4, 9, and 12 in the first rod domain of filamin is necessary to abrogate binding fully. Because filamin is a dimer, filamin binding may promote or stabilize CFTR homodimerization or clustering. This effect is further enhanced if each filamin monomer simultaneously interacts with multiple CFTR N termini. The modest intrinsic affinities of individual CFTR-Ig repeat interactions can allow for such clusters to undergo rapid and dynamic remodeling when required. A similar clustering model has been posited for regulation of integrins by filamin (54). Given that pulmonary epithelia express several integrin β-chains, including integrin β1 (58), “mixed” clusters of CFTR and integrins or other integral membrane proteins may also be organized through filamin binding.

In contrast to the CFTR N terminus, the CFTR C terminus interacts with the PDZ domain-containing protein NHERF1, which connects to the actin cytoskeleton through ezrin (10, 11) and may also bind to microtubules (46). It is currently unclear whether CFTR molecules interact with NHERF1 and filamin simultaneously or if, instead, there are distinct populations of CFTR-NHERF1 complexes and CFTR-filamin complexes at the cell surface. CFTR retained at the surface in our surface biotinylation assays is associated with filamin and RACK1 (Fig. 5C). In preliminary experiments, however, we found that little NHERF1 is present in Flna immunoprecipitates from Calu-3 lysates (supplemental Fig. 8). This suggests that NHERF1 may not associate directly with Flna or with CFTR that is bound to Flna, even though NHERF1 and Flna have distinct binding sites on CFTR. It will be crucial to investigate whether, like NHERF1, filamin can serve both to link CFTR to the cytoskeleton and to act as a scaffold for the recruitment of other regulatory factors.

Our results are highly relevant to CF, a genetic disease characterized by loss of epithelial fluid secretion in the airways, pancreatic duct, and intestine. Disruption of binding of CFTR with Flna profoundly affects surface expression of wild-type CFTR. The most common CFTR mutation, ∆F508-CFTR, results in loss of channel protein in the plasma membrane. Importantly, ∆F508-CFTR recovered from the endoplasmic reticulum using F508-CFTR, results in the transfer of wild-type CFTR into affected organs, such as lung or pancreatic duct.

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Filamin Ig Repeat Interactions with CFTR

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