Published in final edited form as:

Methods Mol Biol. 2011 ; 741: 255–270. doi:10.1007/978-1-61779-117-8_17.

Analysis of CFTR Interactome in the Macromolecular Complexes

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Abstract

Cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel localized primarily at the apical surface of epithelial cells lining the airway, gut, exocrine glands, etc., where it is responsible for transepithelial salt and water transport. A growing number of proteins have been reported to interact directly or indirectly with CFTR chloride channel, suggesting that CFTR might regulate the activities of other ion channels, receptors, and transporters, in addition to its role as a chloride conductor. Most interactions occur primarily between the opposing terminal tails (N or C) of CFTR and its binding partners, either directly or mediated through various PDZ domain-containing proteins. This chapter describes methods we developed to cross-link CFTR into a macromolecular complex to identify and analyze the assembly and regulation of CFTR-containing complexes in the plasma membrane.

Keywords

CFTR; Cross-linking; Macromolecular complex; PDZ protein; Interacting partner

1. Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) is a plasma membrane cAMP-regulated Cl[−] channel that is responsible for transepithelial salt and fluid transport (1–3). It is localized primarily to the luminal, or apical, membranes of epithelial cells in functionally diverse tissues including the airway, intestine, pancreas, kidney, vas deferens, and sweat duct (1–3). CFTR has been implicated in two major diseases: cystic fibrosis (CF) and secretory diarrhea (4, 5). In CF, the synthesis or functional activity of the CFTR Cl[−] channel is reduced. This autosomal recessive disorder affects approximately 1 in 2,500 Caucasians in the United States (4, 5). Excessive CFTR activity has also been implicated in cases of toxin-induced secretory diarrhea (e.g., by cholera toxin and heat stable *E. coli* enterotoxin) that stimulates cAMP or cGMP production in the gut (5, 6). CFTR belongs to the superfamily of ATP-binding cassette transporters, which bind ATP and use the energy to drive the transport of a wide variety of substrates across cellular membrane (7). It is composed of two repeated motifs, each of which consists of a hydrophobic membrane-spanning domain containing six helices and a cytosolic hydrophilic region for binding with ATP (8). These two motifs are linked by a cytoplasmic regulatory domain that contains many charged residues and multiple consensus phosphorylation sites (substrates for various protein kinases, such as PKA, PKC,

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Notes

and PKG). Both the amino and carboxyl terminal tails of this membrane protein are cytoplasmically oriented and mediate the interaction between CFTR and a growing number of binding proteins (5, 9).

1.1. CFTR Forms Macromolecular Signaling Complexes with a Wide Variety of Proteins

A growing body of evidence suggest the existence of various physical and functional interactions between CFTR and many other proteins, including transporters, ion channels, receptors, kinases, phosphatases, signaling molecules, and cytoskeletal elements, and these interactions between CFTR and its binding proteins have been shown to play an important role in regulating CFTR-mediated transepithelial ion transport *in vitro* and most probably *in vivo* (10–24). Among these reported interactions, many are mediated through a physical interaction between these binding proteins with both the amino/carboxyl terminal tails of the CFTR chloride channel (21–24). The amino terminal tail interacts directly with syntaxin 1A and is responsible in part for inhibiting CFTR Cl^- current activity (19, 20). In this chapter, we focus on only the methods and approaches that aid in the study of the interactions between CFTR carboxyl terminal tail, which possesses a protein-binding motif, and a group of scaffolding proteins, which contain a specific binding module referred to as PDZ domains.

PDZ domains are composed of 80–90 amino acid residues that form peptide-binding clefts and mediate interactions, usually with the carboxyl termini of target proteins, which terminate in consensus PDZ-binding sequences (also referred to as PDZ motif) (5, 25–27). So far, six different PDZ scaffolding proteins have been reported to bind to the carboxyl terminal tail of the CFTR channel with various affinities: NHERF1, NHERF2, PDZK1, PDZK2, CAL (CFTR-associated ligand), and Shank2 (28–34). Among these PDZ proteins, four (NHERF1, NHERF2, PDZK1, and PDZK2) possess multiple PDZ domains, whereas CAL and Shank2 have only one PDZ domain (27). NHERF1, NHERF2, PDZK1, PDZK2, and Shank2 have been reported to be localized to the apical membranes of epithelial cells where CFTR also resides, while CAL is localized primarily to Golgi. These CFTR-interacting scaffold proteins demonstrate different tissue distributions, thereby suggesting that different PDZ proteins might interact with CFTR in different tissues (27). The PDZ motif within CFTR that is recognized by PDZ proteins is the last four amino acids at the C terminus (i.e., 1477-DTRL-1480 in human CFTR) (28–34). Interestingly, CFTR can bind to more than one PDZ domain of both NHERFs and PDZK1, albeit with varying affinities (30). This multivalency with respect to CFTR binding has been shown to be functionally significant, suggesting that PDZ proteins may facilitate formation of CFTR macromolecular signaling complexes (21–24). Protein-protein interactions that influence the expression or functional activity of the CFTR channel at the plasma membrane have significant physiological importance, as this channel not only transports Cl^- and HCO_3^- but also regulates the activities of many other transporters and channels (27). The physiological significance of these interactions is that they not only provide a means to link CFTR activity to various epithelial functions and processes but they also coordinate the CFTR chloride channel function with the overall physiologic demands of epithelial cells (5, 27).

1.2. Biochemical Assays Used to Analyze CFTR Interactome for Novel CFTR-interacting Partners

Multiple biochemical assays have been developed to study CFTR-involving protein interactions, such as co-immunoprecipitation, pull-down assay, pair-wise binding assay, colorimetric pair-wise binding assay, and macromolecular complex assembly assay (19–24, 35, 36). Here we focus on the detailed procedures of analyzing and identify the interacting partners in cross-linked CFTR-containing complex (36), which is used extensively by our laboratory to study protein-protein or domain-domain interactions involving CFTR.

2. Materials

2.1. General Reagents

1. pGEX4T-1 vector (Amersham Pharmacia Biotech, Piscataway, NJ).
2. pMAL-C2 vector (New England BioLabs, Ipswich, MA)
3. pET30 vector (Novagen, Gibbstown, NJ)
4. pcDNA3 (Invitrogen, Carlsbad, CA)
5. Glutathione Sepharose beads (Amersham)
6. Amylose resin (New England BioLabs, Ipswich, MA)
7. Talon beads (Clontech, Mountain View, CA)
8. Restriction enzymes (Promega, Madison, WI)
9. PCR reagents (Stratagene, La Jolla, CA)
10. Oligonucleotide primers (Genemed Synthesis, San Antonio, TX)
11. ECL reagents (GE Health Care, Piscataway, NJ)

All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

2.2. Media and Buffers

1. Luria-Bertani containing ampicillin (LB-Amp) or kanamycin (LB-Kan) medium (pH 7.0): 10 g bactotryptone, 5 g bacto yeast extract, and 10 g NaCl per liter. Autoclave, cool, and add filter-sterilized ampicillin (50 $\mu\text{g/mL}$) or kanamycin (30 $\mu\text{g/mL}$).
2. Sucrose buffer: 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM PMSF, and 10% sucrose.
3. Elution buffer for GST fusion proteins: 25 mM Tris-HCl (pH 8.0), 140 mM NaCl, and 20 mM reduced glutathione.
4. Elution buffer for His fusion proteins: 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 200 mM imidazole.
5. Elution buffer for MBP fusion proteins: 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10 mM maltose.
6. Phosphate-buffered saline (PBS) (pH 7.4): 140 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 2.7 mM KCl, and 140 mM NaCl.
7. PBS-C/M: PBS (pH 7.4), supplemented with 1 mM MgCl_2 and 0.1 mM CaCl_2 .
8. Lysis buffer: PBS (pH 7.4) – 0.2% Triton X-100 containing protease inhibitors (1 mM PMSF, 1 $\mu\text{g}/\mu\text{L}$ leupeptin, 1 $\mu\text{g}/\mu\text{L}$ aprotinin, and 1 $\mu\text{g}/\text{L}$ pepstatin).
9. RIPA buffer: 150 mM NaCl, 50 mM Tris-base (pH 8.0), 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS.
10. Sample buffer (5 $\times$): 0.6 M Tris-HCl (pH 6.8), 50% glycerol, 2% sodium dodecyl sulfate (SDS), and 0.1% bromophenol blue; add 5% β -mercaptoethanol (β ME) before use.
11. Buffer-A: 20 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM DTT, and protease inhibitors.

12. Buffer-B: 20 mM HEPES, pH 7.4, 1 mM EDTA, and protease inhibitors.

2.3. Cell Culture

1. All cell culture media and reagents are from Invitrogen (Carlsbad, CA) unless otherwise specified.
2. All cell lines except BHK cells are from American Type Culture Collection (Manassas, VA). BHK-parental, BHK-CFTR and BHK-CFTR_{his10} are from Dr. John Hanrahan (McGill University, Canada)
3. HEK293 (human embryonic kidney 293) cells, COS-7 cells (African green monkey kidney cells), and HT29-CL19A cells (human colonic epithelial origin) are grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).
4. Calu-3 cells (serous gland epithelial cells) are grown in Eagle's minimal essential medium (MEM) supplemented with 15% FBS, 1 mM sodium pyruvate, and 1 mM nonessential amino acids,
5. T84 cells (human colonic epithelial origin), BHK (baby hamster kidney) cells stably expressing CFTR (BHK-CFTR), and C-terminal polyhistidine-tagged CFTR (BHK-CFTR_{his10}) are grown in DMEM and Ham's F-12 supplemented with 15 mM HEPES and 10% FBS.

2.4. Antibodies, Constructs, and Uncommon Reagents

1. CFTR monoclonal antibodies R1104 mab recognizing the regulatory domain of CFTR (epitope at a.a. 722–734), GA1 mab (a.a. 1440–1460), and NBD1-R (a.a. 521–828) polyclonal antibody are custom-made (35, 36). Anti-CFTR IgG MM13-4 (a.a. 25–36) is from Millipore (Temecula, CA).
2. Anti-NHERF1 IgG is from BD-Transduction Laboratories (Lexington, KY).
3. Affinity-purified anti-syntaxin 1A IgG (14D8) is custom-made (19, 20).
4. Affinity-purified rabbit anti-G β IgG is from Santa Cruz Biotechnology (Santa Cruz, CA).
5. Anti-NHERF2 antibody is from Dr. Emanuel Strehler (Mayo Clinic, Rochester, MN).
6. Sulfo-NHS-LC-biotin and various cross-linkers are from Pierce Biotechnology (Rockford, IL).
7. Cross-linkers used in the present chapter are listed in Table 15.1.

3. Methods

3.1. Expression and Purification of Recombinant Tagged Fusion Proteins in Bacteria

1. Amplify defined regions of CFTR C-tail (a.a. 1454–1480 or a.a. 1400–1480) and NHERFs (full-length or PDZ1 or PDZ2 domains) by PCR (containing appropriate restriction enzyme sites). Clone the PCR products into pGEX4T-1 vector (Pharmacia) for GST-NHERFs (or their PDZ1 or 2 domains), pMAL-C2 vector (New England Biolabs) for MBP-CFTR C-tail or MBP-LPA2 C-tail, and pET30 (Novagen) for His-S-CFTR C-tail.
2. Transform in a protease-deficient *E. coli* strain (BL21-DE3) as protein integrity is often an issue.

3. Grow the culture overnight (37°C) in LB with appropriate antibiotics (Amp or Kan). Dilute the overnight culture 1:10 and grow a further 2 h at 37°C. Induce with 0.5–1 mM IPTG for the next 4 h at 30°C. Pellet the cells by centrifugation at 8,000g for 10 min at 4°C.
4. Lyse the cells in sucrose buffer (20 mL for cell pellet originating from 1 L of culture) containing lysozyme (1 mg/mL), 0.2% Triton X-100, and protease inhibitors (*see Subheading 2.2.2.*). Mix on a rotary shaker for 30 min at 4°C.
5. Spin at 20,000g for 30 min at 4°C. Collect the clear supernatant.
6. Into the clear supernatant, add 1 mL of the following resin/agarose beads (50% slurry in sucrose buffer):
 - Glutathione sepharose beads for GST fusion proteins.
 - Talon beads for His-S fusion proteins.
 - Amylose resin for MBP fusion proteins.
7. Mix for 4 h at 4°C on a rotary shaker.
8. Wash the beads by resuspending in PBS (15 mL), mix for 2 min, spin (800g for 2 min), and decant. Repeat this step for six times.
9. Elute the protein from the beads by using respective elution buffer (2 mL elution buffer per 1 mL of beads; *see Subheadings 2.2.3, 2.2.4, and 2.2.5; also see Note 1*).
10. Dialyze the eluted proteins against 2 L of PBS. Change PBS every 4 h for four times. Concentrate the protein using a Centricon filter (10,000 MW cutoff, Millipore) and store as small aliquots at –80°C.
11. Determine the protein concentration by the Bradford method. Assess protein quality by SDS-PAGE using BSA as standard. If the integrity of the protein is not satisfactory, secondary purification procedures such as gel filtration or ion exchange may be used. (e.g., we have used a G-75 Sepharose column to further purify GST-fusion protein).

3.2. Preparation of Cross-linker Working Solutions

1. Always use freshly made cross-linkers to cross-link CFTR and its interacting partners.
2. Prepare the cross-linkers (*see Table 15.1*) on the day of the experiment.
3. Stocks are freshly prepared in DMSO for membrane-permeable cross-linkers (i.e., DSP, DFDNB, etc.) and in PBS-C/M (*see Subheading 2.2.7.*) for membrane-impermeable cross-linkers (i.e., DMP, DTBP, etc.), respectively.
4. For example, to make 1 mM DSP solution, weigh 4.0 mg DSP into a 15-mL tube, add 200 μ L of DMSO (dissolve completely). To this add 9.8 mL of warm PBS-C/M. Incubate at 37°C in dark until use.

3.3. Chemical Cross-linking of CFTR and its Interacting Partners

1. Culture cells in 60- or 100-mm Petri dishes until the cells become confluent.

¹It is important that the pH of the elution buffer be adjusted to 8.0 after adding reduced glutathione. The pH can be as low as 3.0 after the addition of reduced glutathione. If the pH is not adjusted before elution, the efficiency of elution is drastically reduced.

2. Wash cells for ~2 min with PBS-C/M pH 7.4 at 22°C (Alternatively, one could use membrane/vesicular preparation lacking Tris or amino group containing buffers). Repeat two more times.
3. Make cross-linker solution in PBS-C/M and incubate the solution at 37°C (prepare fresh each time, just before the incubation).
4. Incubate cells with cross-linker solution for varying times at 37°C (we typically incubate cells with cross-linkers for 10 min).
5. Treat control cells with prewarmed PBS-C/M (containing no cross-linkers).
6. Rinse cells with 10 mL of chilled TBS (Tris base saline) for 1 min (this step is optional as RIPA has Tris, which acts as a quencher for cross-linking reaction - *see* Note 2).
7. Remove the buffer completely from the plate and add 1–2 mL of RIPA buffer (*see* **Subheading 2.2.9.**) to each plate.
8. Scrape cells off plate and transfer into a microcentrifuge tube (1.5-mL) and mix (10 min at 4°C).
9. Centrifuge at 14,000g at 4°C for 10 min.
10. Take clear supernatant (Immunoprecipitate if necessary).
11. Make two sets of samples, one with sample buffer (no β ME), and the other with 5% β ME. Incubate samples at 37°C for 10–15 min. (*See* Note 3)
12. Run 4–15% or 5% gel (depending on the protein mass) SDS-PAGE.
13. Western blot with anti-CFTR antibody (or look for CFTR-interacting partners using specific antibodies; *see* **Subheading 3.4.**).
14. Representative data are shown in Fig. 15.1 (*also see* ref. 36).

3.4. CFTR:NHERFs Stoichiometry in Apical Plasma Membrane

1. Culture and polarize epithelial cells (Calu-3 or HT29-CL19A) on permeable support (24-mm diameter Transwell in 6-well plates) until they form tight junction (usually takes 10–15 days)
2. Wash cells twice with prewarmed PBS-C/M.
3. Transfer the Transwell to new, clean 6-well plates and wash cells once with prewarmed PBS-C/M.
4. Cross-link cells at both sides (apical/basolateral) with 1 mM prewarmed DSP (dissolved in DMSO, made fresh in prewarmed PBS-C/M) for 10 min at 37°C.
5. Wash cells twice with chilled PBS-C/M on ice.
6. Surface biotinylate the cells only at the apical surface with 0.5 mg/mL sulfo-NHS-LC-biotin (water soluble, made fresh in PBS-C/M), 500 μ L for each Transwell (3 mL for 6 Transwell inserts). Keep the plate on ice, wrap with aluminum foil, and incubate at 4°C for 60 min.
7. Stop the reaction by adding 1–2 mL chilled TBS to the apical surface of cells.

².If using membrane preparation, one could directly add Tris-glycine to a final concentration of 100 mM to quench the reaction.

³.It is strongly recommended not to boil the CFTR-containing complex samples prepared for Western blotting as CFTR aggregation is a common problem. Instead, the samples should be heated at 37°C for 10–15 min prior to loading the gel.

8. Scrape off the cells from the Transwell very carefully with buffer-A (*see Subheading 2.2.11.*), total volume less than 10 mL for 6 Transwell inserts; pool cells into clean 15-mL BD Falcon tube.
9. Spin the cell suspension in the centrifuge at 800g for 5 min at 4°C.
10. Discard the supernatant, and transfer cell pellet into homogenizer (7-mL capacity) with buffer-B (*see Subheading 2.2.12.*). Keep the total volume less than 5 mL. Homogenize the cells twice for 20 strokes on ice.
11. Spin the cell homogenate at 800g for 5 min at 4°C. Collect the supernatant (postnuclear supernatant, PNS), and resuspend PNS with buffer-B to make total volume of 6 mL.
12. Overlay the above-made 6 mL PNS over the 6 mL of 1.16 M sucrose cushion in buffer B. Balance and spin at 100,000g for 1 h at 4°C using SW41 rotor (Beckman).
13. Collect the broad band at the homogenate and sucrose cushion interface (plasma membrane, about 1–2 mL) into a clean 1.5- or 2.0-mL microcentrifuge tube (try to keep less than 1.5 mL). Add 100 μ L streptavidin agarose beads and mix at 4°C for 45–60 min (*See Note 4*).
14. Spin at 1,200g for 1 min.
15. Wash the beads twice with buffer-A.
16. Elute the protein with 500 μ L RIPA buffer containing 2 mM regular biotin [D(+) biotin, ACROS, made as 2.4 mg/5 mL in RIPA buffer] plus protease inhibitors, and mix at 4°C for 5 min. Spin at 1,200g for 1 min and collect the supernatant. Repeat the elution step, and pool the supernatant.
17. Into the supernatant add anti-NBD1-R IgG cross-linked with protein A/G beads (*See Note 5*). Mix at 4°C overnight.
18. Spin down the beads at 1,200g for 1 min, and collect the supernatant (to check the unbound CFTR and its interacting partners later, if necessary)
19. Wash the beads extensively three times with buffer-A (*see Note 6*).
20. Elute the co-immunoprecipitated proteins from beads with 15 μ L sample buffer (+ 2.5% β ME).
21. Resolve the eluted proteins on 10% SDS-PAGE (*See Note 7*).
22. On the same gel, load a wide range of known amounts of purified CFTR_{his10} as a standard to quantitate CFTR, purified GST-NHERF1-PDZ2 as a standard to

⁴.To make fresh streptavidin agarose beads (50% slurry), gently tap the stock beads in the bottle (ImmunoPure® Immobilized Streptavidin, Pierce); take 200 μ L and mark the volume on the tube wall; and spin, discard the supernatant, wash twice with PBS. Then add PBS to the marked volume level (50% slurry) to working concentration.

⁵.Cross-link anti-CFTR IgG to protein A/G agarose beads. Incubate CFTR monoclonal or polyclonal antibodies (1.0 μ g) with 20 μ L of protein A/G agarose beads (Santa Cruz Biotech, Santa Cruz, CA) on ice for at least 1 h, and gently tap the mixture every 10 min. Wash the beads bound with IgG twice with 1 mL of 0.1 M N-ethyl morpholin (NEM; pH 7.5) buffer. Cross-link anti-CFTR IgG to protein A/G beads using 10 mM dimethyl pimelimidate (DMP, Pierce) in 0.5 mL NEM buffer, and mix at 22°C for 30 min. Stop the cross-linking reaction by washing the beads with 1 mL of 100 mM Tris-base (pH 8.0) or TBS buffer. Then the anti-CFTR IgG cross-linked to the protein A/G agarose beads is ready for the subsequent co-immunoprecipitation procedure.

⁶.When co-immunoprecipitating cross-linked CFTR containing complex, the use of 0.5 M urea in the wash buffer facilitates the complete removal of non-crosslinked partners, without significantly affecting CFTR binding to anti-CFTR IgG. This reduces non-specific binding significantly.

⁷.The CFTR protein is run on a 10% gel so that the mature CFTR band can be detected as a sharp band, which can be quantitated by densitometry.

quantitate the co-immunoprecipitated NHERF1, and purified GST-NHERF2 as a standard to quantitate the co-immunoprecipitated NHERF2.

23. Western blot using anti-CFTR (*See Note 8*) and anti-NHERF1 or NHERF2 antibodies. Representative data are shown in Fig. 15.2 (*also see ref. 36*).
24. To analyze and identify other CFTR interacting partners that are co-precipitated with CFTR, 2-dimensional gel electrophoresis can be performed to confirm the known co-immunoprecipitated proteins (*See Note 9*), or mass spectroscopy analysis can also be performed to identify unknown binding partners (*See Note 10*).

3.5. In Vitro CFTR-containing Macromolecular Complex Assembly (CFTR-NHERF2-LPA₂ Complex)

1. Into 200 μ L of lysis buffer (PBS-0.2% Triton-X 100 + protease inhibitors), add MBP-CFTR-C tail (a.a.1454–1480) fusion protein (1 μ M) immobilized on amylose beads (20 μ L) (*See Note 11*).
2. Add various amounts of purified GST-NHERF2 fusion protein, and mix at 22°C for 2 h.
3. During this time, prepare the cell lysates. Lyse the HEK293-parental cells transiently transfected with Flag- LPA₂ via Lipofectamine 2000 (five 60-mm dishes were used); use 500 μ L lysis buffer for each dish (*See Note 12*).
4. Wash the complex once with lysis buffer.
5. Add the above-prepared cell lysates to the beads, and gently mix at 4°C for 3 h or overnight.
6. Wash the beads three times with lysis buffer.
7. Elute the proteins using sample buffer (+ β ME).
8. Incubate in 37°C water bath for 10–15 min, and spin at 5,000g for 30 s to precipitate the beads.
9. Load as much eluate as possible onto 4–15% gel.

⁸If immunoblotting for CFTR, anti-CFTR IgG from a different host origin must be used: use a monoclonal antibody (if polyclonal CFTR antibody is cross-linked on the beads) or polyclonal antibody (if monoclonal CFTR antibody is cross-linked on the beads) raised against CFTR. Heavy- or light-chain contamination is thereby minimized using this method of coupling.

⁹2-dimensional gel electrophoresis can be performed to confirm the known co-immunoprecipitated proteins according to manufacturer's instruction (such as Bio-Rad). In brief, cross-link Calu-3 or HT29-CL19A epithelial cells with 1 mM DSP as described in **Subheading 3.3**, and then lyse the cells with RIPA buffer. Immunoprecipitate CFTR with mouse anti-CFTR antibody (MM13-4) or normal mouse IgG immobilized on protein A/G -agarose beads (Santa Cruz) through constant mixing at 4°C overnight. Rehydrate 7-cm IPG strips with 2-D Protein MW Marker Mix (Pierce) or 150 μ L CFTR proteins extracted with sequential extraction reagent II (8 M urea, 4% CHAPS, 40 mM Tris, 0.2% Bio-Lyte 3/10) supplemented with 2 mM TBP at 20°C for 12–16 h. Perform isoelectric focusing (IEF) at 20°C in an IEF tray by using the following program: 250 V for 15 min, linear ramp, S3=4000 V until 20,000 vhs are reached. Equilibrate the strips for 10 min in a solution containing 6 M urea, 3% SDS, 375 mM Tris-HCl (pH 8.6), 30% v/v glycerol, 2% w/v DTT. Perform a second equilibration step for another 10 min in the same solution containing 3% w/v iodoacetamide instead of DTT. Separate the proteins in the strip in a 10% polyacrylamide gel, and immunoblot with specific affinity-purified antibodies (such as anti-G β IgG or anti-syntaxin 1A IgG). Stain molecular marker gel with GelCode Blue Stain reagent (Pierce) and acquire and analyze the images with Quantity One (Bio-Rad).

¹⁰Mass spectrometry analysis can also be performed to identify unknown binding partners. In brief, cross-link the cells (HEK293 stably overexpressing Flag-CFTR; or Calu-3 or HT29-CL19A cells expressing endogenous CFTR). Purify Flag-CFTR from HEK293 cells by using anti-Flag M2 affinity gel (EZview Red ANTI-FLAG M2; Sigma), or co-immunoprecipitate CFTR from Calu-3 (or HT29-CL19A) cells. Run SDS-PAGE and Commassie stain the gel. Send the respective bands for mass spectrometry analysis to identify the interacting proteins. Using this approach, we identified several scaffolding proteins HSP70 and HSP90 as interacting partners, which are co-immunoprecipitated with CFTR.

¹¹Alternatively, purified His-S-CFTR C-tail (a.a.1400–1480) fusion protein can also be used. Briefly, add 50 μ L of S-protein agarose (Novagen) into 200 μ L of lysis buffer (PBS-0.2% Triton-X-100 + protease inhibitors). Add 50 μ g of His-S-CFTR-C tail, and mix at 22°C for 1 h. Spin at 1,200g and wash three times with lysis buffer.

¹²Alternatively, use purified Flag-LPA₂ at this step instead of the cell lysates, which also contain other components.

10. Western blot with anti-Flag mouse antibody.
11. Representative data are shown in Fig. 15.3 (*also see ref. 22*).

Acknowledgments

We thank Dr. David Armbruster for critically reading the manuscript and Ms. Feng Zhou for formatting the references. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grants DK058545 and DK074996 (to A.P.N.), and American Heart Association (Midwest Affiliate) Beginning-grant-in-aid #0765185B (to C.L.).

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Figure 15.1.

CFTR exists as a higher order complex in plasma membrane through cross-linking. (A) Calu-3 cells were cross-linked as described in the text. The cells were lysed in RIPA buffer and blotted for CFTR (R1104). (B) Calu-3 cells cross-linked with DSP were lysed in RIPA buffer, then 50 μ g of total proteins were solubilized with sample buffer in the absence (left) or presence (right) of 2.5% β -mercaptoethanol (β ME) before immunoblotting for CFTR (R1104). (Reproduced from ref. 36 with permission from American Society for Biochemistry and Molecular Biology.)

Figure 15.2.

Stoichiometry of CFTR:NHERF1 in the complex. (A) Polarized Calu-3 cells were cross-linked with 1 mM DSP and then apically biotinylated with sulfo-NHS-LC-biotin. The plasma membrane was isolated, and the apical plasma membrane was captured using streptavidin-agarose beads and then subjected to co-immunoprecipitation using anti-NBD-R IgG. The immunoprecipitated complex was treated with 2.5% βME to dissociate the cross-linked complex and dissolved on 10% SDS-PAGE. Purified CFTR_{his10} was used as a standard to quantitate CFTR (upper panel), and GST-PDZ2 of NHERF1 was used as a standard to quantitate the co-immunoprecipitated NHERF1 (bottom panel). (B) Calu-3 cells were cross-linked with 1 mM DSP, lysed in RIPA buffer, and subjected to co-immunoprecipitation using mouse anti-CFTR antibody (MM13-4; *bottom panel*) or normal mouse IgG (*upper panel*). The samples were eluted and subjected to 2-D gel electrophoresis (Bio-Rad). IEF (pI: 3–10) was performed in the 1st dimension, and SDS-PAGE was performed in a 10% polyacrylamide gel in the 2nd dimension and blotted with affinity-purified rabbit G_β IgG. (Reproduced from ref. 36 with permission from American Society for Biochemistry and Molecular Biology.)

Figure 15.3.

LPA₂ forms a macromolecular complex with CFTR mediated through NHERF2. **(A)** A pictorial representation of the macromolecular complex assay. **(B)** A macromolecular complex of MBP-C-CFTR, GST-NHERF2, and Flag-tagged LPA₂ that is assembled *in vitro*. (Reproduced from ref. 22 with permission from Rockefeller University Press.)

Table 15.1

Various cross-linkers used to cross-link CFTR with its interacting partners.

Cross-linkers	Acronym	MW	Spacer arm (Å)	Membrane-permeable	Cleavable (by)	Soluble (in)
1, 5-Difluoro 2, 4- Dinitrobenzene	DFDNB	204.1	3.0	Yes	No	DMSO
Disuccinimidyl tartarate	DST	344.24	6.4	Yes	Yes (periodate)	DMSO
Dimethyl pimelimidate•2 HCl	DMP	259.18	9.2	No	No	H ₂ O
Dimethyl 3,3'- dithiobispropionimidate•2 HCl	DTBP	309.28	11.9	No	Yes (thiols)	H ₂ O
Dithiobis [succinimidyl propionate]	DSP	404.42	12.0	Yes	Yes (thiols)	DMSO
3,3'- Dithiobis[sulfosuccinimidyl propionate]	DTSSP	608.51	12.0	No	Yes (thiols)	H ₂ O
Ethylene glycol bis[succinimidyl succinate]	EGS	456.37	16.1	Yes	Yes (hydroxyla mine)	DMSO
Bis-[b-(4- Azidosalicylamido) ethyl] disulfide	BASED	474.52	21.3	Yes	Yes (thiols)	DMSO
Disuccinimidyl suberate	DSS	368.35	11.4	Yes	No	DMSO