

Video Article

In Vitro Analysis of PDZ-dependent CFTR Macromolecular Signaling Complexes

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Abstract

Cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel located primarily at the apical membranes of epithelial cells, plays a crucial role in transepithelial fluid homeostasis¹⁻³. CFTR has been implicated in two major diseases: cystic fibrosis (CF)⁴ and secretory diarrhea⁵. In CF, the synthesis or functional activity of the CFTR Cl⁻ channel is reduced. This disorder affects approximately 1 in 2,500 Caucasians in the United States⁶. 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 gut⁷.

Accumulating evidence suggest the existence of physical and functional interactions between CFTR and a growing number of 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 be critically involved in regulating CFTR-mediated transepithelial ion transport *in vitro* and also *in vivo*⁸⁻¹⁹. In this protocol, we focus only on the methods that aid in the study of the interactions between CFTR carboxyl terminal tail, which possesses a protein-binding motif [referred to as PSD95/Dlg1/ZO-1 (PDZ) motif], and a group of scaffold proteins, which contain a specific binding module referred to as PDZ domains. So far, several different PDZ scaffold proteins have been reported to bind to the carboxyl terminal tail of CFTR with various affinities, such as NHERF1, NHERF2, PDZK1, PDZK2, CAL (CFTR-associated ligand), Shank2, and GRASP²⁰⁻²⁷. The PDZ motif within CFTR that is recognized by PDZ scaffold proteins is the last four amino acids at the C terminus (i.e., 1477-DTRL-1480 in human CFTR)²⁰. Interestingly, CFTR can bind more than one PDZ domain of both NHERFs and PDZK1, albeit with varying affinities²². This multivalency with respect to CFTR binding has been shown to be of functional significance, suggesting that PDZ scaffold proteins may facilitate formation of CFTR macromolecular signaling complexes for specific/selective and efficient signaling in cells¹⁶⁻¹⁸.

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^{16-19,28,29}. Here we focus on the detailed procedures of assembling a PDZ motif-dependent CFTR-containing macromolecular complex *in vitro*, which is used extensively by our laboratory to study protein-protein or domain-domain interactions involving CFTR^{16-19,28,29}.

Video Link

The video component of this article can be found at <http://www.jove.com/video/4091/>

Protocol

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

1. Amplify defined regions of the C-tails (the last 50-100 amino acids containing the PDZ motifs at C-terminus) for CFTR, LPA₂, MRP2, MRP4, β_2 AR, and NHERFs (full-length or PDZ1 or PDZ2 domains) by PCR approach.
2. Clone the PCR products into pGEX4T-1 vector for GST-fusion proteins (such as GST-NHERFs, GST-MRP4 CT), pMAL-C2 vector for MBP-fusion proteins (such as MBP- β_2 AR CT, MBP-CFTR CT), and pET30 for His-S-fusion proteins (such as His-S-CFTR CT, His-S-PDZK1).
3. Transform in a protease-deficient *E. coli* strain (BL21-DE3) to minimize possible recombinant protein degradation.
4. Grow the culture overnight at 37 °C in Luria-Bertani medium (pH 7.0) containing appropriate antibiotics (such as ampicillin or kanamycin). Dilute the overnight culture at 1:10 and grow for another 2 hr at 37 °C. Induce with 0.5-1 mM IPTG for the next 4 hr at 30 °C. Pellet the cells by centrifugation at 8,000 × g for 10 min at 4 °C.
5. Lyse the cells in Sucrose Buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, and 10% sucrose) containing lysozyme (1 mg/ml), 0.2% Triton X-100, and protease inhibitors. Use 20 ml for cell pellet originating from 1 L of culture.
6. Mix on a rotary shaker for 30 min at 4 °C.

7. Spin at $20,000 \times g$ for 30 min at 4 °C. Collect the clear supernatant.
8. Into the clear supernatant, add 1 ml of the following resin/agarose beads (50% slurry in Sucrose Buffer):
 - Glutathione agarose beads for GST fusion proteins.
 - Talon beads for His-S fusion proteins.
 - Amylose resin for MBP fusion proteins.
9. Mix for 4 hr at 4 °C on a rotary shaker.
10. Wash the beads by resuspending in 1x PBS (15 ml), mixing for 2 min, spinning at $800 \times g$ for 2 min, and discarding the supernatant. Repeat this step for 6 times.
11. Elute the protein from the beads by using respective elution buffer (2 ml elution buffer per 1 ml of beads).
 - Elution buffer for GST fusion proteins: 25 mM Tris-HCl (pH 8.0), 140 mM NaCl, and 20 mM reduced glutathione.
 - Elution buffer for His fusion proteins: 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 200 mM imidazole.
 - 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.
12. Dialyze the eluted proteins against 2 L of 1x PBS at 4 °C (to avoid possible protein degradation). Change PBS every 4 hr for 4 times. Concentrate the protein using a Centricon filter (10,000 MW cutoff, Millipore) and store as small aliquots at -80 °C.
13. Determine the protein concentration by the Bradford method. Assess protein quality by SDS-PAGE using BSA as standards. 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).

2. Cell Culture and Cell Lysate Preparation

1. Culture baby hamster kidney (BHK) cells that stably over-express CFTR-wt and CFTR^{-his10} or BHK cells that transiently over-express Flag-LPA₂-wt or Flag-LPA₂-ΔSTL, and Madin-Darby canine kidney (MDCK) cells that stably over-express MRP2 in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum and penicillin/streptomycin in polystyrene flasks at 37 °C with 5% CO₂.
2. Culture human embryonic kidney 293 (HEK293) cells that stably over-express CFTR-wt and CFTR^{-his10} in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin in polystyrene flasks at 37 °C with 5% CO₂.
3. Lyse the cells in Lysis Buffer (PBS - 0.2% Triton-X-100 supplemented with a mixture of protease inhibitors containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin); use 500 μl lysis buffer for each 60-mm Petri dish, and 1000 μl lysis buffer for each 100-mm Petri dish.
4. Rock the cell lysates for 20 min at 4 °C, and remove the insoluble material by centrifugation at $16,000 \times g$ for 15 min at 4 °C. Determine the protein concentration by the Bradford assay.

3. In Vitro Assembly of a CFTR-containing Macromolecular Complex (CFTR-PDZK1-MRP4)

1. Into 200 μl of lysis buffer (PBS - 0.2% Triton-X 100 + protease inhibitors), add 20 μg purified GST-MRP4-C terminal 50 a.a. (CT50) fusion protein.
2. Add various amounts (0-40 μg) of purified His-S-PDZK1 fusion protein.
3. Mix the two proteins on a rotary mixer at 22 °C for 1-2 hr.
4. Add 20 μl glutathione beads (50% slurry) into the protein mixture, and continue to mix for another 1 hr. This step is also referred to as pairwise binding.
5. During this time, prepare the HEK293 cell lysates that overexpress Flag-CFTR (wt) as described above in Step 2).
6. Wash the complex twice with lysis buffer. Spin at $800 \times g$ for 1 min for each wash and carefully aspirate the supernatant after each wash. Use caution not to suck off the beads in the bottom.
7. Add the above-prepared HEK293 cell lysates to the beads, and gently mix at 4 °C for 3 hr (or overnight).
8. Wash the beads extensively 3 times with lysis buffer as described in Step 3.6).
9. Elute the proteins using 30 μl Sample Buffer (5×): 0.6 M Tris-HCl (pH 6.8), 50% glycerol, 2% SDS, and 0.1% bromophenol blue; containing 5% β-mercaptoethanol.
10. Incubate in 37 °C water bath for 10-15 min, and spin at $5,000 \times g$ for 30 s to precipitate the beads.
11. Load all the eluate onto 4-15% gel.
12. Run SDS-PAGE for about 30-40 min.
13. Transfer protein bands to the PVDF membrane, for 1.5 hr.
14. Block the membrane in TBS-Tween containing 5% non fat milk.
15. Incubate the membrane with primary antibody (such as anti-CFTR IgG, R1104) at 4 °C, overnight.
16. Wash the membrane with TBS-Tween for 5 min, 6 times.
17. Incubate the membrane with secondary antibody (such as goat anti-mouse HRP-conjugated 2nd antibody) for 45 min at 22 °C.
18. Wash the membrane with TBS-Tween for 5 min, 6 times.
19. Visualize the protein bands via ECL.
20. Representative data are shown in **Figure 1**.

4. Representative Results

An example of CFTR-containing macromolecular signaling complex that was assembled *in vitro* is shown in **Figure 1**. A macromolecular complex was formed between MRP4 C-terminal 50 amino acids (MRP4-CT50), PDZK1, and full-length CFTR (**Figure 1, bottom**). The complex formation increased dose-dependently with increasing amounts of the intermediary protein, PDZK1 (**Figure 1, bottom**)¹⁸.

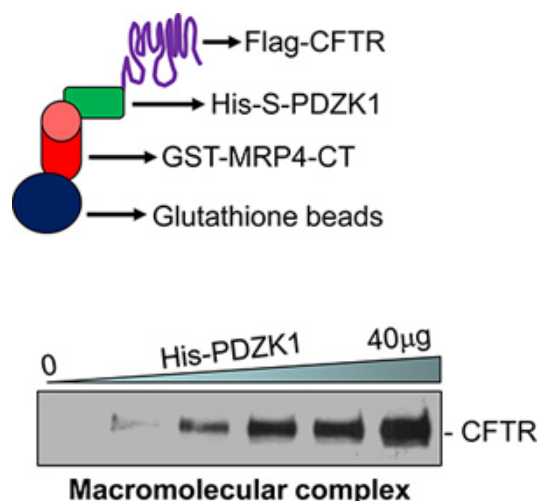


Figure 1. A pictorial representation of the macromolecular complex assay (*top*). A macromolecular complex was detected *in vitro* with three proteins (GST-MRP4-CT50, His-S-PDZK1, and Flag-CFTRwt) in a dose-dependent manner (*bottom*)¹⁸.

	CFTR-NHERF1- β_2 AR (ref. ¹⁴)	CFTR-NHERF2-LPA ₂ (ref. ¹⁵)	CFTR-PDZ proteins-MRP ₂ (ref. ¹⁷)	CFTR-PDZK1-MRP4 (ref. ¹⁶)
Affinity beads	Amylose resin	S-protein agarose	Amylose resin	Glutathione agarose
Purified protein-1	MBP- β_2 AR CT	His-S-CFTR CT	MBP-CFTR CT	GST-MRP4 CT
Purified protein-2	GST-NHERF1	GST-NHERF2	GST-PDZ proteins	His-S-PDZK1
Purified protein-3 (or cell lysates)	CFTR-wt or CFTR ^{his10} (BHK or HEK cell lysates)	Flag-LPA ₂ -wt or Flag-LPA ₂ - Δ STL (BHK cell lysates)	MRP2 (MDCK cell lysates)	Purified Flag-CFTR-wt or CFTR ^{his10} (or cell lysates)
Antibody	Anti-CFTR IgG	Anti-Flag HRP	Anti-MRP2 IgG	Anti-Flag HRP

Table 1. Summary of various CFTR-containing macromolecular complexes assembled *in vitro*.

Discussion

In this protocol we demonstrated a method for *in vitro* assembling and detection of a CFTR containing macromolecular signaling complex using purified proteins (or protein fragments) and/or cell lysates as reported previously^{16-19,29,30}. To achieve best results the following critical points during the preparation process require special attention:

- It is important that the pH of the elution buffer be adjusted to 8.0 after adding reduced glutathione when purifying the GST-fusion protein as described in step 1). 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.
- When protein is eluted from the beads, they need to be dialyzed right away; otherwise, the purified protein might come out of the solution. If protein dialysis is not possible right after the elution process, do not elute the protein and leave them with the agarose/beads.
- It is strongly recommended not to boil the CFTR-containing macromolecular 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 before loaded in the gel.
- For the protein complex with low affinity interaction, the immunoblot can be visualized using SuperSignal West Femto (or Pico) Maximum Sensitivity Substrate (Pierce) to increase the signal.

The technique demonstrated here provides a convenient and reproducible assay to biochemically define a CFTR-containing tertiary protein complex. There are multiple ways to assemble the *in vitro* CFTR macromolecular complexes as outlined in **Table-1**.

- In order to obtain compelling results, one should try to assemble the complex in both ways, *i.e.*, i) CFTR - PDZ scaffold protein(s) - the third protein; ii) the third protein - PDZ scaffold protein(s) - CFTR.
- In order to demonstrate the PDZ motif-dependency of the macromolecular complex formation, CFTR^{his10} (CFTR^{his10} contains 10 His residues in the C-terminal tail; this protein has an internal PDZ motif and cannot bind NHERFs²⁹), proteins with their PDZ motifs mutated or deleted (such as β_2 AR-L413A; LPA₂- Δ STL; etc) can be used in parallel in the macromolecular complex assay as well.
- Instead of using the whole lysates of cells that also contain many other components, one can use purified proteins (as the third protein) at the step-3.7) (such as in the assembly of MRP4-PDZK1-CFTR, we also used the purified Flag-CFTR; ref.¹⁸). This provides compelling result of a solely tri-protein complex, as there are only 3 purified proteins contained in the whole assembly system.

However, detection of the CFTR macromolecular complex *in vitro* using purified proteins (or protein fragments) or lysates from cells that overexpress CFTR or the interacting proteins does not indicate whether the macromolecular signaling complex exists in cells that endogenously express these interacting proteins. To address this issue, co-immunoprecipitation can be performed to assess the endogenous CFTR complexes in cells such as airway epithelial cells¹⁶ and gut epithelial cells^{17,18}. Moreover, mass spectrometry analysis and 2-dimensional

gel electrophoresis²⁹ can be performed to identify unknown binding partners for CFTR. However, it is beyond the scope of this protocol to demonstrate these techniques.

In addition, the subcellular localization of interacting proteins *in vivo* may also affect molecular interactions. For instance, MRP4 has been shown to be expressed in both apical and basolateral membranes, whereas CFTR is localized primarily to the apical membrane, though they have been demonstrated to interact physically and functionally with each other via the PDZ scaffold protein PDZK1¹⁸. Furthermore, overexpression of the recombinant proteins, as used in the current protocol, may affect their subcellular localization and thus enable interactions that would otherwise not occur. These possibility should also be taken into consideration when interpreting the data and extrapolating the findings.

Although the current protocol focuses on the procedure to assemble PDZ-dependent CFTR-containing macromolecular complexes, this protocol also has potential applications in assembling non-CFTR involved multi-protein complex (either PDZ-dependent or independent). Most recently, using this macromolecular assembly assay, we have demonstrated that a chemokine receptor CXCR2 physically forms a protein complex with its downstream effector PLC β 2 mediated via NHERF1 in neutrophils, and this CXCR2 macromolecular complex has functional relevance as disrupting the complex (via using an exogenous CXCR2 peptide) significantly attenuated neutrophil functions³¹.

Disclosures

No conflicts of interest declared.

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