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Analysis of CFTR Folding and Degradation in Transiently Transfected Cells

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Abstract

Misfolding and premature degradation of F508del-CFTR is the major cause of cystic fibrosis. Components of the ubiquitin-proteasome system function on the surface of the endoplasmic reticulum to select misfolded proteins for degradation. The folding status of F508del-CFTR is monitored by at least two ER quality control checkpoints. The ER-associated Derlin-1/RMA1 E3 complex appears to recognize folding defects in CFTR that involve misassembly of NBD1 into a complex with the R-domain. In contrast, the cytosolic Hsp70/CHIP E3 complex appears to sense folding defects that occur after synthesis of NBD2. Herein we describe methods that allow for the study of how modulation of these ER quality control factors by siRNA impacts CFTR folding and degradation. The experimental system described employs transiently transfected HEK293 cells and is utilized to monitor the biogenesis of CFTR by both Western blot and pulse chase studies. Methods to detect complexes formed between CFTR folding intermediates and ER quality control factors will also be described.

Keywords

F508del; pulse chase; Western blot; folding; degradation

1. Introduction

Proper folding and interdomain contact formation between membrane and cytosolic sub-domains of CFTR is critical for channel activity (1–3). CFTR synthesis takes around 10 min and affords chaperones and ER quality control factors sufficient time in which to monitor the progression of CFTR folding intermediates (4). However, CFTR folding is a relatively inefficient process with approximately 70–85% of wild-type and 99% of the F508del mutant being partitioned from a folding to the ubiquitin-proteasome degradation pathway (5).

The attempt to fold CFTR into a native conformation that is no longer recognized by the ER quality control machinery involves the cooperation of both ER-localized and cytosolic chaperones. The Hdj-2/Hsp70 chaperone pair mediates early or co-translational folding of CFTR sub-domains (4). Calnexin's contribution to CFTR folding involves facilitating proper interactions between the membrane and cytosolic domains of CFTR (6). Terminal steps of CFTR folding appear to be mediated by Hsp90 and its co-factors (7, 8).

Selection of nascent forms of CFTR and F508del-CFTR for proteasomal degradation is also facilitated by molecular chaperones. Misfolded forms of CFTR are recognized by at least two ER quality control complexes. The cytosolic E3 ubiquitin ligase CHIP interacts with Hsp70 to form a quality control machine that utilizes the polypeptide binding activity of Hsp70 to target misfolded CFTR for proteasomal degradation (9). In addition, the ER-associated E3 RMA1/RNF5 acts in association with Derlin-1 and the E2 Ubc6e to ubiquitinate CFTR (10). Other co-factors, including Gp78, BAP31, and p97, are then responsible for delivering the ubiquitinated CFTR to the proteasome (11–13). How selection of CFTR for degradation by the RMA1 E3 machinery and the CHIP E3 complex is synergized is not entirely clear. However, the RMA1 E3 complex may act cotranslationally to recognize folding defects in CFTR that involve misassembly of NBD1 into a complex with the R-domain (6, 10). In contrast, the CHIP E3 may act post-translationally to recognize misfolded regions of CFTR that include NBD2 (10).

Herein we describe methods that allow for modulation of the ubiquitin ligase complexes which select F508del-CFTR for degradation that can be used in efforts to permit cell surface expression of F508del-CFTR. The experimental system described employs transiently transfected HEK293 cells to study the bio-genesis of CFTR and its folding mutants. We describe how to deplete levels of ER quality control factors by siRNA. Next, biochemical methods to analyze CFTR steady-state levels by Western blot and folding/degradation kinetics by pulse chase studies are detailed. Finally, methods to detect complexes formed between CFTR folding intermediates and ER quality control factors will also be described.

2. Materials

2.1. General Reagents

1. Phenylmethylsulfonyl fluoride (PMSF): 100 mM stock of PMSF in 100% molecular-grade ethanol. Store at -20°C .
2. Protease inhibitor cocktail (PI): CompleteTM Protease inhibitor cocktail (Roche, 11697498001).
3. Phosphate-buffered saline (PBS), pH 7.4: 135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 . Store at 4°C . Supplement PBS with 1% Triton X-100 (PBS-Tr (1%)), 1 mM PMSF, and PI just prior to use in cell lysis.
4. 10% bovine serum albumin (BSA) in PBS.
5. Antibodies: CFTR clone MM13-4 (Millipore, 05-581) and RMA1 (Santa Cruz Biotechnology, sc81716).
6. Protein G-agarose (PG beads) (Roche, 11243233001): resuspend PG beads in PBS supplemented with 1% Triton and 1% BSA. Incubate PG beads on a rotator for 24 h at 4°C to block non-specific binding sites on the beads. Pellet the beads with a microcentrifuge and resuspend them as a 50% v/v slurry in PBS supplemented with 1% Triton. Store at 4°C .

2.2. Reagents and Buffers

2.2.1. SDS-PAGE

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 2× sample buffer (2× SB): 125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 0.05% of Coomassie blue, and distilled water. This solution can be stored at room temperature. Prior to use add 80 µl of β-mercaptoethanol and 10 µl PI to 1 ml of 2× SB.
2. SDS-PAGE electrode buffer, pH 8.3: 10 mM Tris–HCl, 75 mM glycine, and 0.1% SDS.
3. Acrylamide-*bis*-acrylamide solution: 30% acrylamide, 0.8% *N,N*-methylene *bis*-acrylamide. Store in an aluminum foil-covered glass bottle at 4°C.
4. 4× SDS-PAGE resolving gel buffer, pH 8.8: 1.5 M Tris–HCl, 8 mM EDTA, and 0.4% SDS. Adjust to pH 8.8 and store at room temperature.
5. 4× SDS-PAGE stacking gel buffer, pH 6.8: 0.5 M Tris–HCl, 8 mM EDTA, and 0.4% SDS. Adjust to pH 6.8 and store at room temperature.
6. 10% ammonium persulfate (APS).
7. *N,N,N',N'*-Tetramethylethylenediamine (TEMED) (Fisher Scientific, BP150).
8. Different percentage SDS-PAGE gels are used to resolve proteins of varying size. SDS-PAGE gels are made by combining the above SDS-PAGE buffers and reagents as follows: 2.35 ml acrylamide, 5.15 ml distilled water, and 2.5 ml 4× resolving gel buffer for a 7% gel; 3.2 ml acrylamide, 4.3 ml distilled water, and 2.5 ml 4× resolving gel buffer for a 10% gel; and 4.2 ml acrylamide, 3.23 ml distilled water, and 2.5 ml 4× resolving gel buffer for a 12.5% gel. To polymerize these gel mixtures add 100 µl of 10% APS and 7.5 µl of TEMED. The combined solution is mixed and then poured between the glass plates of a mini-gel apparatus (Bio-Rad). A layer of water-saturated *N*-butanol is added to the top surface of the gel to obtain a flat smooth surface and to prevent the resolving gel from drying out. Polymerization should be complete by 30 min. When the resolving gel is polymerized, pour off the butanol layer, rinse top of gel with distilled water, and then add the stacking gel mixture. The stacking gel is composed of the following: 0.6 ml of acrylamide, 2.35 ml of distilled water, 1 ml of 4× stacking gel buffer, 75 µl of 10% APS, and 5 µl of TEMED. The stacking gel mixture should be mixed well and then added to the top of the resolving gel. Immediately insert well combs and stacking gel polymerization should occur within 15 min.
9. SDS-PAGE gel stain: 25% methanol, 10% glacial acetic acid, 2.5 g/l of Coomassie blue. In order to minimize the formation of precipitates use distilled water to prepare this stain.
10. SDS-PAGE gel destain: 10% methanol, 10% acetic acid, and distilled water.
11. Fluorography reagent: 0.5 M sodium salicylate, pH 7.4.

2.2.2. Western Blot

1. Western blot transfer buffer: 20 mM Tris-base, 150 mM glycine-HCl, 20% methanol, and 0.02% SDS.
2. Ponceau S protein stain: Dissolve 1 g of Ponceau S in 2 ml of glacial acetic acid and 198 ml of water.
3. PBSTrX-100: PBS supplemented with 0.1% Triton X-100.
4. Western blot blocking solution: PBSTrX-100 and 10% nonfat dry milk.
5. Antibody solutions: antibody diluted into PBSTrX-100 supplemented with 3% BSA and 0.2% sodium azide.
6. 0.45- μ m nitrocellulose membrane (GE Water and Process Technologies, WP4HY00010).
7. 3M Whatman filter paper.

2.3. Cell Culture

1. HEK293 cells are grown in Dulbecco's modified Eagle's medium (Sigma, D6429) supplemented with 10% fetal bovine serum (FBS; Mediatech, 35010CV) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin; Mediatech, 30002CI) (DMEM) at 37°C in an atmosphere of 5% CO₂ to approximately 100% confluency in T-75 flasks (Corning, 430641). Each flask contains approximately 8.4×10^6 cells.
2. Citric saline: 135 mM KCl, 15 mM Na-citrate, pH 7.4.
3. Minimum essential medium without L-methionine (MEM) (Gibco, 21013) supplemented with 5 ml glutamine (Cell-gro, 25-0005-CI) and 5 ml pyruvate (Mediatech; 25000CI); pre-warm media to 37°C prior to use. Cells are incubated in this media to deplete intracellular methionine prior to incubation with Trans ³⁵S-Label.
4. Trans ³⁵S-Label (1200 Ci/mmol; MP Biomedicals, 51006). Trans ³⁵S-Label is a mixture of ³⁵S-methionine and ³⁵S-cysteine utilized for radiolabeling of cellular proteins. Supplement MEM with 100 μ Ci Trans ³⁵S-Label per 35 mm well.
5. 0.05% trypsin-EDTA (Gibco, 25300).
6. Transfection reagents: Effectene (Qiagen, 301427); Lipofectamine 2000 (Invitrogen, 11668-019).
7. High-quality plasmid DNA (*see* Note 1).

¹Transfection efficiency is dependent on the quality of the DNA expression constructs. We suggest using Qiagen reagents to obtain high-quality plasmid DNA. Expression can vary between different preparations of the same plasmid; therefore, we also recommend preparing a large quantity of the expression plasmid of interest and to use the same material for each experiment.

3. Methods

3.1. Expression of CFTR in HEK293 Cells

3.1.1. Growth of HEK293 Cells for Transfection

1. Grow HEK293 cells to approximately 100% confluency in T-75 flasks.
2. Aspirate growth media and gently rinse cells with 6 ml of PBS. Remove PBS from cells.
3. Add 1.5 ml of trypsin-EDTA and incubate cells for approximately 1 min at 37°C to detach cells from the bottom of the flask.
4. After the cells have detached, dilute the trypsin-EDTA with 32 ml of DMEM. Pipet several times to break up large clumps of cells.
5. Pipet 2 ml of cell suspension into each 35-mm well. A confluent T-75 flask will provide enough cells for 16 wells. Allow 12–14 h for these cells to adhere to the bottom of the 35-mm wells.
6. The cells will be at approximately 40–50% confluency and are now ready to transfect.

3.1.2. Transfection of HEK293 Cells for Overexpression Analysis

1. The mammalian expression plasmid pcDNA3.1(+)-CFTR or F508del-CFTR is introduced into HEK293 cells with the Effectene transfection reagent. For each 35-mm well of cells add 1 µg of pcDNA3.1(+)-CFTR to 100 µl of EC buffer and mix well. Next, add 4 µl of Enhancer reagent/µg of DNA, mix the cocktail well, and incubate the mixture at room temperature for 5 min. Add 5 µl of Effectene/µg of DNA, vortex for 10 s, and incubate for 20 min. Dilute the transfection mixture with 800 µl of DMEM.
2. Aspirate the DMEM from the HEK293 cells and gently add the transfection mixture down the side of the 35-mm well of cells. Incubate the cells with the transfection mixture for 4–5 h at 37°C and 5% CO₂. Replace the transfection mixture with DMEM and incubate for 18–24 h (*see* Note 2).

3.1.3. Isolation of Transfected HEK293 Cells

1. To harvest HEK293 cells remove the DMEM and add 1 ml citric saline.
2. Detach the cells by repeatedly pipetting the citric saline over the cells. Once the cells have become detached remove them from the 35-mm well and place in a microcentrifuge tube.
3. Rinse the 35-mm well with 0.5 ml of citric saline and add the solution to the microcentrifuge tube.

²Continually overgrowing HEK293 cells (exceeding 100% confluence) can result in reduced transfection efficiencies. In addition, surpassing 30 passes of HEK293 cells can result in variable transfection efficiency.

4. Pellet the cells by centrifugation at 3,000×g for 2 min. Aspirate supernatant. Cell pellets can either be placed on ice prior to lysis or frozen in liquid nitrogen for storage and later lysis.

3.2. Impact of ER Quality Control Factors on CFTR Biogenesis

The fate of CFTR and F508del-CFTR is dependent on ER quality control factors. Modulation of the levels of known quality control factors allows us to assess how each protein impacts the folding of CFTR. Here we describe the general procedure for depleting endogenous quality control proteins, using the RMA1 E3 ligase as an example.

1. Prepare HEK293 cells for transfection as described in **Section 3.1.1**.
2. Introduce siRNA oligonucleotides (oligos) into HEK293 cells using Lipofectamine 2000 as the transfection reagent. In tube A, oligos directed against RMA1 (sequence 1, GCGCGACCUUCGAAUGUAA; sequence 2, CGGCAAGAGUGUCCAGUAU) or a non-specific control (Dharmacon) are mixed with 250 µl/reaction of Opti-MEM (Invitrogen). In tube B, 10 µl/reaction of Lipofectamine 2000 and 240 µl/reaction of Opti-MEM are combined.

The siRNA oligos and Lipofectamine 2000 reagent can be added to the tubes outside of a tissue culture hood but Opti-MEM addition and subsequent transfection steps should be carried out within the hood. Incubate tubes A and B for 7 min. Combine 250 µl/reaction of tube B with 250 µl/reaction of tube A and incubate for 25 min. During this incubation remove media from cells in 35-mm wells and replace with 2 ml DMEM supplemented with 10% FBS but lacking antibiotics (no penicillin or streptomycin). Add 500 µl of the combined tube A and B mixture to each well. We have found that using a final total concentration of 100 nM with the RMA1 siRNA oligos is sufficient to obtain greater than 90% knockdown of endogenous RMA1 levels.

3. Incubate the cells with the transfection mixture for 4–5 h at 37°C and 5% CO₂. Replace the transfection mixture with DMEM supplemented with FBS and antibiotics.
4. Twenty-four hours post-transfection, remove the media and wash the cells with 1 ml PBS.
5. Aspirate off the PBS, add 200 µl trypsin-EDTA, and incubate cells at 37°C for approximately 1 min to detach cells from the bottom of the wells.
6. Add 800 µl DMEM to the cells and pipet several times to break up clumps of cells.
7. Take 350–400 µl of resuspended cells and add to a new 35-mm well. Add 1.5 ml of DMEM to the new wells. Thus, one well of siRNA cells can be divided into two new wells. Allow the cells to attach to the bottom of the wells overnight.
8. Transfect siRNA cells with pcDNA3.1(+)-CFTR or pcDNA3.1(+)-F508del-CFTR and harvest these cells as described in **Sections 3.1.2 and 3.1.3**.

9. For CFTR steady-state analysis, harvest the siRNA cells 24 h post-transfection and add 250 μ l 2 $\times$ SDS SB. To lyse the cells sonicate the samples (intensity 5) for 10 s $\times$ 2. To prevent overheating of the samples it is important to keep them on ice during sonication. Normalize samples to contain the same total amount of protein (*see* Note 3). Analyze the levels of CFTR and the efficiency of RMA1 knockdown via Western blot as described in **Section 3.3.1**.
10. For CFTR pulse chase studies follow the procedure outlined in **Section 3.3.2**.

3.3. Analysis of CFTR and F508del-CFTR Folding and Degradation in HEK293 Cells

3.3.1. Analysis of CFTR Levels by Western Blot—Steady-state levels of transiently transfected CFTR and F508del-CFTR in HEK293 cells can be determined by Western blots (Fig. 15.1).

1. Transfect HEK293 cells with pcDNA3.1(+)-CFTR or pcDNA3.1(+)-F508del-CFTR and harvest these cells as described in **Sections 3.1.1 – 3.1.3**.
2. Add 200 μ l 2 $\times$ SB to each cell pellet, sonicate as described in **Section 3.2**, and normalize samples to contain the same total amount of protein (*see* Note 3).
3. Heat samples at 37°C for 10 min. Load an equal volume from each sample to a 10% SDS-PAGE gel and electrophorese samples by applying a constant voltage of 120 V to gels for approximately 90 min.
4. To prepare for the wet transfer of proteins in the SDS-PAGE gel to a nitrocellulose membrane using a Bio-Rad mini-gel transfer apparatus
 - a. Cut 3 M Whatman filter paper to the same size as the apparatus sponges and soak the paper in Western blot transfer buffer.
 - b. Soak the transfer apparatus sponges in Western blot transfer buffer.
 - c. Cut a nitrocellulose membrane to the size of the mini-gel and soak this membrane in Western blot transfer buffer.
5. On completion of the electrophoresis, assemble the gel into the transfer apparatus with a nitrocellulose membrane.
6. Transfer the protein in the gel to the nitrocellulose by applying a constant voltage of 110 V for 70 min. The transfer can be run either at room temperature or at 4°C.
7. After completion of the transfer, remove the nitrocellulose membrane from the transfer apparatus. Stain the proteins on the membrane with Ponceau S for 2–3 min and then destain with distilled water. This staining step allows for comparison of protein loads in each lane.

³To determine the protein concentration of our samples we use the colorimetric detergent compatible (DC) protein assay (Bio-Rad). To minimize the amount of sample that is used for protein determination we follow the modified assay for a 96-well plate as supplied by Bio-Rad. A standard protein curve is generated using dilutions of BSA ranging from 0.5 to 2.5 mg/ml that are prepared in the same buffer as the sample. As a side note, if the samples that need to be normalized are to be lysed in 2 $\times$ SB, leave the β -mercaptoethanol out of the SB as the DC protein assay is incompatible with this chemical. The β -mercaptoethanol can be added after the samples are normalized.

8. Block sites on the nitrocellulose membrane that bind antibodies non-specifically via incubation of the membrane on a shaker with Western blot blocking solution. This blocking step can be carried out at room temperature for minimally 1 h and can be extended overnight with incubation at 4°C.
9. Rinse membrane with PBSTrX-100 a few times to remove excess block solution.
10. Incubate the membrane with the monoclonal primary antibody α -CFTR MM13-4 at a 1:1000 dilution in PBSTrX-100 supplemented with 3% BSA and 0.2% sodium azide. To determine the efficiency of RMA1 knockdown use the α -RMA1 monoclonal antibody at a 1:1000 dilution. α -tub (1:2000 dilution) can be used to indicate loading consistency. The nitrocellulose membrane(s) and primary antibody solution(s) should be incubated on a shaker for minimally 1 h at room temperature or overnight at 4°C.
11. Wash the membrane with PBSTrX-100 3×5 min on a shaker.
12. Incubate the membrane with goat anti-mouse sera conjugated to horseradish peroxidase (Bio-Rad) that is diluted 1:3000 with PBSTrX-100 and 1% non-fat dry milk on a shaker for 60 min at room temperature.
13. Wash the nitrocellulose membrane 3×5 min with PBSTrX-100 on a shaker.
14. Incubate the membrane with enhanced chemiluminescent reagent (GE Healthcare). Wrap the membrane in saran wrap, expose membrane to X-ray film, and develop the film. Exposure times may vary to observe a signal in the linear range; thus we typically take a few exposures usually between 30 s and 2 min.

3.3.2. Pulse Chase Analysis—Pulse chase experiments are used to analyze the folding efficiency of CFTR folding and the rate of F508del-CFTR degradation (Fig. 15.1b).

1. Transfect HEK293 cells with pcDNA3.1(+)-CFTR or pcDNA3.1(+)-F508del-CFTR as described in **Sections 3.1.1** and **3.1.2**.
2. Approximately 18–24 h post-transfection remove the media, wash the cells with 0.5 ml of pre-warmed MEM minus methionine, and then methionine-starve the cells with 1 ml MEM for 20 min at 37°C in a 5% CO₂ atmosphere.
3. Replace the MEM minus methionine with 750 μ l MEM minus methionine supplemented with 100 μ Ci Trans ³⁵S-Label. Incubate the cells for 20 min at 37°C and 5% CO₂.
4. Remove the labeling media and start the chase by adding 1 ml of DMEM supplemented with 5 mM methionine. Incubate the ³⁵S-labeled cells for 0–3 h at 37°C and 5% CO₂.
5. At each chase time point, remove chase media from the cells and add 1 ml ice-cold citric saline. Collect the detached cells and place into pre-chilled 1.5 ml microcentrifuge tubes. Pellet the cells and aspirate off the supernatant. Freeze the cell pellets with liquid nitrogen and store in freezer until all time points have been collected.

6. When all time points have been harvested, cells can be lysed in 400 μ l of cold PBS-Tr (1%) supplemented with PI and 1 mM PMSF. Cells are resuspended in the PBS-Tr (1%) buffer by mixing with a pipet. Incubate samples for 1 h at 4°C on a rotator.
7. Obtain the soluble cell lysate by centrifuging the samples at 36,000 $\times$ g for 10 min at 4°C. Transfer the supernatant to a new precooled microcentrifuge tube.
8. Normalize each sample to contain the same total amount of protein (*see* Note 3).
9. Add 3 μ l of CFTR MM13-4 antibody and 0.2% BSA to the normalized samples and incubate for 30 min on a rotator at 4°C.
10. Add 30 μ l of a 50% Protein G-agarose slurry to each sample and incubate for 30 min on a rotator at 4°C.
11. Pellet the Protein G beads by centrifugation at 1,500 $\times$ g for 2 min at 4°C.
12. Aspirate off the supernatant being careful not to disturb the beads. Resuspend the pelleted beads with 500 μ l cold PBSTr (1%) and transfer to a new 1.5 ml microcentrifuge tube. Changing the tubes helps to get rid of any radiolabeled proteins that may be stuck to the sides of the microcentrifuge tube.
13. Pellet the Protein G beads again, remove the supernatant, then resuspend and wash the pelleted beads two times with 500 μ l cold PBS-Tr (1%) supplemented with 0.2% SDS. After the last wash, aspirate off the buffer and remove all remaining buffer from around the Protein G beads using a Hamilton syringe.
14. To each pellet add 15 μ l 2 $\times$ SB and heat samples at 55°C for 10 min.
15. Pellet the Protein G beads by centrifugation at 1,500 $\times$ g for 1 min at room temperature. Load all the supernatant using a Hamilton syringe onto a 7% SDS-PAGE gel.
16. Electrophorese the samples by applying a constant voltage of 120 V for 70 min.
17. After electrophoresis, fix and stain the protein in the gel by incubation in SDS-PAGE gel stain for 5 min.
18. Remove the stain, rinse the gel with distilled water, and then soak the gel with SDS-PAGE gel destain until protein bands on the gel are clearly visible.
19. Rinse the gel with distilled water and then soak the gel in 0.5 M sodium salicylate for at least 20 min (*see* Note 4).
20. Rinse the gel with distilled water and place it on a wet piece of Whatman paper to be dried on a slab type gel dryer for 1 h at 80°C.
21. Expose the dried SDS-PAGE gel to X-ray film for 1–3 days at –80°C. Develop the film with a processor.

⁴After the SDS-PAGE gel has been destained, rinsing the gel with distilled water is a necessary step. Failure to sufficiently remove the acetic acid (a component of the destain solution) may cause a precipitate to form on the gel when it is soaked in sodium salicylate. This precipitate is caused by the low pH of the gel and can be re-dissolved by adding 1 M Tris-HCl, pH 8.0. After the precipitate is dissolved, add fresh sodium salicylate to the gel.

3.4. Co-immunoprecipitation of CFTR with ER quality control factors

Complexes formed between CFTR folding intermediates and ER quality control factors can be detected by co-immunoprecipitation studies (Fig. 15.2).

1. Follow **Section 3.1.2** for the transient transfection of HEK293 cells with CFTR and epitope-tagged ER quality control factors. For co-immunoprecipitation reactions we usually transfect 1 μ g of CFTR and 0.2 μ g of the other factors into the HEK293 cells. If necessary, use the empty vector pcDNA3.1(+) to ensure equal microgram quantities of DNA are used in all transfection reactions.
2. Metabolically label transfected cells with ^{35}S as described in **Section 3.3.2**.
3. After the ^{35}S -labeling step, harvest the cells as indicated in **Section 3.1.3**. All subsequent steps are carried out at 4°C.
4. Lyse the samples with 250 μ l of cold PBS-Tr (1%) supplemented with PI, 1 mM PMSF, and 0.2% BSA. Incubate samples for 1 h on a rotator.
5. Soluble cell lysate is obtained by centrifuging samples at 36,000 $\times$ g for 10 min. Transfer supernatant to a new microcentrifuge tube.
6. Add antibody to the soluble cell lysate and incubate for 30 min on a rotator. For this experimental setup, we typically use 2 μ l of antibody against the protein we want to immunoprecipitate. For example, to assess which factors are interacting with RMA1 we would use 2 μ l of anti-RMA1. To monitor the background binding of overexpressed proteins a good control to include is co-immunoprecipitation samples that do not have antibody added to them.
7. Add 30 μ l of 50% Protein G-agarose slurry to each sample and rotate for 30 min.
8. Pellet the Protein G beads by centrifugation, wash the isolated complex 3 $\times$ 500 μ l with PBS-Tr (1%), add 15 μ l 2 $\times$ SB to each sample, heat samples at 55°C for 10 min, and load all the supernatant onto a SDS-PAGE gel. Finish processing the gel as detailed in **Section 3.3.2**.
9. To determine where a given protein runs on the SDS-PAGE gel, a direct immunoprecipitation of the protein under denaturing conditions can be included in the experimental design. For this control, add SDS (0.2% final concentration) to the buffer in the wash steps.
10. In the case that the co-immunoprecipitation step results in the presence of a large number of radiolabeled protein bands, a re-immunoprecipitation reaction can be used to identify the other potential components of the isolated complex. Add 2 $\times$ SB to the complex isolated by co-immunoprecipitation and heat the samples at 55°C to disrupt the interacting proteins. Dilute the samples with PBS-Tr (1%) supplemented with PI, 1 mM PMSF, 0.2% SDS, and 0.5% BSA. Antibodies against the proteins of interest are next added to the samples and the remainder of this immunoprecipitation reaction is processed as described in **Section 3.3.2**.

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Fig. 15.1.

Impact of RMA1 siRNA on CFTR folding in HEK293 cells. RMA1 siRNA and transient transfection of CFTR or F508del-CFTR are described in **Section 3.2**. Steady-state levels (**a**) and stability (**b**) of CFTR and F508del-CFTR in the presence or absence of the E3 ligase RMA1 are indicated by Western blot (**Section 3.3.1**) and pulse chase assays (**Section 3.3.2**), respectively. Bands B and C represent the ER-localized immaturely glycosylated form and the maturely glycosylated plasma membrane form of CFTR, respectively. The band marked with a * denotes a background band.

Fig. 15.2.

Co-immunoprecipitation of components of the RMA1 E3 complex with F508del-CFTR. Using the method described in **Section 3.4**, the complex of interest was isolated by co-immunoprecipitation through FLAG-RMA1. The other potential components of this complex were then identified by a re-immunoprecipitation step.