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Molecular and Cellular Characterization of GCC185: A Tethering Protein of the *Trans*-Golgi Network

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Abstract

Transport vesicle tethers are proteins that link partner membranes together to permit subsequent SNARE protein pairing and fusion. Despite the identification of a relatively large number of tethering proteins, little is known about the precise mechanisms by which they act. Biochemical isolation of tethers permits direct analysis of their physical characteristics and molecular interactions. Here, we describe the expression and purification of GCC185, a *trans*-Golgi-localized, 190-kDa coiled-coil tethering protein. In addition, we present a gene rescue approach to analyze the function of this tether after its depletion from cells using siRNA.

Keywords

Golgi complex; Membrane traffic; Tethering factor; Secretion; Protein purification

1 Introduction

Transport vesicle tethering is the process that physically links incoming vesicles to target membranes [1–3]. To date, two classes of tethers have been described: (1) multi-subunit complexes such as the exocyst, COG, Dsl1, and TRAPP complexes [4] and (2) larger, coiled-coil-containing proteins such as golgins and EEA1 [5, 6]. Despite significant progress, how vesicle tethering occurs remains poorly understood. One of the major limitations is the availability of purified tethering proteins. There are expression and purification protocols available for smaller tethering complexes that function at the *cis*-Golgi or for truncated versions of longer proteins [7, 8]. However, the very large size of some tethering proteins (>100 kDa) [9] makes bacterial expression difficult, if not impossible to achieve.

Here we demonstrate the expression and purification of the *trans*-Golgi-localized, coiled-coil tether, GCC185, using a mammalian expression system. The constructs presented here include both the full length and the N-terminal half of the protein; both contain an N-terminal GFP tag, followed by a FLAG-tag, prior to the start codon. Purification of GCC185 highly relies on affinity chromatography using anti-FLAG resin and a size exclusion step to remove eluting FLAG peptides and residual contaminants. The N-terminal GFP can be used for multiple purposes: (1) as a fluorescent marker to follow protein expression in cells or to detect the protein molecules in fluorescence-based assays, (2) as a globular marker to

identify protein's orientation in high-resolution microscopic techniques, and (3) as an affinity tag for immobilization on resins to capture binding partner(s).

Cellular characterization of the tethering function of GCC185 is based on its role in the retrograde transport of the mannose 6-phosphate receptors (MPRs) from late endosomes to the *trans*-Golgi network (TGN) [10, 11]. MPRs bind newly synthesized lysosomal hydrolases in the Golgi complex and deliver them to late endosomes. After releasing these enzymes, MPRs are recycled back to the TGN [12]. The cellular distribution of MPRs can be assessed by immunofluorescence microscopic staining using anti-cation-independent MPR antibody. Normally, at steady state, MPRs are in perinuclear late endosome and to a lesser extent in the TGN [13]. When the tethering is impaired, MPR staining is much more dispersed and distributed in cell periphery. This lab has shown that GCC185 is required for the transport of MPRs from late endosomes to the TGN: in the absence of GCC185, MPRs accumulate in peripheral Rab9⁺ and AP-1⁺ vesicles [14–16]. In this manner, the functionality of a tethering protein and its mutants can be examined by plasmid rescue after siRNA–protein depletion, in this case using immunofluorescence microscopic staining of MPRs to determine their intracellular localization.

2 Materials

2.1 Protein Expression in Mammalian Cells in Suspension

1. Orbital shaker housed in a 37 °C incubator with a humidified atmosphere supplied with 5 % CO₂.
2. Erlenmeyer cell culture flasks or glass flasks (*see* Note 1).
3. FreeStyle™ 293-F cells and FreeStyle™ 293 Expression Medium (GIBCO, Grand Island, NY).
4. Expression constructs: Ligate the cDNA encoding the tethering protein of interest (here we use human GCC185 full length and its N-terminal half (residues 1–889)) in the EGFP-C1 plasmid; insert a FLAG-tag (DYKDDDDK) downstream of the GFP flanked by the GGATCC linker (AS) for purification purposes.
5. Opti-MEM® I Reduced Serum Media (GIBCO) or other medium without serum.
6. Polyethylenimine (PEI) (Polysciences Inc., Warrington, PA): 1 mg/ml PEI pH 7.4. Dissolve 50 mg of PEI in 40 ml sterile water. Mix the solution on a magnetic stirrer at 50–60 °C until the solution turns colorless. Adjust the pH to 7.4. Add water to a final volume of 50 ml such that the final concentration is 1 mg/ml and sterile-filter the solution through a 0.22-μm membrane (*see* Note 2). Prepare 0.5 ml aliquots and store them at –20 °C (*see* Note 3).

¹Glass flasks may be used after thorough cleaning and autoclaving to avoid potential contamination. Glass flasks can be first soaked in dilute bleach (5–10 %) overnight. Rinse the flasks with water to remove bleach and any residual detergent. Fill each flask with water up to 50 % of the total volume before the first autoclaving. Pour out the water and autoclave the flasks for a second time.

²Filtering the PEI solution is both for sterility and to enhance the efficiency of transfection, as the presence of undissolved PEI can interfere with transfection by precipitating DNA plasmids.

³Thawed PEI can be kept at 4 °C and should be used within 2 weeks. Test the transfection efficiency before using thawed PEI that has been kept at 4 °C for more than 2 weeks.

2.2 Protein Purification

Except for the stock solutions, all buffers in this section should be prepared fresh before use and kept on ice (unless otherwise indicated).

1. 10× phosphate buffered saline (PBS) stock: 1.35 M NaCl, 17.5 mM KH₂PO₄, 100 mM Na₂HPO₄, 27 mM KCl, pH 7.4.
2. 1× PBS: Dilute the 10× PBS stock with distilled water.
3. Protease inhibitor stocks: Phenylmethanesulfonyl fluoride (PMSF, 100× stock solution), 0.1 M in isopropanol; aprotinin (100× stock), 0.1 mg/ml in ddH₂O; leupeptin (100× stock), 0.1 mg/ml in ddH₂O; pepstatin (1,000× stock), 1 mg/ml in methanol. All the protease inhibitors are used together as a 1× cocktail in lysis buffer. The stock solutions are stored at −20 °C (stable for months).
4. Lysis buffer: 50 mM Tris–HCl pH 7.4, 250 mM NaCl, 0.5 % Triton X-100, 1 mM EDTA, 10 % glycerol (v/v), and protease inhibitors.
5. Anti-FLAG M2 Affinity Gel (Sigma-Aldrich, St. Louis, MO) (*see* Note 4).
6. High-salt wash buffer: 50 mM Tris–HCl pH 7.4, 1 M NaCl, 0.5 % Triton X-100, 10 % glycerol.
7. Storage buffer: 50 mM Tris–HCl pH 7.4, 250 mM NaCl, 10 % glycerol.
8. 3× FLAG peptide (Sigma-Aldrich): Stock solution can be prepared by dissolving the peptide in 50 mM Tris–HCl pH 7.4, 150 mM NaCl at a concentration of 5 mg/ml (*see* Note 5).
9. Sepharose CL-4B (Sigma-Aldrich).
10. Basic buffers and apparatus for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

2.3 Reagents for Transfections in Functional Rescue Experiment

1. HeLa cells (ATCC) and Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 7.5 % fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.
2. Coverslips (22 × 22 mm (or smaller) that fit into the wells of a six-well plate).
3. Opti-MEM[®] I Reduced Serum Media (GIBCO) or other medium without serum.
4. Oligofectamine (Invitrogen, Grand Island, NY) or other siRNA transfection reagent.
5. FuGENE6 (Roche, Indianapolis, IN) transfection reagent.

⁴Anti-FLAG M2 is a mouse monoclonal antibody that binds FLAG-tag (or tandem FLAG) at the N-terminal, Met-N-terminal, C-terminal, and internal locations of fusion proteins. The calcium-dependent anti-FLAG M1 is an alternative, if working with N-terminal (not Met-N-terminal) FLAG fusion proteins. In addition to the use of FLAG peptide, proteins can also be eluted with a buffer containing EDTA when using the M1 resin.

⁵Construct containing 3× FLAG fusion must be eluted with 3× FLAG peptide. If the construct contains only 1× FLAG-tag fusion, FLAG peptide can be used for elution.

6. siRNA targeting the sequence 5'-GGAGTTGGAACAATCACAT-3' of GCC185 [14].
7. Constructs for rescue experiments (here, GCC185 wild type): cDNA encoding rescue plasmid ligated into pcDNA3.1(+) modified with an N-terminal Myc tag. It contains eight silent mutations in the siRNA-targeted region to prevent destruction [14, 15].

2.4 Cell Fixation and Immunofluorescence Staining

1. 1× PBS (as in Subheading 2.2, **item 2**).
2. Fixative: 3.7 % paraformaldehyde (PFA) in 200 mM HEPES pH 7.4 (*see* Note 6).
3. Permeabilization buffer: 0.2 % Triton X-100 in PBS (prepared fresh from 20 % stock).
4. Blocking/washing buffer: 1 % bovine serum albumin in PBS. It is better to prepare the blocking buffer fresh every time and filter the buffer before use.
5. Primary antibodies: monoclonal mouse anti-cation-independent MPR (2G11; [15]) and chicken anti-Myc (Bethyl Laboratories, Inc., Montgomery, TX) antibodies.
6. Secondary antibodies: Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-chicken (Invitrogen).
7. Mowiol mounting medium (*see* Note 7).

2.5 Imaging Equipment

1. Fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan): fitted with a 60×/NA 1.4 plan apochromat objective lens, a Sedat Quad filter set (Chroma Technology Corp., Bellows Falls, VT), and a charge-coupled device camera (CoolSnapHQ, Photometrics, Tucson, AZ) at room temperature.
2. MetaMorph imaging software (Molecular Devices, Sunnyvale, CA) or other software to control the instrument.
3. softWoRx (v.4.1.0; Applied Precision, Inc., Issaquah, WA) or other image deconvolution software.

3 Methods

3.1 Protein Expression and Purification

Expression scale: 100 ml.

⁶Caution: PFA is toxic. To prepare 3.7 % PFA, dissolve 1.48 g of PFA in 30 ml sterile water by adding four drops of 10 N KOH and heat the mixture at 50 °C in a hood for 15 min; vortex gently a few times. Add 8 ml 1 M HEPES pH 7.4, mix well, and filter the solution through a 0.22-μm membrane. 3.7 % PFA can be prepared fresh before immunofluorescence staining experiments; it can also be stored at −20 °C.

⁷To prepare Mowiol mounting medium, add 2.4 g Mowiol 4–88 to 6 g of glycerol, and mix by stirring thoroughly, followed by the addition of 6 ml distilled water. Incubate the mixture at room temperature for 2 h. Add 12 ml of 0.2 M Tris pH 8.5 and incubate at −53 °C until the Mowiol has dissolved. Clarify by centrifugation at 5,000 × g for 20 min. Aliquot the supernatant into vials and store at −20 °C.

1. FreeStyle™ 293-F cells are maintained in a 37 °C humidified incubator on an orbital shaker rotating at 125 rpm (*see* Note 8). Use a 500-ml Erlenmeyer cell culture flask for 100 ml culture (*see* Note 9). Depending on the type of flask used, loosen the cap of the flask if needed, to allow CO₂ exchange.
2. One day before transfection, determine cell viability and density. Seed cells at a density of 0.6×10^6 cells/ml (target cell density on the day of transfection should be $\sim 1 \times 10^6$ viable cells/ml).
3. On the day of transfection, take a small aliquot of cells to determine the viability and density before transfection.
4. Spin down the rest of the cells at $\sim 200 \times g$ for 5 min and remove the medium. Resuspend the cell pellet and dilute cells to 1×10^6 viable cells/ml in fresh, pre-warmed medium. Return cells to the incubator.
5. To prepare the transfection mixture, dilute 100 µg of DNA in 9.5 ml of Opti-MEM® I Reduced Serum Medium; mix well and add 500 µl PEI into the mixture without touching the wall of the tube. Incubate at room temperature for 15 min (*see* Note 10).
6. Add the transfection mixture dropwise to the cells and incubate the cells on a shaker at 37 °C for 22–24 h (*see* Note 11).
7. Before purifying the protein, put a small aliquot (50–100 µl) of the culture into a well plate and check the transfection efficiency or the expression of the GFP fusion protein using a fluorescence microscope. The green fluorescence signal should be readily apparent.
8. To harvest, spin down the cells at $\sim 200 \times g$ for 5 min at 4 °C and discard the medium. Wash cells gently by resuspending the pellet with 20 ml of PBS and spin down the cells again at $\sim 200 \times g$ for 5 min at 4 °C; discard supernatant.
9. Prepare lysis buffer and keep it cold on ice. Add protease inhibitors to lysis buffer right before use.
10. Lyse cells by resuspending the pellet in 30 ml lysis buffer. Incubate the lysate on ice for 15 min.

⁸The FreeStyle™ 293-F cells are mammalian cells derived from the HEK293 cell line that is adapted to growth in suspension, with a doubling time of about 24 h depending on the age and handling of the cells. Cells should be split to $0.2\text{--}0.5 \times 10^6$ cells/ml when the density reaches $1\text{--}3 \times 10^6$ cells/ml. Cell viability and density can be assessed by trypan blue staining and hemocytometer counting.

⁹The culture volume should always be about 20 % of the flask volume. The performance of the cells may be affected if the culture volume is 30 % or more of the flask volume.

¹⁰The amount of DNA required for transfection depends on the volume of culture, the expression construct, and the size of the protein. A strong promoter or a smaller protein usually requires less DNA and shorter transfection time to reach a similar level of protein production. A recommended starting condition is 1 µg DNA/ml of culture; further optimization may be required. In addition to PEI, 293fectin™ reagent (Invitrogen) can be used for transfecting FreeStyle™ 293-F cells. In our hands, 293fectin™ reagent gives higher protein production for secreted proteins when compared with the use of PEI, but the two reagents appear to perform almost equally well for cytosolic proteins. Please refer to the manufacturer's protocol for details regarding the 293fectin™ reagent.

¹¹Time required for optimal expression varies between proteins. If the protein is expressed in FreeStyle™ 293-F cells for the first time, harvest cells at multiple time points to determine the shortest time required to get the highest level of protein and whether the protein is intact or degraded.

11. Spin the lysate at $20,000 \times g$ for 30 min at 4 °C in a Fiberlite F15 (8 × 50c) (Thermo Scientific, Waltham, MA) or comparable rotor.
12. Equilibrate 100 µl of anti-FLAG M2 affinity gel with 30 column volumes of lysis buffer (1 ml × three times).
13. Incubate supernatant from **step 11** with equilibrated anti- FLAG M2 affinity gel on a rocking platform for 4 h at 4 °C.
14. Wash affinity gel three times with ten column volumes of high-salt wash buffer (*see* Note 12).
15. Wash affinity gel two times with ten column volumes of storage buffer.
16. Elute bound FLAG fusion protein by incubating the affinity gel with 300 µl of 0.2 mg/ml 3× FLAG peptide overnight at 4 °C with rotation.
17. Perform a final elution with 200 µl of 0.1 mg/ml 3× FLAG peptide in storage buffer at 4 °C for 30 min (*see* Note 13).

The FLAG peptide eluate of GFP-FLAG-GCC185 full length and N-terminal half has a purity of about 90 % when assessed by Coomassie Blue staining of SDS-PAGE gels. The smaller, N-terminal half of GCC185 has higher yield than the full-length protein (Fig. 1). The next step is designed to remove 3× FLAG peptide and residual contaminants by size exclusion.

18. Prepare a 12.5 ml Sepharose CL-4B column (*see* Note 14) and equilibrate the column with two column volumes (25 ml) of storage buffer (*see* Note 15).
19. Carefully load 300 µl of the FLAG peptide eluent without disturbing the resin and allow the sample to enter the packed bed completely (*see* Note 16). Discard the flow-through.
20. Place tubes for sample collection under the column.
21. Elute with a total of one column volume (12.5 ml) of storage buffer and collect 0.5 ml fractions.
22. Perform SDS-PAGE to detect fractions containing the target protein and to evaluate purity (*see* Note 17). The N-terminal half of GCC185 elutes at 6.0–6.5 ml from a 12.5 ml Sepharose CL-4B column with high purity (Fig. 1).

¹²High-salt wash can help reduce nonspecific, ionic binding.

¹³The elution conditions vary between proteins. Please refer to Note 5 for the choices of FLAG peptides. A total of five column volumes of peptide elution are sufficient to elute most FLAG fusion proteins. Proteins with high thermal stability can be eluted at room temperature for shorter times. Allow longer incubation times when working with larger proteins.

¹⁴Sepharose CL-4B has a fractionation range of 6×10^4 – 2×10^7 for globular proteins.

¹⁵The storage/elution buffer in the size-exclusion step depends on the buffer requirement of the assay(s) that the purified protein will be used for. Check the pI of the protein, since basic proteins at low ionic strengths may adsorb to the resin due to the presence of a small number of ionic sulfate and carboxyl groups on Sepharose; in this case, high ionic strength buffer may be necessary.

¹⁶A sample volume of 1–3 % of the column volume usually gives the maximum resolution in size exclusion chromatography; do not exceed 5 %.

¹⁷The protein is ready to use. Depending on the concentration and the amount of protein needed, concentration of the final product or larger-scale expression may be required.

3.2 Monitoring Tethering Function by Immunofluorescence Microscopic Staining

In this type of functional rescue experiment, cells are depleted of the tether with siRNA for a total of 72 h. Rescue constructs are transfected into cells 24 h after initial siRNA treatment (*see* Note 18) so that the time permitted for rescue is 48 h (*see* Note 19) overall.

1. HeLa cells are cultured in DMEM supplemented with 7.5 % fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 37 °C tissue culture incubator supplied with 5 % CO₂.
2. One day before transfection, plate HeLa cells in a 10-cm dish at ~30 % confluency in medium without antibiotics.
3. On the day of transfection, check to make sure the cells are healthy and viable at ~50–60 % confluency.
4. Prepare the transfection mixture by diluting 800 pmol siRNA (20 µl from 40 µM stock) in 625 µl of Opti-MEM® I Reduced Serum Medium. In a separate tube, dilute 16 µl of Oligofectamine in 44 µl of Opti-MEM® I Reduced Serum Medium. After incubating the two tubes at room temperature for 5 min, mix the two tubes and incubate the mixture at room temperature for another 20 min to allow formation of siRNA–Oligofectamine complex.
5. Slowly add the siRNA–Oligofectamine complex dropwise to cells using a 200-µl micropipette tip, and incubate the transfected cells in a 37 °C incubator for 22 h.
6. Place sterile coverslips into each well of a six-well dish. 22 h after siRNA transfection, trypsinize the transfected cells in the 10-cm dish and seed them into each well of the six-well plate containing sterile coverslips, to achieve a confluency of ~40 %.
7. Return the plate to the incubator and allow cells to adhere to the coverslips for at least 3 h before transfection of rescue constructs.
8. Set up transfection reactions in sterile polystyrene tubes:
 - a. Calculate the volume of DNA to be added in each reaction. The amount of DNA needed for each well of a six-well plate is 1 µg.
 - b. To each reaction tube, add pre-warmed Opti-MEM® I Reduced Serum Medium (volume of medium = 100 µl, volume of DNA to be added).
 - c. Add 3 µl of FuGENE® 6 transfection reagent to the medium (transfection reagent: DNA = 3:1), mix immediately, and incubate at room temperature for 5 min (*see* Note 20).

¹⁸Cells depleted of GCC185 appear more elongated and larger in size; this can be seen 22 h after siRNA transfection.

¹⁹The time required for incubating the cells with rescue constructs depends on the phenotype being assessed, the size of the protein being expressed, and the half-life of the endogenous protein being depleted. MPR retrieval phenotype is usually assessed 48 h post-transfection.

²⁰The ratio between transfection reagent and DNA should be optimized as it varies with the cell line and construct used. Always add transfection reagents directly into medium without touching the sides of the tube.

- d.** Add 1 μ g of Myc-tagged siRNA-resistant rescue constructs to the mixture from step (c); mix well and incubate at room temperature for 15 min (*see* Note 21).
- 9.** Slowly add the 100 μ l transfection reactions dropwise to each well of the six-well using a 200- μ l micropipette tip. After 48 h, remove the medium and wash each well three times with 2 ml PBS.
- Procedures below are performed at room temperature unless specified.
- 10.** Incubate the cells in 3.7 % formaldehyde in 200 mM HEPES pH 7.4 for 20 min for fixation. Remove the fixative and wash cells twice with PBS.
- 11.** Permeabilize the cells with 0.2 % Triton X-100 in PBS for 5 min. Remove the permeabilization buffer and wash cells twice with PBS.
- 12.** Incubate the cells in blocking buffer for 15 min.
- 13.** Prepare primary antibodies by diluting the chicken anti-Myc antibody at 1:1,000 in monoclonal mouse anti-cation-independent MPR antibody culture supernatant.
- 14.** Add the diluted primary antibodies on top of each coverslip and incubate at room temperature for 1 h (*see* Note 22). Make sure the antibody solution covers the entire surface of the coverslips.
- 15.** Remove primary antibodies and wash coverslips three times for 5 min each with wash buffer on a Belly Dancer or other platform shaker.
- 16.** Prepare secondary antibodies by diluting the Alexa Fluor 488 goat anti-mouse antibody 1:2,000 and the Alexa Fluor 555 goat anti-chicken antibody 1:2,000 in wash buffer.
- 17.** Add secondary antibodies on top of each coverslip and incubate at room temperature for 1 h (*see* Note 22).
- 18.** Remove the secondary antibodies and wash the coverslips two times for 5 min each with wash buffer and once with PBS for 5 min on a Belly Dancer.
- 19.** Clean glass slides and put a drop of Mowiol on top.
- 20.** Transfer 500 ml of distilled water into a beaker. Dip the coverslip into distilled water for 10 s; absorb any residual water droplets by placing the edge of the coverslip onto a piece of Kimwipe or other tissue.
- 21.** Carefully put the coverslip (cells facing down) on top of the Mowiol droplet; avoid bubbles and clean away excessive Mowiol using a Kimwipe. Let the glass slides dry in the dark overnight at room temperature before imaging. Glass slides can be transferred to 4 °C in the dark for long-term storage.
- 22.** Acquire images using a fluorescence microscope at 60 $\times$ [13]:

²¹Long incubation times (e.g., >45 min) may affect the performance of transfection.

²²Fixed cells on coverslips can be incubated in primary and secondary antibodies at 4 °C for longer times (at least 4 h).

- a. Acquire 3–5 Z-sections with a Z-axis drive (MFC-2000, Applied Scientific Instrumentation, Eugene, OR) in 0.2-mm steps.
 - b. Use software to deconvolve images using a theoretical point spread function.
23. Score rescue experiment images. Rescued cells display a more compact perinuclear staining for MPRs, whereas cells depleted of the tethering protein or non-rescued cells show a more dispersed punctate staining that extends to the cell periphery (Fig. 2) (*see* Note 23).

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²³When scoring for the MPR dispersal phenotype, do not include dividing cells or multinucleated cells.

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Fig. 1. Purification of the *trans*-Golgi-localized tethering protein GCC185. Coomassie-stained SDS-polyacrylamide gel of lysate input (*lane 1*), high-salt washes (*lanes 2 and 3*), purified GFP-FLAG-GCC185 full length (*lanes 4 and 5*), and N-terminal half (1–889) before (*lane 6*) and after gel filtration on Sepharose CL-4B (*lane 7*)

Fig. 2. Immunofluorescence micrographs of HeLa cells depleted of GCC185 by siRNA for 72 h followed by transfection of GCC185 rescue plasmid. *Left column*, expression of the indicated rescue construct detected using chicken anti-Myc and Alexa Fluor 555 goat anti-chicken antibodies. *Right column*, MPR localization detected by 2G11 mouse anti-cation-independent MPR and Alexa Fluor 488 goat anti-mouse antibodies. Approximate cell outlines are indicated