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Expression of functional myc-tagged conserved oligomeric Golgi (COG) subcomplexes in mammalian cells

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Abstract

Docking and fusion of transport carriers in eukaryotic cells is regulated by a family of multi-subunit protein complexes (MTC) that sequentially and/or simultaneously interact with other components of vesicle fusion machinery, such as SNAREs, Rabs, coiled-coil tethers and vesicle coat components. Probing for interactions of multi protein complexes has relied heavily on the method of exogenously expressing individual proteins and then determining their interaction stringency. An obvious pitfall of this method is that the protein interactions are not occurring in their native multi-subunit state. Here, we describe an assay where we express all eight subunits of the conserved oligomeric Golgi (COG) complex that contain the same triple-myc epitope tag, and then assay for the (sub) complex's interaction with known protein partners. The expression of all eight proteins allows for the assembled complex to interact with partner proteins, and by having the same tag on all eight COG subunits, we are able to very accurately quantify the interaction with each subunit. The use of this assay has highlighted a very important level of specificity of interactions between COG subcomplexes and their intracellular partners.

Keywords

COG; conserved oligomeric Golgi complex; Golgi; SNARE; vesicle tethering; multi-expression; co-immunoprecipitation; subcomplexes; protein-protein interaction

1. Introduction

The conserved oligomeric Golgi (COG) complex is a peripheral membrane protein complex in the sub-family of multi-subunit tethering complexes (MTC) (1). The COG complex functions to tether retrograde intra-Golgi vesicles to the Golgi cisternae, a critical step in vesicle docking that occurs prior to SNARE mediated membrane fusion (2). The COG complex is required for the proper recycling of Golgi localized glycosylation enzymes (3–5), with defects in COG subunits resulting in a class of disorders known as congenital disorders of glycosylation (6). According to the current “maturation” model of the Golgi (7), vesicle-mediated recycling of Golgi enzymes is essential for proper glycosylation of glycoconjugates that traffic through the Golgi apparatus. Vesicle tethering is hypothesized to occur in two steps, the initial capturing of a vesicle, achieved by the sub-family of long

coiled-coil tethers, and then the organization of the vesicle on the acceptor membrane, achieved by the MTC's (8). Currently there is little understanding of how the steps of vesicle tethering and fusion are coordinated.

The COG complex is comprised of eight different protein subunits (named COG1-8) (9, 10) that are organized into two functionally distinct lobes; COG1-4 in lobe A, and COG5-8 in lobe B (11, 12). The two lobes, or subcomplexes, are bridged together via an interaction between lobe A subunit COG1 and lobe B subunit COG8 (13, 11). Previous studies on the interactome of the COG complex has revealed interactions with many different families of trafficking regulatory proteins including SNARE's, SNARE-interacting proteins, Rab GTPases, coiled-coil tethers, and COPI subunits (14–22), and COG membrane attachment relies heavily on these interactions (22). COG interactions with its partners are likely to be transient and tightly regulated, and as a result are difficult to detect and measure in biochemical assays with the endogenous proteins. Therefore, insight into the COG complex function/mechanism has relied heavily on the use of exogenous overexpression of individual subunits (23–25, 19). Furthermore, the COG subunits are not thought to exist in the cell in a monomeric state (Willett and Lupashin, in preparation), thus interactions with COG subunits are believed to be occurring with the assembled complex/subcomplexes.

In this chapter, we describe an assay based on simultaneous expression of all eight COG subunits in mammalian cells. Importantly, we express all eight proteins with the same C-terminus triple-myc epitope tag (Figure 1A), which allows for the accurate quantification of each COG subunit in relation to the entire COG complex (a task that is not possible with detection by individual antibodies). Using this multi-expression assay we can probe for COG interactions in the COG's native state. Indeed, when we express all eight COG subunits in HEK293T cells, we see that the complex is both soluble and bound to the Golgi (P15) and vesicle (P100) membranes, indicating that our expressed proteins are physiologically similar to the endogenous complex (26, 27). In a previous study, we and others (24, 23) have demonstrated that the *trans*-Golgi SNARE Syntaxin 6 (STX6) very specifically interacted with COG6. Now, using our multi-expression assay we show that there is a clear preference for GFP-STX6 to bind to lobe B, and not lobe A or the assembled complex (Figure 2A, B). Similarly, we see that there are certain proteins which prefer to bind to either lobe A, or the fully assembled complex (22). This demonstrates specificity in the subcomplexes' interactions, and is further indicative that the subcomplexes have different functional activities in the cell. Finally, we propose that this assay is a more physiologically relevant method to study interactions of the COG complex, and perhaps all other multi-subunit protein complexes.

2. Materials

2.1 Constructing multi-expression plasmid of COG components

Mammalian expression constructs were generated using standard molecular biology techniques.

1. Individual COG ORF's were cloned into a vector plasmid containing three sequential Myc epitope tags (See Note 1).

2. Primers designed to add AscI and PacI restriction digestion sites on the COG-3Myc genes:
 - a. Forward Primer: GATAGGCGCGCCTGACCGCCCAACGACCC
 - b. Reverse Primer: GAGCTTAATTAAGGGACCCCGTCCCTAACCCACGG
3. Multi-label kit from ATG Biosynthetics (Germany): contains donor and acceptor fusion vectors ML-DGZ2x and ML-DSZ2cx, donors and ML-AAZ6 and ML-AKZ1, acceptors, as well as competent piRHC+ bacteria for cloning.
4. Cre recombinase (New England Biolabs; Ipswich, MA)
5. AscI and PacI restriction enzymes (New England Biolabs; Ipswich, MA)

2.2 Lipofectamine 2000 Transfection components

1. Culture dishes: 12 well tissue culture plates (TPP).
2. HEK293 T (ATCC CRL-3216) cells grown on 12 well plate to 90% confluency.
3. Lipofectamine 2000 Transfection Reagent (Invitrogen; Carlsbad, CA).
4. Plasmids: Rat Syntaxin6 (rSTX6) cloned into pEGFP-C1. See section 3.1 for COG's with Myc tag.
5. Dulbecco's Phosphate Buffered Saline (dPBS 1X) without calcium and magnesium (Thermo Fisher Scientific Inc; Waltham, MA).
6. Transfection media: Opti-MEM® I Reduced Serum Media buffered with HEPES and sodium bicarbonate and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements and growth factors (Invitrogen; Carlsbad, CA).
7. Growth Media: dilute 50 mL of heat inactivated Fetal Bovine Serum (FBS) (Atlas Biological; Fort Collins, CO) in 450 mL of in DMEM/F-12 50/50 medium supplemented with 15 mM HEPES, 2.5 mM L-glutamine (Invitrogen; Carlsbad, CA). Filter solution in 0.45 µm PES Corning (Lowell, MA) filtration system.
8. Gibco® 0.25% Trypsin-EDTA (1x) phenol red (Invitrogen; Carlsbad, CA).

2.3 Immunoprecipitation of components

1. HEK293T cells transfected with COG complex multi expression plasmids for 24 h (See Methods 3.1).
2. 1.7 mL microcentrifuge tubes
3. Centrifuge equipped with microcentrifuge rotor.
4. Dulbecco's Phosphate Buffered Saline (dPBS 1X) without calcium and magnesium (Thermo Fisher Scientific Inc; Waltham, MA).

¹COG ORF's were cloned as previously described (22, 19, 24).

5. Immunoprecipitation (IP) lysis buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 μ L/mL Protease inhibitor cocktail, 2 μ L/mL 20% PMSF in DMSO.
6. GFP binding protein (GBP)-conjugated glyoxal agarose beads (ABT; Tampa, FL) (see Note 2–³)
7. Clip-Bar model microcentrifuge rotator
8. Washing buffer: 0.05% Triton X-100 in PBS.
9. 6X Laemmli sample buffer: 30% Glycerol, 12% SDS, 303 mM Tris-HCl pH 6.8, 0.006% Bromophenol Blue, 5% 2-mercaptoethanol.
10. Vacuum apparatus for collecting waste: Büchner flask, with extended intake tubing, connected to a vacuum source.
11. Elution buffer: 2X Laemmli sample buffer (BioRad) with 10% 2-mercaptoethanol.

2.4 Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot components

1. 7.5% SDS-PAGE mini gels
2. Vertical mini gel electrophoresis unit (BioRad)
3. SDS-PAGE running buffer: 25 mM Tris, 0.192 M Glycine, 0.1% SDS
4. 0.2 μ m Whatman Protran Nitrocellulose Blotting Membranes (GE Life Sciences; Pittsburgh, PA)
5. Blotting paper
6. Sponges
7. Transfer Chamber: Wet-Tank blotting unit (BioRad)
8. Transfer buffer: 25 mM Tris, 0.192 M Glycine, 20% Methanol, 0.02% SDS
9. PBS
10. Flat platform model rotator
11. OdysseyTM blocking buffer (LI-COR Biosciences; Pittsburgh, NE) containing 0.01% Tween-20
12. Secondary antibody incubation solution: PBS containing 5% milk.
13. Antibodies:

²GFP binding protein (GBP) (28) with a six histidine tag (pET24b vector) was transformed into the BL21 strain of competent *E. coli*. The bacteria were induced with 1mM IPTG for 4 hrs at OD 0.4. The bacteria were lysed and the GBP protein was purified on a column containing Talon Resin (Clontech; Mountain View, CA) and eluted with 0.1 M Imidazole. The purified protein was then dialyzed in a 0.1 M bicarbonate buffer with 10% glycerol (pH 8.5) overnight. The next day the purified protein was dialyzed in 0.1 M bicarbonate buffer with 10% glycerol pH 10.5, and then the protein was conjugated to glyoxal agarose beads (Agarose Bead Technologies; Tampa, FL) using the manufacturer's protocol.

³GBP beads are not required for this protocol. Additionally, we have used the standard method of GFP antibodies and Protein G beads to capture the protein interactions (22, 19, 24).

- a. Primary antibodies: c-Myc (A190-205A: Bethyl Laboratories; Montgomery, TX), GFP (MMS-118P: Covance; Princeton, NJ)
- b. Secondary antibodies: LI-COR Donkey anti Mouse 680 (926-32222), LI-COR Donkey anti Rabbit 800 (926-32213) (LI-COR Biosciences; Lincoln, NE)

14. LI-COR Odyssey® imaging system.

3. Methods

3.1 Construction of multi-COG expression plasmid

1. COG ORF's with the triple Myc epitope tag were amplified by PCR to add AscI and PacI restriction digestion sites at the 5' and 3' ends of the gene, respectively, and then inserted into either donor or acceptor vectors (See Notes 4–5).
2. Using the manufacturer's protocol, donor and acceptor vectors were fused using cre recombinase into combinations of one, two, or three COGs: hCOG5-3Myc/hCOG7-3Myc, hCOG6-3Myc/hCOG8-3Myc, hCOG1-3Myc/hCOG2-Strep-3Myc/-hCOG4-3Myc, hCOG3-3Myc.
3. Isolate DNA from bacteria using the QIAprep Spin Miniprep Kit (Qiagen)

3.2 Transfection

1. Plate HEK293T cells on 12 well culture dishes one day prior to transfection in 10% FBS DMEM/F-12 media that does not contain any antibiotics so that the cells are 90% confluent and evenly spread on the day of the transfection (see Note 6). Grow cells at 37°C and 5% CO₂ in a 90% humidified incubator.
2. Prepare transfection solutions with modified manufacturer's protocol. For a 12 well plate: in a 1.5 mL microcentrifuge tube dilute 3 µL of Lipofectamine™ 2000 in 50 µL of Opti-MEM®, set aside and let incubate for 5–10 minutes (See Note 7).
3. In a separate tube, combine 1.6 µg total DNA (multi-expression COG DNA, 0.2 µg of each plasmid, and 0.8 µg of GFP tagged partner protein) with 50 µL of Opti-MEM® gently mixing the solution (See Note 8).
4. After 5–10 minutes, combine the diluted DNA with the diluted Lipofectamine™ 2000 and incubate for 20 minutes.

⁴Because we were only successful in combining 2 or 3 COGs into one plasmid, we chose to combine them by their specific interactions with the other COG proteins. Lobe B COG5 and COG7 were combined into one plasmid because they are known to form a stable dimer (11). COG6 and COG8 were combined because they are strong protein partners (19), and COG1, COG2, and COG4 were combined into one plasmid.

⁵Multi-expression plasmids were generated as per the manufacturer's protocol. Positive clones were selected on dual resistance media and were confirmed to contain the COG's both by restriction enzyme digestion and western blot.

⁶HEK293T cells are loosely adhered to the plate. It is important not to add media directly to the cells as they will detach.

⁷We found that by decreasing the volume of the OPTI-MEM used to dilute the transfection reagent and DNA we achieved higher transfection efficiency.

⁸The amount of GFP-rSTX6 was kept even with the total concentration of Myc tagged COGs (0.8 µg each). For the COG's, the 0.8 µg was divided evenly between the 4 plasmids that contain all 8 COG subunits.

5. After 20 minutes of incubation, add in drop wise manner the DNA-Lipofectamine™ 2000 complexes to their corresponding wells. Mix gently by rocking the plate back and forth (See Note 9).
6. Incubate the cells for 8–12 hours at 37°C, 5% CO₂, and 90% humidity, then remove transfection solution and replace with 10% FBS DMEM/F-12 growth media and allow cells to recover.
7. 24 hours after the transfection proceed to harvesting for Co-Immunoprecipitation.

3.3 Co-Immunoprecipitation

All steps of the Immunoprecipitation are done at room temperature, with room temperature reagents.

1. HEK293T cells that have been transfected for 24h with COG multi-expression plasmids were collected by gentle resuspension in 1mL of growth media (See Note 10) and placed in a 1.7 mL microcentrifuge tube.
2. Centrifuge cells for 2 min at 1000g.
3. Remove growth media from tube using a vacuum and re-suspend the cell pellet in 1 mL of 1X PBS (See Note 11).
4. Centrifuge cells for 2 min at 1000g.
5. Remove PBS from tube re-suspend the cell pellet in 0.5 mL of Immunoprecipitation lysis buffer.
6. Incubate the cells in lysis buffer and allow lysis to proceed for 30 min.
7. Centrifuge cell lysate for 10 min at 20,000g.
8. Aliquot 50 µL of lysate supernatant (S20) to a new 1.7 mL microcentrifuge tube for analysis. Add 5 µL of 6X sample buffer and boil for 5min.
9. Transfer remaining S20 to a new microcentrifuge tube.
10. Add 25 µL of 50% washed GBP-bead suspension (See Note 12).
11. Incubate beads and lysate sample for 2 h on rotator.
12. After 2 h, centrifuge bead and sample mixture for 1min at 3000g to pellet beads.
13. Remove sample supernatant by pipette and set aside.
14. Wash beads by resuspension in 0.5 mL of washing buffer (See Note 13)
15. Centrifuge beads for 1min at 3000g to pellet beads.

⁹Add the Lipofectamine/DNA solution very gently to the cells in a drop wise fashion so as not to disrupt them.

¹⁰Because the HEK293T cells are only loosely adherent, the cells can be collected directly in the growth media by gentle resuspension with a pipette.

¹²Before adding the GBP-beads to the lysate, it is important to wash the beads 3 times in PBS to remove any unbound GBP from the beads. Unbound GBP protein will decrease the overall efficiency of the IP because it will readily bind the GFP tagged proteins. To wash the beads, simply add PBS to the beads, spin for 1min at 3000g, remove PBS by vacuum aspiration, and repeat 2 more times.

¹³When washing beads add washing buffer gently to the side of the tube. Gently tap the tube to completely re-suspend the bead pellet.

16. Remove wash buffer by vacuum aspiration (See Note 11)
17. Repeat wash step repeated more times (step 12–14).
18. After the 3rd wash, remove wash buffer using vacuum aspiration. Then, using a fine pipette tip, carefully remove the residual wash buffer from beads.
19. Re-suspend the beads in 45 μ L of elution buffer. Boil sample for 5 min at 95°C.

3.4 SDS-PAGE electrophoresis and Western blot

1. Load boiled samples onto SDS-PAGE gel and run at 150V until the dye front reaches the bottom of the gel.
2. When gel is almost finished running, prepare the transfer apparatus: Soak sponges (2) and filter paper (2) in transfer buffer. Soak nitrocellulose membrane in 20% methanol solution.
3. After electrophoresis, pry open the glass plates and gently place the gel onto the filter paper/sponge soaked in transfer buffer.
4. Gently place the nitrocellulose membrane on top of the gel (See Note 14).
5. On top of the membrane add the second layer of filter paper and sponge that was soaked in transfer buffer.
6. Close the cassette and place into the transfer chamber.
7. Fill the chamber with transfer buffer and insert stir bar and ice block.
8. Run the transfer at 100V for 1h on top of a stir plate running at maximum speed.
9. After transfer, trim the membrane and place inside a clean blotting box with PBS (See Note 15).
10. Block the membrane for 30 min using Odyssey blocking buffer containing 0.01% Tween. Incubate membrane at room temperature on table rocker (See Note 16).
11. After block, add primary antibodies (Myc: 1:10000, GFP: 1:2000) and incubate for 1hr while rocking at RT.
12. After primary antibody incubation, wash the membrane four times by adding 5–10 mL of PBS and rocking for 5min.
13. After wash, add secondary antibody solution diluted in PBS containing 5% milk (both secondary antibodies diluted 1:40000). Let incubate for 1h while rocking at room temperature.
14. Repeat wash step (Step12).

¹¹When using a vacuum, be careful not to get too close to the cell pellet/bead pellet. Leave approximately 25–50 μ L of volume to protect the pellet.

¹⁴Remove all air bubbles between the gel and membrane by gently swiping your fingers across the top of the membrane or with a plastic roller.

¹⁵Before placing the membrane in the blotting box you may choose to do a Ponceau S stain to determine the efficiency of the transfer.

¹⁶The amount of buffer to be added is dependent on the size of the container used for blotting. Use enough solution to completely cover the membrane

15. Scan in blots using LI-COR Odyssey® system.

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Figure 1. Expression of all eight COG subunits in mammalian cells

A) HEK293T cells were transfected with 4 plasmids containing all 8 COG subunits with c terminal 3 Myc epitope tag. Cells were lysed, loaded on SDS-PAGE gel and then blotted with anti-Myc antibodies. B) HEK293T cells transfected with lobe B plasmids were homogenized by passage through a needle and then fractionated into cytosol and membrane by differential centrifugation. Lobe B subunits can be detected both on Golgi membranes (P15) and vesicular membranes (P100), indicating that expression of the full subcomplex is physiologically similar to the endogenous sub-complex

Figure 2. COG subcomplexes selectively interact with COG partner protein Syntaxin 6

A) Single COG subunit hCOG6-3Myc, 2 plasmids containing 4 Lobe B COG-Myc subunits, 2 plasmids containing 4 Lobe A COG-Myc subunits, or 4 plasmids containing all 8 COG-Myc subunits were transfected in HEK293T cells along with GFP-tagged STX6. Cells were lysed in IP lysis buffer and then incubated with GFP beads. Bead eluates were run on an SDS-PAGE gel and blotted with anti-Myc and anti-GFP antibodies. There is significantly more of the lobe B COG subunits that are co-IP'd by GFP-STX6 compared to individual COG6, lobe A, or even the fully assembled COG complex (B). These results indicate a preference and level of selectivity of COG interactions with assembled (sub)complexes.