Molecular Insights into Vesicle Tethering at the Golgi by the Conserved Oligomeric Golgi (COG) Complex and the Golgin TATA Element Modulatory Factor (TMF)*

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Background: Delivery of the vesicle into the pre-fusion state during tethering is not understood.

Results: Interactions between the COG complex, golgins and Rabs were mapped. Two ends of the golgin TMF both bind COG and different Rabs, the middle binds the target membrane.

Conclusion: COG may reel the vesicle into docking along the golgin.

Significance: Mechanistic link between tethering complex and coiled tether established.

Protein sorting between eukaryotic compartments requires vesicular transport, wherein tethering provides the first contact between vesicle and target membranes. Here we map and start to functionally analyze the interaction network of the conserved oligomeric Golgi (COG) complex that mediates retrograde tethering at the Golgi. The interactions of COG subunits with members of transport factor families assign the individual subunits as specific interaction hubs. Functional analysis of selected interactions suggests a mechanistic tethering model. We find that the COG complex interacts with two different Rabs in addition to each end of the golgin “TATA element modulatory factor” (TMF). This allows COG to potentially bridge the distance between the distal end of the golgin and the target membrane thereby promoting tighter docking. Concurrently we show that the central portion of TMF can bind to Golgi membranes that are liberated of their COPI cover. This latter interaction could serve to bring vesicle and target membranes into close apposition prior to fusion. A target selection mechanism, in which a hetero-oligomeric tethering factor organizes Rabs and coiled transport factors to enable protein sorting specificity, could be applicable to vesicle targeting throughout eukaryotic cells.

Vesicular transport sorts proteins between organelles, thereby ensuring their distinct biochemical compositions. This requires that vesicles carrying different cargoes are delivered to the appropriate target compartment. Vesicle tethering is believed to play a critical role in determining targeting specificity (1, 2). Rather than a physical attachment per se, vesicle tethering is defined as the collection of protein interaction steps that a vesicle undergoes leading to SNARE-mediated membrane fusion (3). Tethering factors, either extended coiled-coil dimers or multisubunit hetero-oligomers, are the organizers of vesicle targeting. Whereas the coiled-coil dimers (known as golgins at the Golgi) supposedly capture the vesicle while it is distant from its target (4–6), multisubunit complexes are thought to coordinate the protein interactions of tethering (7, 8).

Vesicle transport within the Golgi is a particularly useful system for studying the targeting specificity and tethering of vesicles. The mammalian Golgi typically consists of four to eight cisternae, each of these containing a unique set of modifying enzymes. Most of the transport through the Golgi is believed to occur by cisternal maturation (9), which means that secretory proteins always remain within the cisternae, while resident enzymes are recycled via retrograde vesicles (10). This sorting model implies that several distinct types of retrograde coat protein I (COPI) vesicles with differing enzyme content generate the differential enzyme distribution of the Golgi. Indeed, such vesicles have been shown to use different members of the golgin family for tethering (2).

Tethering factors have been shown to interact with Rab GTPases (4, 11), a protein family responsible for the specificity of membrane organization (12). For example, the golgins GM130 and p115 synergistically interact with Rab1, whereby the GM130-Rab1 binding enhances the p115-Rab1 interaction (13). These interactions are necessary for the tethering of COPI vesicles at the cis-Golgi (14) and likely involve an induced con-

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The abbreviations used are: COPI, coat protein I; COG, conserved oligomeric Golgi; 3AT, 3-amino triazole; Y2H, yeast two hybrid; BFA, Brefeldin A; TMF, TATA element modulatory factor; CATCHR, complexes associated with tethering containing helical rods.
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formational change of GM130 (15). Moreover, the C-terminal coil of the golgin TMF has been shown to interact with Rab6, this interaction being necessary for Golgi localization of this golgin (16, 17). Many golgins bind more than one Rab in Droso-
phila (18) as well as mammals (19–21), suggesting either a combinatorial Rab-code for golgin-assisted vesicle targeting and/or the need for multiple Rabs for moving vesicles through the mesh of golgins covering the organelle (6). The questions remain: what are the molecular details of these golgin-Rab interactions during tethering, and how do interactions between Rabs and golgins lead to a specific vesicle targeting reaction?

The central coordinator for retrograde vesicle tethering at the Golgi is the multisubunit conserved oligomeric Golgi (COG) complex (8, 22). Indeed, mutations in the COG complex cause glycosylation defects in both model organisms and patients (23–25). COG localization has no cis-trans preference (26), implying that it coordinates vesicle targeting at every cis-
terna. Yet, lobe A (subunits 1–4) is necessary for maintaining proteins in the early/medial Golgi, and lobe B (subunits 5–8) is needed primarily for the targeting of late-Golgi processing enzymes (27–31). Given that the only protein motifs in COG are predicted coiled coils (32), a plausible molecular function for COG is to act as a protein interaction hub. Indeed, the yeast ortholog was found in a direct physical interaction with the Rab Ypt1p and the vesicle coat COPI (33), whereas mammalian COG has been found to interact with the SNAREs syntaxin5 and 6 (34, 35), the SM protein Sly1 (36), several Rabs (37), and the golgins p115 and golgin-84 (38, 39). Development of a general model for COG mediated tethering is hampered by the lack of knowledge about the full set of protein interactions that the complex is involved in. We therefore determined the map of interactions between the complex and other proteins implicated in vesicle tethering. A number of Rabs, golgins, and sub-
units of the COPI coat combinatorially interact with different COG subunits. Upon further functional dissection of a subset of these interactions we discovered a network between COG, the Rabs1 and 6, and different regions of the golgin TMF. Since we also find that TMF binds to Golgi membranes cleared of COPI, and its COG interacting regions dominantly inhibit protein gly-
coylation, we propose a model in which interactions between a tethering complex and a coiled coil tether could jointly reel in cosylation, we propose a model in which interactions between a and its COG interacting regions dominantly inhibit protein gly-

EXPERIMENTAL PROCEDURES

Antibodies—For immunoblotting affinity-purified anti-Cog4 (1:300), anti-His-HRP (1:10000, Sigma), anti-GST (1:1000, Invitrogen monoclonal), anti-Cog3 (1:2000, 40, monoclonal), affinity-purified anti-TMF (1:500, Lowe laboratory), anti-
HA.11 (1:500, Covance monoclonal), anti-GFP (1:2000, Invit-
rogen monoclonal), and anti-myc (1:1000, Cell Signaling monoclonal) were used. The anti-Cog4 antibodies (Oka et al., 27) were affinity purified against recombinant insect cell pro-
duced His6-Cog4 (Richardson et al., 43). The anti TMF anti-
bodies were raised against GST-tagged head and tail regions in sheep, and affinity purified against these same proteins. Serum was pre-cleared against GST before affinity purification against immunogen. Anti-HA 3F10 (Roche) or anti-GFP (Invitrogen) was used for immunoprecipitation. For immunofluorescence mouse monoclonal anti-GalNAcT2 (1:5, tissue culture super-
natant, kind gift from H. Clausen), anti-GFP (1:500, Invitrogen polyclonal), anti-Rab6 (1:50, Santa Cruz Biotechnology, C19), anti-β-COP (1:3000, MAD, T.E. Kreis laboratory) were used.

Yeast Two-hybrid Assays—COG subunits, golgins, and Rabs were expressed as Gal4 binding or activation domain fusion proteins from pGAD/pGBD/pGBDU plamids (66). Rab binding domain fusion proteins contained mutations to lock the protein in the active (T) or inactive (D) conformation (67). Pairs of fusion constructs were introduced into yeast (genotype: trp1–901 leu2–3,112 ura3–52 his3–200 gal4Δ gal80Δ LYS2:: 

GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) by mating of hap-
loid single transformants (Fig. 1A) or pairwise co-transforma-
tion (Figs. 1B and 2A and C). Cells were subsequently scored for growth on selective media, typically following 1 week of incubation at 30 °C or 2 weeks at 14 °C. For COPI subunits the pACT2 vector was used.

GST Pull-down Experiments—GST-Rab or GST-TMF frag-
ment constructs were expressed in the Escherichia coli BL21strain, induced at A600 = 0.8 with 0.5 mM IPTG followed by overnight incubation at 18 °C. Rab30 lacked the C-terminal 19 amino acids to aid solubility (PDB structure 2EW1). After harvesting, cells were lysed in GST-lysis buffer (20 mM Heps, 200 mM KCl, 2 mM MgCl2, 1 mM DTT, pH 7.4 with additional protease inhibitors) and the lysate cleared by centrifugation (15,000 rpm for 15 min). Glutathione-agarose (Clontech) equil-
ibrated with GST-lysis buffer was added and rotated for 1 h at 4 °C. Beads were pre-blocked with 0.1% BSA for COG binding experiments. The beads were washed with GST-lysis buffer before pull-down experiments.

For GST-Rabs, beads were washed with nucleotide buffer (20 mM Heps, 200 mM KCl, 5 mM MgCl2, 1 mM DTT) with 1 mM nucleotide and 10 mM EDTA, and incubated overnight at 4 °C. The beads were then incubated for 30 min in the same buffer lacking EDTA. Nucleotide loading was confirmed by reverse phase HPLC analysis. 60 μg of recombinant His6-Cog4 purified from insect cells (Richardson et al., 43) or 8 mg COG-containing sample purified from bovine brain by ammonium sulfate precipitation and butyl Sepharose chromatography (Ungar et al., 22) were added. For GST-TMF fragments, the beads were incubated with either His6-Rab1(T), His6-Rab1(D), or partially purified COG. His6-Rab1a was purified in GTP or GDP locked mutant forms from E. coli using Ni2+-chelate chromatography, diazylized into GST-lysis buffer, then incubated with 1 mM of the appropriate nucleotide and 5 mM EDTA for 1.5 h. Subsequently 10 mM MgCl2 and 0.5 mM of nucleotide were added, and following 30 min of incubation the Rab sample mixed with TMF-fragment containing beads.

For all GST pull-downs, after 1 h of incubation at 4 °C with rotation and washing with nucleotide buffer, bound proteins were eluted into SDS-PAGE buffer. All samples were analyzed using SDS-PAGE followed by immunoblotting using the Immobilon HRP substrate (Millipore) and the Syngene Gene-Genius ECL imaging system.
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**Immunoprecipitation—GFP-TMF** expression plasmids were transfected into HEK-293 cells stably expressing HA-tagged Cog1 using the GeneCellin transfection reagent (BioCellChallenge). For COG-COP1 co-immunoprecipitations YFP-βCOP and HA-Cog2 (Sohda *et al.*, 2007) were co-transfected into HeLa cells. After 36–48 h, cells were washed with IP buffer (20 mM Hepes, 110 mM KOAc, 1 mM MgCl2, pH 7.4) then scraped into 1 ml of IP lysis buffer (IP buffer with 1% Triton X-100). All steps were performed at room temperature. The cell slurry was rotated for 30 min prior to 30 min of centrifugation. The lysate obtained was incubated with 1.5 μl anti-HA 3F10 (0.1 μg/μl) or the equivalent amount of IgG for 1 h, then 10 μl of pre-equilibrated HiTrap Protein G beads (GE Healthcare) were added for a further 25 min. Beads were washed four times with buffer (IP buffer + 0.1% Triton X-100) and the bound proteins eluted with SDS-PAGE buffer, followed by immunoblot analysis.

**Immunofluorescence and Lectin Staining**—Hela cells grown on coverslips were fixed 72 h after transfection for lectin or 16–30 h after transfection for immunofluorescence. BFA treatment was performed for 5 min using 5 μg/ml BFA immediately prior to fixation. Cells were washed with PBS were fixed using MES-buffered ice cold methanol (90% methanol, 10 mM MES, 0.1 mM EDTA, 1 mM MgCl2, pH 6.9) for 5 min at −20 °C before washing with PBS. For lectin-staining cells were blocked for 1 h in P-L (PBS with 0.1% BSA) and incubated with 10 μg/ml GNL-FITC (Vector labs) in P-L for 1 h. Immunofluorescence cells were blocked in P-B buffer (PBS with 0.5% (w/v) saponin, 2% (w/v) BSA, 2% (v/v) goat serum) for 1 h and incubated with primary antibody in P-B for 1 h, washed in PBS and then incubated with Alexa-488 and/or 568 labeled secondary antibodies (1:400, Invitrogen) in P-B for 1 h. All coverslips were washed with PBS, and water before mounting for microscopy. Lectin-stained cells were imaged with a Leica IRB epifluorescence microscope, other imaging was done using confocal microscopy on an Olympus BX60 upright microscope. For Rab6 RNAi a SMARTpool oligo mix (Dharmacon) was transfected using oligofectamine. For mitochondrial localization experiments fluorescently tagged chimeras of full-length COG proteins were generated using pmCherry-C1-ActA. HeLa cells were co-transfected with COG and Rab constructs, fixed 20 h after transfection and imaged with the 63× oil 1.4 NA objective of a LSM510 Zeiss Laser inverted microscope outfitted with confocal optics.

**RESULTS**

**COG-Rab Interactions**—COG is a large hetero-oligomeric complex whose tethering function in the cell likely involves a series of protein interactions. The main aim of our study was to generate a full interaction map of COG that can later form the basis for functional dissection of retrograde vesicle tethering at the Golgi. Rab GTases are involved in all steps of vesicle transport, and several tethering complexes (including COG) have been shown to interact with Rabs (33, 37, 41). Using a directed yeast two-hybrid (Y2H) approach we tested the interactions between COG subunits and 14 Rab-GT Pases. 10 interactions involving three COG subunits (4, 5, and 6) and seven Rabs (1a, 2a, 4a, 6a, 14, 30, 36) were found of the 184 possible combinations (Fig. 1A). All observed interactions were with Rabs locked in the active conformation (T) as opposed to the inactive (D) conformation.

Encouraged by these initial results, we extended our survey to test a more complete set of 57 constitutively active mammalian Rabs (42) against the three COG subunits found to interact with Rabs in the initial assay (Fig. 1B). This experiment used increased stringency of detection, and revealed additional interactions with Rab1b, 6b, 10, 39, and 41. Combining the results, and rejecting interactions that were positive in one but negative in the other experiment, we identify 8 interactions: Cog4 with Rab30; Cog5 with Rab2a and 39; and Cog6 with Rab1a, 1b, 6a, and 41. Except for the previously reported Rab1a, 6a and 30 interactions (37) all these interactions are novel.

To validate our Y2H findings, we tested the ability of GST-tagged wild type Rabs to pull down partially purified bovine brain COG complex (22). Rab1a, 2a, 6a, and 30 loaded with either GDP or GTP were tested; GTP-loaded endosomal Rab5a was included as a negative control (Fig. 1C). Corroborating the high stringency Y2H results (Fig. 1B), active (GTP-loaded) Rab1a, 6a, and 30 displayed an increased affinity for COG in comparison to the inactive (GDP-loaded) forms (Fig. 1C). Binding of COG to GST-Rab2 was not observed (data not shown), suggesting that this interaction is not sufficiently long-lived for an *in vitro* pull-down experiment. Finally, two Rabs, 4a and 14, that interacted with Cog6 only in the less stringent Y2H experiment (Fig. 1A) were tested; one of them, GTP-Rab4a, was able to pull down significant quantities of the COG complex (Fig. 1C). Although Rab4a has not been implicated in Golgi-transport, these results suggest that this Rab might in fact be a COG interaction partner.

To investigate COG subunit interactions in isolation we used recombinant Cog4-His6 (to-date the only mammalian COG subunit that has been purified in a well-folded form (43)). Immobilized GST-Rab1a and GST-Rab30 both bound Cog4 in their active forms (Fig. 1D), whereas GST-Rab2 did not. Cog4 also bound to inactive Rab30 as previously reported (37). In contrast to the previous report though, binding to the GTP form (normalized to the amount of Rab30 on the beads) was still superior (Fig. 1D). Thus the pull-down experiments confirm the nucleotide-state specific binding of Rabs to the COG complex, indicating that COG is a *bona fide* effector of several Golgi Rabs.

As an additional test for the Cog5 and Cog6 interacting Rabs, for which isolated COG subunits were not available for pull-downs, *in vivo* relocation experiments were carried out. We made use of an assay that moves proteins tagged with the *Listeria* derived ActA sequence (and their interaction partners) to mitochondria (44). This assay was recently used to relocalize COG-interacting SNARE proteins to mitochondria (45). When co-transfected with GFP-tagged Rabs, ActA-tagged Cog6 relocalized Rab1a, Rab4a, and Rab6a, whereas ActA-tagged Cog5 relocalized Rab2a to mitochondria (Fig. 1E). The relocation of these Rabs was observed only for the GTP-locked versions (Fig. 1E and data not shown).

In summary, interactions were found between COG subunits and five different Rabs: Rab1a, 2a, 4a, 6a, and 30, using at least two independent methods. In addition, robust Y2H interac-
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A

Rab GTPase BD-fusions

| COG subunit AD-fusions | 1a-D | 1b-T | 2a-D | 3a-D | 3b-D | 4a-D | 5a-D | 6a-D | 7-D | 7L | 8a-D | 8b-D |
|------------------------|------|------|------|------|------|------|------|------|-----|----|------|------|
| vector                 |      |      |      |      |      |      |      |      |     |    |      |      |

B

| Cog4 | Cog5 | Cog6 |
|------|------|------|
| 1a   | 1b   | 2a   |
| 2b   | 3a   | 3b   |
| 3c   | 4a   | 4b   |
| 5a   | 5b   | 6a   |
| 6b   | 7    | 7L   |
| 8a   | 8b   |      |

C

| Input Beads | Rab1a | Rab4a | Rab5 |
|-------------|-------|-------|------|
| α-Cog4      | GDP   | GTP   | GDP  |
| α-GST (Rabs)|       |       |      |

D

| Input Beads | Rab1a | Rab2a |
|-------------|-------|-------|
| α-His (Cog4)| GDP   | GTP   |
| Beads       | Rab30 | GDP   |
| α-GST (Rabs)|       | GTP   |

E

COG5 Act, COG6 Act, COG6 ActA, myc Rab2a, GFP Rab1a, GFP Rab4a, GFP Rab6a, giantin, giantin, giantin, giantin, giantin, giantin, giantin.
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FIGURE 2. COG-golgin and COG-COPI interactions. A, Y2H interactions between COG subunit bait constructs and golgin prey constructs on -His or, where indicated - Ade media. To suppress self-activation 2-amino triazole (2AT) was added at the indicated concentrations to -His media. B, co-immunoprecipitation of the golgin TMF and the COG complex from HEK293 cells stably expressing HA-tagged Cog1 transiently transfected with GFP-TMF. Immunoprecipitation was with 3F10 anti-HA antibodies (HA IP) or the same amount of nonspecific IgG (IgG IP). The precipitates were probed with anti-TMF (top row) or anti-Cog3 (bottom row). Input is 0.1% of the precipitate for TMF and 3% for Cog3. C, yeast two hybrid interactions between COG subunit bait constructs and βCOP or vector only prey constructs. All other COPI subunits gave negative Y2H results with COG subunits. D, co-immunoprecipitation of co-transfected HA-Cog2 and βCOP-YFP or GFP from Hela cells using anti-GFP antibodies. Inputs and precipitates were probed with anti-GFP and anti-HA.11 at the same time. Input is 10%.

COG Subunit Interactions with Golgins and the Vesicle Coat—

A third class of proteins central to tethering at the Golgi are the golgins. COG has been shown to interact directly with two golgins, p115 and golgin-84 (38, 39). Given the interactions with a number of Rabs, we investigated whether additional golgins also interact directly with COG. Three representative golgins were chosen: GM130, acting at the cis-Golgi (55); CASP, a partner of golgin-84 that functions at the medial Golgi (2, 56); and TMF, a golgin with reported trans-Golgi functions but a localization throughout the Golgi stack (16, 17). The Y2H method was again used to test for interactions between these golgins and COG subunits (Fig. 2A). As shown previously by co-immunoprecipitation, golgin-84 interacts with Cog7 (39). Yet a stronger interaction was detected with Cog2 (Fig. 2A) that has been reported to interact with the golgin p115 (38). Indeed, the Cog2 subunit interacted with all tested golgins (Fig. 2A). These interactions are not promiscuous, since Cog2 did not interact with any of the Rabs tested (Fig. 1A). Cog2 is not a generic coiled-coil-binding protein either, since it failed to bind most other COG subunits (31) that have a strong propensity to form coiled coils close to their N termini (32). The tested golgins also interact with other COG subunits; namely, CASP with Cog8, GM130 with Cog3 and 5, and TMF with Cog6 (Fig. 2A).

All of the tested golgins, except for TMF, have previously been co-immunoprecipitated with COG (34, 38, 39). GFP-TMF was therefore tested for co-immunoprecipitation with COG from HEK293 cells stably expressing HA-tagged Cog1 that can complement the absence of endogenous Cog1 (26), and was expressed at close to endogenous levels (27 and data not shown). The anti-HA precipitation recovers the full COG complex from these cells, as shown by probing for Cog3 (Fig. 2B, lower panel) that is only present in cells as part of the full eight subunit complex (22). Anti-HA antibodies specifically co-precipitated GFP-TMF with COG (Fig. 2B, upper panel). Thus, all tested golgins that interact with COG subunits by Y2H also co-immunoprecipitate with the complex.

A further important player in intra-Golgi vesicle transport is the COPI protein complex (57, 58) that has been shown to interact with COG in yeast (33). To test whether this interaction is conserved in mammalian cells, we examined the pairwise interactions of COG and all COPI subunits using Y2H (Fig. 2C). Using high-stringency selection, we found robust interactions between βCOP and Cog2, 5, and 8. None of the other COPI subunits interacted with COG (data not shown). The COG-
COPI interaction was confirmed by co-immunoprecipitation of HA-tagged Cog2 using anti-GFP antibodies following co-transfection with YFP-H9252 COP (Fig. 2D).

Mapping the COG/TMF Interactions—Interactions between COG and golgins have been observed before (38, 39), yet it is not known how these interactions support vesicle tethering. Golgins have been suggested to tether vesicles that are distant from the target. We thus wondered whether a golgin-COG interaction could help bring the golgin-tethered vesicles into closer proximity with the target membrane. TMF, which requires its C-terminal coils for binding to the Golgi via Rab6 (16; see also Fig. 4A), was chosen to map the COG binding site(s) along its length. Utilizing Y2H with TMF fragments whose boundaries were based on coiled-coil predictions (59, Fig. 3A), we found that both Cog2 and Cog6 interacted with the two C-terminal TMF-coils (Fig. 3B, Cterm-C fragment). Although this is the same region previously mapped as the Rab6 binding site (16), we in this study were able to narrow down this Rab binding site to the terminal coil of the golgin (Fig. 3B, Cterm-Cfragment). The COG binding site therefore could overlap but is not identical to the Rab6 binding site on the TMF C terminus.

To confirm the interaction between the TMF C-terminal region and COG by co-immunoprecipitation, Cog1HA cells transfected with GFP-tagged TMF truncation constructs (Fig. 3A) were used. As expected from the Y2H results, the Cterm but not the Cterm-mid fragment co-precipitated with COG using HA-specific antibodies (Fig. 3C, top and middle panels). Surprisingly, TMF lacking the C-terminal region, termed ΔCterm, very efficiently co-precipitated with COG as well (Fig. 3C, bottom panel). This was not due to heterodimerization with endogenous full-length TMF, since endogenous TMF was not detected in the precipitate (data not shown). It is unlikely that
the lack of a positive Y2H signal with the N-terminal fragments (Nterm and central coil) of TMF is due to the lack of interaction strength, since the efficiency of the ΔCterm co-IP is better than the one with the Cterm fragment. The absence of Y2H signal could instead be due to the requirement of multiple COG subunits or a further endogenous factor.

The ΔCterm TMF construct consists of a predicted coiled coil (central coil) and a globular head domain (Nterm) (Fig. 3A). To further define the region involved in COG binding, we pulled down partially purified COG complex using recombinant GST-Nterm and -central coil fragments (Fig. 3D, left panel). Whereas the central coil fragment did not pull COG down significantly above the levels observed with beads only, the Nterm fragment did. Indeed, the TMF Nterm fragment also efficiently co-immunoprecipitated with COG from Cog1HA cells (Fig. 3D, right panel), demonstrating that this interaction exists in vivo. The COG complex thus has two separate interactions with the golgin TMF, one binding site being in the C-terminal, the other in the N-terminal region of the golgin.

Many golgins have been shown before to interact with multiple Rabs (18, 20, 21). We therefore asked whether, besides Rab6, TMF also interacted with other COG-partner Rabs, and found that both Rab1a and Rab2a interacted with full TMF by Y2H (Fig. 3E). Neither Rab, however, interacted with the TMF fragments used in Fig. 3B (data not shown); therefore, the binding of His-tagged Rab1a to GST-tagged TMF fragments was tested. Nterm-TMF bound specifically to the active form of Rab1 (Fig. 3F, top). The two opposing ends of TMF thus not only bind COG, but also two distinct Rabs.

Multiple Regions of TMF Can Bind to the Golgi—Previous work has shown that TMF is recruited to the Golgi via Rab6 (16, 17). In line with this, we find that depletion of Rab6 by RNAi results in loss of Golgi association of TMF (Fig. 4A, middle row). To investigate the possibility that TMF may associate with other Golgi factors in addition to Rab6, we performed the same experiment but this time incubated the cells with BFA for 5 min to remove the COPI coat covering the Golgi rims (57) prior to fixation. Under these conditions, strikingly, we observe that TMF is efficiently recruited to the Golgi even when Rab6 is depleted (Fig. 4A, bottom row). This result indicates an additional binding partner at the Golgi membrane, and strongly suggests that binding to this additional factor is masked by the COPI coat. Note that recruitment of TMF in the presence of Rab6 is unaffected by BFA treatment or βCOP depletion (data not shown), suggesting that Rab6 binding is the dominant recruitment mechanism when the COPI coat is unperturbed. In addition, neither this short BFA treatment, nor depletion of Rab6 perturbs COG localization to the Golgi (data not shown), in line with the multiple contacts COG makes with Golgi localized proteins.

To identify the domain of TMF that binds to this new putative binding partner, we first analyzed recruitment of the ΔCterm TMF truncation mutant lacking the C-terminal Rab6 binding site. Again, this construct was efficiently recruited to
the Golgi membrane after BFA treatment that removed COPI from the Golgi as expected (Fig. 4B). This result indicates that binding is via a region distinct from the C terminus. Further mapping indicated that the central coil region was sufficient to mediate Golgi recruitment under conditions where COPI had been removed by a short BFA treatment (Fig. 4C). This implies that during vesicle tethering TMF can bind to Golgi membranes via its central coil domain once the coat is locally cleared off the Golgi rim.

Overexpression of TMF Fragments Leads to Changes in Glycosylation—After finding an in vivo role for the central coil fragment of TMF in Golgi targeting we wondered whether the protein interactions of the N- and C-terminal regions identified had in vivo relevance too. We reasoned that overexpression of these TMF fragments could act in a dominant fashion, since these overproduced fragments may saturate their cognate binding partners. Therefore, TMF fragments were overexpressed and glycosylated, as a readout of Golgi function, examined in HeLa cells. We used the lectin GNL that shows increased affinity to HeLa cells depleted of COG4 (43) by binding to terminal α1–3 mannose (60). This sugar is generally presented on incompletely processed high-mannose and hybrid glycans. Expression of full-length TMF or the central coil domain did not significantly increase GNL staining compared with mock-transfected HeLa cells (Fig. 5). However, overexpression of the Cterm and especially the Nterm regions, which interact with COG and Rab6 or Rab1 respectively, significantly increased GNL affinity (Fig. 5). This suggests that the COG-Rab-TMF interactions may play a critical role in the vesicular sorting of enzymes responsible for eliminating the GNL epitope.

**DISCUSSION**

The COG complex functions as the CATCHR family tethering complex for retrograde intra-Golgi vesicle transport (22, 24, 61). In this study, we have begun the molecular dissection of COG-mediated vesicle tethering by defining the protein interaction network of COG, and starting to dissect part of it. It is important to consider that a variety of different vesicles will be subject to COG-mediated tethering while being directed to different destinations within the Golgi. Previous work suggests that COG facilitates all retrograde intra-Golgi-targeting reactions, since it localizes to every cisterna (26), and mediates the localization of enzymes in different Golgi regions (28–30, 40). Therefore COG is likely to require a large number of interactions to guide all the distinct tethering reactions. As an example, although TMF null mutant mice and COG lobe B mutant fruit flies are both male sterile due to a spermatogenesis defect (62–64), the defect during spermatogenesis occurs much earlier in this process for the COG mutants, possibly because some of the COG-mediated steps that do not require TMF are more critical for spermatogenesis than the one involving TMF.

**Physiological Importance of COG Interactions**—COG binding is in all cases stronger to the GTP-bound Rab, as expected for a true effector (33, 41). Even more compellingly, although we tested the full range of human Rabs for interaction with COG, of those that we identify as COG interactors, all but
Rab4a have been assigned a function at the Golgi (12). Although previous work assigns a recycling endosomal function to Rab4a, the combination of our Y2H, GST pull-down, and mitochondrial relocation results strongly implicate COG as a Rab4a effector. Future work will need to clarify the physiological context of this particular COG-Rab interaction. It is important to note that although Rab1 has predominantly been regarded to function in anterograde transport, we presume that its interaction with COG fulfills an additional retrograde transport function. The specificity of the interactions is particularly highlighted by the fact that Cog2 shows interactions with all tested golgins; yet none of the Rabs or SNAREs tested showed interactions with Cog2. In contrast, Cog4 and Cog6, which show interactions with many Rabs, only interact with one golgin (Figs. 1, 2, and 6A).

The resulting interaction diagram (Fig. 6A) lends itself to some generalization that, for the first time, may be used to assign functions to different COG subunits. As such, interaction hubs within COG can be outlined for the different classes of transport factor partners (Fig. 6A), including the interactions between SNAREs and COG subunits (45). We stress that the assignment of these hubs is based solely on interaction data, and will undoubtedly be refined by future mechanistic experiments. All golgins tested so far, including p115 (38), interact...
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with Cog2 that in turn does not interact with Rabs or SNAREs, therefore we term this the golgin hub. Rabs in contrast, interact with Cog4, Cog5, and Cog6. Since only one SNARE (34) but no golgins interact with Cog4, and one golgin but no SNAREs with Cog5, we call Cog4 and 5 Rab hubs. The hub for SNAREs is Cog8 (45) since the only other relevant interactor here is the golgin CASP. This leaves Cog6 to serve as a hub for both Rabs and SNAREs. The vesicle coat component βCOP can bind to the hubs of all interaction partner families by interacting with Cog2, 5, and 8. A possible reason could be the need for redundancy. While the interactions of COG with other types of transport factors are combinatorial, it is quite likely that only a subset of those factors will interact with the complex during any one tethering event. However, the COG-COP interaction is necessary for all tethering reactions, independently of the set of Rab, golgin, and SNARE partners in question. Thus alternative COG-COP binding sites could be necessitated by the incompatibility of a single site with all possible COG-Rab, COG-golgin, and COG-SNARE interactions.

The Relationship between the Interaction Map and COG Lobe A/Lobe B Phenotypes—Defects in the two halves of COG, lobes A (Cog1–4) and B (Cog5–8), result in different phenotypes. Lobe A defects have more severe consequences than lobe B defects (24, 30). One suggested explanation for these differences has been that lobe A is important for early/medial, whereas lobe B for late Golgi enzyme targeting (29, 40). An alternative suggestion has been that lobe B has an enzyme targeting role, whereas lobe A plays a role in Golgi organization (28). Both of these earlier suggestions are compatible with the found interaction set. The hub for golgins is the lobe A subunit Cog2. Golgin interactions could well determine the Golgi integrity phenotype associated with lobe A defects, since golgins have been strongly associated with Golgi integrity (4). Alternatively, the one Rab hub in lobe A (Cog4) could be responsible for the targeting of some early Golgi enzyme subsets through the Rabs 1, 30, and/or 41, while the remaining COG partners could determine lobe B phenotypes, mainly affecting the targeting of late Golgi enzymes.

Model of Vesicle Tethering at the Golgi—The observed COG-TMF-Rab interactions, together with the BFA-induced Golgin recruitment of TMF fragments, prompt us to propose a tentative molecular model for COG-mediated tethering (Fig. 6B).

TMF is known to attach to the Golgi by its C-terminal tail binding to Rab6 (Fig. 4, A and B and Ref. 17). Rab1, in contrast, binds TMF at the opposite end (Fig. 3F), and thus could facilitate the binding of an approaching vesicle to TMF. The fact that a significant fraction of COG has been localized to vesicles by EM (26) supports an initial interaction of COG with the approaching vesicle, this may require the observed COG-βCOP binding. Long range tethering could then be completed by binding of both Rab1 and COG to the Nterm region of TMF (Fig. 6B, panel 1). Approach of the vesicle to the target could be further facilitated by COG binding to Rab6 and the TMF-Cterm, while still bound to the opposite end of the golgin, thereby restricting the golgin into a bent conformation (Fig. 6B, panel 2). EM images of purified bovine COG show that the most splayed out conformations of the complex can reach at least 80 nm (22), a length sufficient to bridge the head and tail of a partially bent golgin. Further interaction studies involving all four proteins together, as well as structural studies will be necessary to validate such a proposal. However, since the COG subunits that bind Rab6 and the Cterm of TMF are different from the ones that bind Rab1, such an arrangement is compatible with our current knowledge.

The binding of COG to the membrane proximal region of TMF would also bring the complex into proximity of the COPI coat present on the cisternal rim (57, 58), providing a further occasion for COG-βCOP binding. Once TMF is close enough the central coil region can bind to a factor that is normally concealed by the COPI coat covering the Golgi (Fig. 6B, panel 3). The short BFA treatment that moves the TMF central coil to the Golgi (Fig. 4C), has likely revealed this binding site by removing COPI, although the effect of BFA on other Arf1-mediated processes could be involved here too. The tight TMF binding could thus complete docking.

It is clear that our interaction data are but static representations of the proposed mechanistic model. Yet it is intriguing that both the Nterm and Cterm regions of TMF, which we suggest to participate in critical binding events, perturb glycan processing in a dominant fashion (Fig. 5). In contrast, the central coil region, which binds a cryptic site that is secluded until the machinery is primed, is ineffective in the same assay.

Our data and the model presented above link hetero-oligomeric and coiled-coil tethers. These protein families have been shown to interact, yet this is to our knowledge the first molecular model for their combined mode of action. In this model vesicle and target membranes could be marked by combinatorial sets of Rabs, and thus targeting could be achieved through specific golgins that have combinatorial Rab interactions themselves (18). CATCHR family complexes acting at other compartments (e.g. exocyst, Dsl1 complex) could use a similar mechanism to reel a vesicle into close proximity of the target membrane, albeit their protein partners would certainly be different.

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REFERENCES
1. Ungar, D., Oka, T., Krieger, M., and Hughson, F. M. (2006) Retrograde transport on the COG railway. Trends Cell Biol. 16, 113–120
2. Malsam, J., Satoh, A., Pelletier, L., and Warren, G. (2005) Golgin tethers define subpopulations of COPI vesicles. Science 307, 1095–1098
3. Waters, M. G., and Pfeffer, S. R. (1999) Membrane tethering in intracellular transport. Curr. Opin. Cell Biol. 11, 453–459
4. Ramirez, I. B., and Lowe, M. (2009) Golgins and GRASPs: holding the Golgi together. Semin. Cell Dev. Biol. 20, 770–779
5. Sapperstein, S. K., Walter, D. M., Grosvenor, A. R., Heuser, J. E., and Waters, M. G. (1995) p115 is a general vesicular transport factor related to the yeast ER-Golgi transport factor Uso1p. Proc. Natl. Acad. Sci. U.S.A. 92, 522–526
6. Munro, S. (2011) The Golgin coiled-coil proteins of the Golgi apparatus. Cold Spring Harb. Perspect. Biol. 3, a005256
7. Yu, I. M., and Hughson, F. M. (2010) Tethering factors as organizers of
intracellular vesicular traffic. *Annu. Rev. Cell Dev. Biol.* **26**, 137–156

8. Miller, V. J., and Ungar, D. (2012) Re'COG'nition at the Golgi. *Traffic* **13**, 891–897

9. Emr, S., Glick, B. S., Linstedt, A. D., Lippincott-Schwartz, J., Luini, A., Malhotra, V., Marsh, B. J., Nakano, A., Pfeffer, S. R., Rabouille, C., Rothman, J. E., Warren, G., and Wieland, F. T. (2009) Journeys through the Golgi—taking stock in a new era. *J. Cell Biol.* **187**, 449–453

10. Glick, B. S., Elston, T., and Oster, G. (1997) A cisternal maturation mechanism can explain the asymmetry of the Golgi stack. *FEBS Lett.* **414**, 177–181

11. Ungar, D., and Hughson, F. M. (2003) SNARE protein structure and function. *Annu. Rev. Cell Dev. Biol.* **19**, 493–517

12. Hutagalung, A. H., and Novick, P. J. (2011) Role of Rab GTPases in membrane traffic and cell physiology. *Physiol. Rev.* **91**, 119–149

13. Beard, M., Satoh, A., Shorter, J., and Warren, G. (2005) A cryptic Rab1-binding site in the p115 tethering protein. *J. Biol. Chem.* **280**, 25840–25848

14. Allen, B. B., Moyer, B. D., and Balch, W. E. (2000) Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COPII vesicles for fusion. *Science* **289**, 444–448

15. Diao, A., Frost, L., Morohashi, Y., and Lowe, M. (2008) Coordination of golgin tethering and SNARE assembly: GM130 binds syntaxin 5 in a p115-regulated manner. *J. Biol. Chem.* **283**, 6957–6967

16. Fridmann-Sirks, Y., Siniossoglou, S., and Pelham, H. R. (2004) TMIF is a golgin that binds Rab8 and influences Golgi morphology. *BMC Cell Biol.* **5**, 18

17. Yamane, J., Kubo, A., Nakayama, K., Yuba-Kubo, A., Katsuno, T., Tsukita, S., and Tsukita, S. (2007) Functional involvement of TMIF/ARA16 in Rab6-dependent retrograde membrane traffic. *Exp. Cell Res.*** **313**, 3472–3485

18. Sinka, R., Gillingham, A. K., Kondylis, V., and Munro, S. (2008) Golgi-coiled-coil proteins contain multiple binding sites for Rab G protein partners. *J. Cell Biol.* **183**, 607–615

19. Short, B., Preisinger, C., Körner, R., Kopajtich, R., Byron, O., and Barr, F. A. (2001) A GRASP55-rab2 effector complex linking Golgi structure to membrane traffic. *J. Cell Biol.* **155**, 877–883

20. Hayes, G. L., Brown, F. C., Haas, A. K., Nottingham, R. M., Barr, F. A., and Pfeffer, S. R. (2009) Multiple Rab GTPase binding sites in GCC185 suggest a model for vesicle tethering at the trans-Golgi. *Mol. Biol. Cell* **20**, 209–217

21. Rosing, M., Ossendorf, E., Rak, A., and Barnekow, A. (2007) Giantin interacts with both the small GTPase Rab6 and Rab1. *Exp. Cell Res.*** **313**, 2318–2325

22. Ungar, D., Oka, T., Brittle, E. E., Vasile, E., Lupashin, V. V., Chatterton, J. E., Heuser, J. E., Krieger, M., and Waters, M. G. (2002) Characterization of a mammalian Golgi-localized protein complex, COQ, that is required for normal Golgi morphology and function. *J. Cell Biol.* **157**, 405–415

23. Kingsley, D. M., Kozarsky, K. F., Segal, M., and Krieger, M. (1986) Three types of low density lipoprotein receptor-deficient mutant have pleiotropic defects in the synthesis of N-linked, O-linked, and lipid-linked carbohydrate chains. *J. Cell Biol.* **102**, 1576–1585

24. Whyte, J. R., and Munro, S. (2002) Vesicle tethers in membrane traffic. *J. Cell Sci.* **115**, 2627–2637

25. Wu, X., Steet, R. A., Bohorov, O., Bakker, J., Newell, J., Krieger, M., Spaepen, L., Kornfeld, S., and Freeze, H. H. (2004) Mutation of the COG complex subunit gene COG7 causes a lethal congenital disorder. *Nat. Med.* **10**, 518–523

26. Vasile, E., Oka, T., Ericsson, M., Nakamura, N., and Krieger, M. (2006) IntraGolgi distribution of the Conserved Oligomeric Golgi (COG) complex. *Exp. Cell Res.* **312**, 3132–3141

27. Oka, T., Ungar, D., Hughson, F. M., and Krieger, M. (2004) The COG and COPII complexes interact to control the abundance of GEArs, a subset of Golgi integral membrane proteins. *Mol. Biol. Cell* **15**, 2423–2435

28. Peanne, R., Legrand, D., Dufet, S., Mir, A. M., Matthijs, G., Rohrer, J., and Foulquier, F. (2011) Differential effects of lobe A and lobe B of the Conserved Oligomeric Golgi complex on the stability of β1,4-galactosyltransferase I and [α]2,3-sialyltransferase I. *Glycobiology* **21**, 864–876
Mellman, I. (1991) The small GTP-binding protein rab4 is associated with early endosomes. Proc. Natl. Acad. Sci. U.S.A. 88, 6313–6317

50. Opdam, F. J., Echard, A., Croes, H. J., van den Hurk, J. A., van de Vorstenbosch, R. A., Ginsel, L. A., Goud, B., and Fransen, J. A. (2000) The small GTPase Rab6B, a novel Rab subfamily member, is cell-type specifically expressed and localised to the Golgi apparatus. J. Cell Sci. 113, 2725–2735

51. Goud, B., Zahraoui, A., Tavitian, A., and Saraste, J. (1990) Small GTP-binding protein associated with Golgi cisternae. Nature 345, 553–556

52. Kelly, E. E., Giordano, F., Horgan, C. P., Jollivet, F., Raposo, G., and McCaffrey, M. W. (2012) Rab30 is required for the morphological integrity of the Golgi apparatus. Biol. Cell 104, 84–101

53. Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T. E., and Warren, G. (1995) Characterization of a cis-Golgi matrix protein, GM130. J. Cell Biol. 131, 1715–1726

54. Duden, R., Griffiths, G., Frank, R., Argos, P., and Kreis, T. E. (1991) Beta-COP, a 110 kDa protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to beta-adaptin. Cell 64, 649–665

55. Beck, R., Rawet, M., Ravier, M., Wieland, F. T., and Cassel, D. (2009) The COPI system: molecular mechanisms and function. FEBS Lett. 583, 2701–2709

56. Shibuya, N., Goldstein, I. J., Van Damme, E. J., and Peumans, W. J. (1988) Binding properties of a mannose-specific lectin from the snowdrop (Galanthus nivalis) bulb. J. Biol. Chem. 263, 728–734

57. Shibuya, N., Goldstein, I. J., Van Damme, E. J., and Peumans, W. J. (1988) Binding properties of a mannose-specific lectin from the snowdrop (Galanthus nivalis) bulb. J. Biol. Chem. 263, 728–734