Genetic Analysis of the Subunit Organization and Function of the Conserved Oligomeric Golgi (COG) Complex

STUDIES OF COG5- AND COG7-DEFICIENT MAMMALIAN CELLS*‡

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The conserved oligomeric Golgi (COG) complex is an eight-subunit (Cog1–8) peripheral Golgi protein involved in Golgi-associated membrane trafficking and glycoconjugate processing. We have analyzed the structure and function of COG using Cog1 or Cog2 null Chinese hamster ovary cell mutants, fibroblasts from a patient with Cog7-deficient congenital disorders of glycosylation, and stable Cog5-deficient HeLa cells generated by RNA interference. Although the dilation of some Golgi cisternae in Cog5-deficient cells resembled that observed in Cog1- or Cog2-deficient cells, their global glycosylation defects (less severe) and intracellular processing and function of low density lipoprotein receptors (essentially normal) differed from Cog1- and Cog2-deficient cells. Immunoblotting, gel filtration, and immunofluorescence microscopy analyses of the COG-deficient cells and cell extracts indicated that 1) Cog2–4 and Cog5–7 form stable subcomplexes, 2) Cog1 mediates Golgi association of a Cog2–4 plus Cog8 subcomplex, 3) Cog8 associates stably with both Cog5–7 and Cog1–4 subcomplexes, and thus 4) Cog8 helps assemble the Cog1–4 and Cog5–7 subcomplexes into the complete COG complex. This model of the subunit organization of COG is in excellent agreement with in vitro data presented in an accompanying paper (Ungar, D., Oka, T., Vasile, E., Krieger, M., and Hughson, F. M. (2005) J. Biol. Chem. 280, 32729–32735). Only one or two of the seven Cog1- or Cog2-dependent Golgi membrane proteins called GEARs are also sensitive to Cog5 or Cog7 deficiency, indicating that the COG subunits play distinctive roles in controlling Golgi structure and function.

Multisubunit peripheral membrane protein complexes appear to play important roles in facilitating Golgi-associated membrane trafficking and glycoconjugate processing (1–10). One of these is the conserved oligomeric Golgi (COG)2 complex comprising eight distinct subunits, Cog1–8 (11–27). The initial identification and characterization of low density lipoprotein receptor-deficient mammalian cell mutants with defects in Cog1 and Cog2 subunits that affect the structure and function of the Golgi apparatus (11–13, 18, 24) were followed by genetic and biochemical analysis in yeast (14–16, 19–23, 26, 28–31) and Drosophila melanogaster (32), as well as purification of the COG complex from bovine brain (17, 24).

Defects in COG function can cause abnormalities in glycoconjugate synthesis, intracellular protein sorting, protein secretion, and, in some cases, cell growth. In mammalian Cog1 (Idlb cells) or Cog2 (IdIC cells) null Chinese hamster ovary (CHO) cell mutants, multiple Golgi cisternae are dilated (24), and there are pleiotropic defects in a number of medial- and trans–Golgi-associated reactions affecting virtually all N-, O-, and lipid-linked glycoconjugates (12). The pleiotropy and heterogeneity of the glycosylation defects suggested that COG influences the regulation, compartmentalization, or activity of multiple Golgi glycosylation enzymes and/or their substrate transporters without substantially disrupting secretion or endocytosis (12, 33). Because the activities of many glycosylation-related proteins depend on their proper intra–Golgi localization and appropriate intraluminal environments (7, 34–42), it was proposed that COG might play a role directly or indirectly in resident Golgi proteins’ transport to, retention at, or retrieval to appropriate sites, or that it might otherwise determine the Golgi’s structure and/or luminal environment (12, 43). This proposal was supported by the finding that a subset of Golgi type II membrane proteins, called GEARs, is mislocalized and/or abnormally rapidly degraded in Cog1- and Cog2-deficient CHO mutants (43). As expected from the analysis of the CHO cell mutants, a mutation in a gene encoding one of the human COG subunits (COG7) has been shown to be responsible for a rare form of lethal congenital disorders of glycosylation (CDG) (44). In the mutant fibroblasts from the Cog7-deficient patients, which exhibit global glycosylation defects less severe than those in the Cog1 and Cog2 null CHO cells, trafficking of a Golgi-localized glycosylation enzyme, Galβ1,3GalNAc α2,3-sialyltransferase, is impaired (44). Abnormal Golgi-associated glycosylation also has been reported in yeast mutants with defects in COG subunits (22, 29–30), as has the mislocalization of two yeast Golgi mannosyltransferases, Och1p and Mnn1p, in cog3 mutants (31).

In addition to the global glycosylation defects, yeast cog5 mutants have shown a temperature-sensitive defect in mating projection (28), suggesting that yeast Cog5 is involved in polarized cell growth. It is, therefore, striking that loss of Cog5 function in D. melanogaster causes failure of spermatogenesis and male sterility but no other overt gross abnormal phenotypes (32). It is clear that the COG complex plays an important role in controlling multiple aspects of intracellular function.

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from yeast to humans, although the precise molecular mechanisms underlying COG activities have not been elucidated.

Biochemical and genetic studies in yeast and mammalian systems led to the proposal that COG is composed of two lobes, each containing four distinct subunits, lobe A (Cog1–4) and lobe B (Cog5–8), (24, 45). In yeast, deletion of any one of the four lobe A subunits causes a severe growth defect, whereas disruption of the remaining genes (COG5–8) does not substantially interfere with normal cell growth (22). Electron microscopic images of the COG complex purified from bovine brain reveal a bilobed structure with two similarly sized globular domains connected by small extensions (24). A recent in vitro translation and co-immunoprecipitation study also supports this model of COG structure (Ref. 46, see below for further discussion).

When the mammalian COG complex was initially purified and characterized and the two-lobed low resolution model of its architecture proposed (24), the only four antibodies available for analyzing the complex recognized Cog1, 2, 3, and 5. The subsequent generation of additional anti-COG antibodies (43) that recognize all eight COG subunits provide powerful new tools for the analysis of COG structure and function. Here, we have used these reagents and generated stable Cog5-deficient human HeLa cell lines using the RNA interference technique (57) to further explore the structure and function of COG. These studies suggest that the Cog1 and Cog8 subunits play important roles in assembling Cog2–4 and Cog5–7 subcomplexes into the intact, Golgi-associated COG complex. They also emphasize the distinct structural and functional roles of the Cog5 and Cog7 subunits.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents and sources were: ECL Plus detection kit (Amersham Biosciences), Alexa 488-conjugated peanut lectin (Molecular Probes), Ham’s F-12 (Invitrogen), RPMI1640 medium (Invitrogen), concanavalin A, wheat germ agglutinin, and phytohemagglutinin (Sigma). The following antibodies for immunoblotting and immunofluorescence experiments were used as described previously (43): anti-Cog1(LDLB) antibody, anti-GS15 antibody, anti-GOS-28 antibody, anti-golgin-84 antibody, anti-GM130 antibody, anti-Vitla antibody, anti-Bet1 antibody, and anti-Sec8 antibody (BD Biosciences), anti-golgin-84 antibody, anti-GM130 antibody, anti-Vit1a antibody, concanavalin A, wheat germ agglutinin, and phytohemagglutinin (Sigma). When the mammalian COG complex was initially purified and characterized and the two-lobed low resolution model of its architecture proposed (24), the only four antibodies available for analyzing the complex recognized Cog1, 2, 3, and 5. The subsequent generation of additional anti-COG antibodies (43) that recognize all eight COG subunits provide powerful new tools for the analysis of COG structure and function. Here, we have used these reagents and generated stable Cog5-deficient human HeLa cell lines using the RNA interference technique (57) to further explore the structure and function of COG. These studies suggest that the Cog1 and Cog8 subunits play important roles in assembling Cog2–4 and Cog5–7 subcomplexes into the intact, Golgi-associated COG complex. They also emphasize the distinct structural and functional roles of the Cog5 and Cog7 subunits.

**Immunoblotting**—SDS-polyacrylamide gel electrophoresis and immunoblotting were carried out with the indicated antibodies at the concentrations described previously (43). Signals were detected using the ECL Plus detection kit (Amersham Biosciences) according to the manufacturer’s instructions.

**Gel Filtration**—Cytosol fractions were prepared from CHO, ldlB, and ldlC cells and analyzed by gel filtration chromatography with a Superose 6 column. Samples in the fractions were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-COG subunit antibodies.

**Construction of pcDNA U6-COG5**—The siRNA expression vector pcdNA3.1-U6-Zeo includes the U6 promoter with the addition of an Apal site at the transcription start site. It was digested with Apal, blunted, and digested with EcoRI. The following oligomers were used: 5’-CTACAGTCCGGGAACTCTTTCTAAGAGAAGAGGTTCCTCC-3’ (sense) and 5’-AATTAAAAACTACA-GTCCGGGAACTCTTTCTAAGAGAAGAGGTTCCTCC-3’ (antisense). The annealed oligomers were cloned into the digested pcdNA3.1-U6-Zeo to construct pcdNAU6-COG5.

**Cell Culture**—Wild type CHO, ldlB, ldlC, ldlB(COG1), and ldlC(COG2) cells were cultured as described previously (13, 18). Human fibroblast and the COG7 mutant (P1) cells were maintained as described (44). HeLa cells were cultured at 37 °C in medium A (RPMI1640 supplemented with 2% glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin) containing 10% fetal bovine serum.

**Transfection and Establishment of Stable Cog5 Knockdown Cells**—HeLa cells were transfected with the pcDNAU6-COG5 plasmid using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions, and colonies were selected in medium B (medium A supplemented with 0.2–0.4 mg/ml Zeocin (Invitrogen)). In two independent transfections, three individual colonies (kd-COG5#1, #2, and #3) were isolated, grown to mass culture, and used for further experiments. The relative decreases of Cog5 were determined by immunoblotting using a dilution series of cell lysates from wild type cells and the three individual colonies.

**Immunofluorescence**—Cells were grown on glass coverslips, fixed with 2% paraformaldehyde, and stained with the indicated antibodies as described previously (24, 43). Images were obtained with a Zeiss LSM510 confocal microscope.

**Immunoprecipitation**—Cells were suspended in buffer A (20 mM Hepes-KOH, pH 7.2, and 125 mM magnesium acetate) supplemented with Complete™ EDTA-free protease inhibitors (Roche Applied Science), disrupted by passing 30 times through a 30-gauge needle with a 5-ml syringe, and then clarified by centrifugation (~20,000 × g, 10 min, 4 °C). The resultant supernatant was further subjected to ultracentrifugation (100,000 × g, 60 min, 4 °C). The protein concentration of the cytosol fraction was determined using BCA protein assays (Pierce).

For immunoprecipitations, 5 μg of the indicated monoclonal antibody were added to 400 μg of cytosol from either HeLa or kd-COG5#1 cells in buffer B (phosphate-buffered saline with 5% (w/v) nonfat dried milk) and incubated overnight at 4 °C. After further incubation with protein A-Sepharose CL-4B (Amersham Biosciences), the mixture was washed four times with buffer B and then once with phosphate-buffered saline. The immunoprecipitates were analyzed by immunoblotting with the indicated anti-COG subunit antibodies.

**Peanut Lectin Staining**—Cells grown on glass coverslips were fixed with 2% paraformaldehyde and then permeabilized with 0.2% saponin. After washing with phosphate-buffered saline, the cells were incubated with 1–5 μg/ml of PNA-Alexa488 in phosphate-buffered saline containing 0.1% bovine serum albumin. Images were collected with a Nikon Eclipse TE200 microscope and RT Spot CCD camera (Diagnostic Instruments, Inc.).

**Electron Microscopy**—Wild type and Cog5 knockdown HeLa cells were grown on plastic culture dishes. Cells were rapidly rinsed with Dulbecco’s complete phosphate-buffered saline (containing calcium and magnesium), fixed with 2% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate–HCl, pH 7.2, for 60 min at 4 °C, and postfixed with 2% osmium tetroxide for 60 min at 4 °C, followed by dehydration in graded alcohol up to 70% and dehydration in graded alcohol up to 70% and 80%.

**Ultrastructural Analysis**—Wild type and Cog5 knockdown HeLa cells were cut with a diamond knife, poststained with uranyl acetate and Reynold’s lead citrate, and viewed with a Philips 400 electron microscope operated at 80 kV.

**Organization of COG in Cog5-deficient Cells**

When the mammalian COG complex was initially purified and characterized and the two-lobed low resolution model of its architecture proposed (24), the only four antibodies available for analyzing the complex recognized Cog1, 2, 3, and 5. The subsequent generation of additional anti-COG antibodies (43) that recognize all eight COG subunits provide powerful new tools for the analysis of COG structure and function. Here, we have used these reagents and generated stable Cog5-deficient human HeLa cell lines using the RNA interference technique (57) to further explore the structure and function of COG. These studies suggest that the Cog1 and Cog8 subunits play important roles in assembling Cog2–4 and Cog5–7 subcomplexes into the intact, Golgi-associated COG complex. They also emphasize the distinct structural and functional roles of the Cog5 and Cog7 subunits.
Organization of COG in Cog5-deficient Cells

RESULTS

Characterization of the COG Subunits in ldlB and ldlC Cells—Previous studies using biochemical purification, somatic cell mutants, and antibodies to the Cog1–3 and Cog5 subunits suggested that in wild type mammalian cells and tissues the COG complex is composed of eight polypeptide chains (Cog1–8) that form two lobes (lobe A (Cog1–4) and lobe B (Cog5–8)) and that a smaller subcomplex that contains Cog5, but not Cog1–3, is also present (Fig. 1D). We have extended those earlier studies using a set of antibodies that recognizes all eight COG subunits.

Fig. 1 shows an immunoblotting analysis of a total cell lysate (panel A, lane 1) and size fractionation of cytosol from wild type CHO cells (panel B, left) for all eight COG subunits. The Golgi-associated protein GM130 is included in panel A as a loading control. All eight subunits co-eluted in a large complex (panel B, left, open triangle), centered in fractions 2 and 22; asterisk indicates an apparently unrelated protein that cross-reacts with the anti-Cog4 antiserum. In addition, Cog5–7 and Cog8 co-eluted in what appear to be distinct, smaller complexes (solid triangle, centered in fractions 24 and 25 in CHO cells), consistent with our previous findings (24). In light of additional findings using Cog1 (ldlB)- and Cog2 (ldlC)-deficient CHO cell mutants (12, 13, 18), and Cog1 expression was virtually abolished. Pulse-chase analysis showed that Cog1 was synthesized normally but was rapidly degraded in the ldlC cells compared with wild type CHO cells (data not shown). All COG subunits were present in normal or slightly greater than normal levels in the mutants stably transfected with the corresponding complementing cDNAs, ldlB[COG1] and ldlC[COG2] cells (also see Refs. 13, 18, 24, 43), which indicates that the reduced steady state levels, presumably due to instability, of the COG subunits in the ldlB and ldlC mutants are a consequence of the mutations in the genes encoding Cog1 or Cog2, respectively. These results indicate that loss of Cog2 influences the stability of all of the other subunits, whereas the steady state levels of Cog2–4 are unaffected by the absence of Cog1.

ldlB and ldlC, cells a small amount of Cog7 was always found without the other COG subunits in fractions 27–29, in which proteins larger than Cog7 monomers are expected to elute (18, 24). This raises the possibility that Cog7 may independently form an oligomer or form a complex with other as yet unidentified proteins.

We also examined the remaining COG subunits and subcomplexes in Cog1 (ldlB)- and Cog2 (ldlC)-deficient CHO cell mutants (12, 13, 18). In Cog1-deficient ldlB cells, the steady state levels of Cog5–8 determined by immunoblotting (Fig. 1A, lane 2) were dramatically reduced relative to the wild type control CHO cells, whereas the levels of Cog2–4 were essentially unchanged. In Cog2-deficient ldlC cells (Fig. 1A, lane 4) there was an equally striking decrease in the levels of six subunits (Cog3–8), and Cog1 expression was virtually abolished. Pulse-chase analysis showed that Cog1 was synthesized normally but was rapidly degraded in the ldlC cells compared with wild type CHO cells (data not shown). All COG subunits were present in normal or slightly greater than normal levels in the mutants stably transfected with the corresponding complementing cDNAs, ldlB[COG1] and ldlC[COG2] cells (also see Refs. 13, 18, 24, 43), which indicates that the reduced steady state levels, presumably due to instability, of the COG subunits in the ldlB and ldlC mutants are a consequence of the mutations in the genes encoding Cog1 or Cog2, respectively. These results indicate that loss of Cog2 influences the stability of all of the other subunits, whereas the steady state levels of Cog2–4 are unaffected by the absence of Cog1.

FIGURE 1. Immunoblotting (A), gel filtration chromatographic (B), and immunofluorescence microscopic (C) analyses of COG subunits in wild type, mutant, and transfected CHO cells. A, total cell lysates (20 μg/lane) from CHO, ldlB, ldlC, ldlB[COG1], and ldlC[COG2] cells were subjected to SDS-PAGE and immunoblotting using antibodies to the indicated COG subunits and GM130, a Golgi-associated protein used as a loading control. B, cytosols prepared from CHO (left), ldlB (center), and ldlC (right) cells were fractionated by Superose 6 gel filtration chromatography. The indicated fractions were analyzed by SDS-PAGE and immunoblotting using anti-COG subunit antibodies. Open, solid, and gray triangles represent positions of the peaks of complexes and subcomplexes (see “Results”). Asterisks indicate an unknown protein recognized by the anti-Cog4 antiserum. Elution positions of size standards: 669 kDa, fraction 26; 440 kDa, fractions 29 and 30; and void volume, fractions 18 and 19. C, the indicated cells were fixed and stained with antibodies to Cog1 (top) or Cog3 (bottom), and confocal images were acquired as described under “Experimental Procedures.” Scale bar, 10 μm. D–F, working models of the subcomplexes within the bilobed (A and B) COG complex (see “Results”). White numerals indicate the corresponding COG subunits.

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Fig. 1B, center and right, shows as previously described (24) that the absence of one or more subunits of the COG complex alters the sizes of the residual complex components. In ldlB (Cog1-deficient) and ldlC (Cog2-deficient) cytosols, the other COG subunits were no longer present in the largest complex (centered in fractions 21 and 22). In the ldlB cells lacking Cog1, Cog2–4 co-eluted in fractions centered on fraction 26 (Fig. 1B, center, gray triangle), suggesting that these subunits form a stable subcomplex in the absence of Cog1 (see model in Fig. 1F). Despite the substantial reductions in the steady state levels of Cog5–8 in both ldlB and ldlC cells, it was possible to detect their co-elution centered in fractions 24 and 25 (Fig. 1B, center and right, solid triangles), suggesting that they form the same subcomplexes in these mutant cells without Cog1 and/or Cog2 as seen in wild type CHO cells (Fig. 1B, left).

The COG complex has been shown to be associated with the cytosolic surface of the Golgi apparatus (13, 17, 18, 24, 25, 27, 44). To determine whether the Cog2–4 subcomplex in ldlB cells retained the ability to associate with the Golgi, we used immunofluorescence microscopy and anti-Cog1 and anti-Cog3 antibodies to locate these components in the mutant and control cells (Fig. 1C). Staining with both antibodies exhibited perinuclear Golgi distributions in wild type CHO and stably transfected control ldlB[Cog1] and ldlC[Cog2] cells. Similar findings have been reported previously (13, 18, 25, 27). As expected because of the very low steady state expression levels, there was only a very weak, apparently nonspecific background, staining of Cog1 in ldlB and ldlC cells and of Cog3 in ldlC cells. In the ldlB cells, the anti-Cog3 antibody exhibited a substantial staining throughout the cytoplasm with little, if any, Golgi-like perinuclear staining. The results suggest that the Cog2–4 subcomplex is unable to stably associate with the Golgi membrane in the absence of Cog1, raising the possibility that Cog1 and/or the lobe B subunits might directly mediate Cog2–4 association with the Golgi.

Establishment of Stable HeLa Cell Cog5 Knockdown Lines by Plasmid-based RNA Interference—To explore further the organization and function of the COG complex, we examined the phenotypes of cells with deficiencies in the expression of two lobe B COG subunits: Cog5-deficient HeLa cells generated for this study by siRNA knock down (kd) and Cog7-deficient human fibroblasts isolated from a patient with CDG type-IIe, one form of COG-deficient CDG (44).

To identify effective RNA interference sequences for COG5, we transiently transfected small double-stranded RNAs corresponding to several distinct segments of the human COG5 coding sequence into HeLa cells. Lysates were prepared from cells harvested 48 h after transfection and subjected to SDS-PAGE and immunoblotting analysis using antibodies to the indicated COG subunits. A, total cell lysates (20 μg/lane) from HeLa cells and three individual knockdown (kd) clones (kd-COG5#1–3) were subjected to SDS-PAGE and immunoblotting analysis using antibodies to the indicated COG subunits. B, HeLa and Cog5 knockdown (kd-COG5#1) cells were fixed and stained with antibodies to Cog1 or Cog3, and confocal images were acquired as described under “Experimental Procedures.” Scale bar, 10 μm. C, cytosols isolated from HeLa and kd-COG5#1 cells were subjected to immunoprecipitation either with anti-Cog1 (α-Cog1) or control anti-Bet1 (α-Bet1) antibodies as indicated. The immunoprecipitates (lanes 3–6) and untreated cytosols (5% of the samples subjected to immunoprecipitation, lanes 1 and 2) were analyzed by SDS-PAGE and immunoblotting using antibodies to the indicated proteins. D, HeLa and Cog5-deficient cells were fixed and stained with anti-Cog8 antibody, and confocal images were acquired. Scale bar, 10 μm. E, model of the association of the Cog1–4 plus Cog8 subcomplex with the Golgi in Cog5-deficient cells.
ibly exhibited an inhibitory effect on Cog5 protein expression. Therefore, we established stable cell lines carrying an expression plasmid for the double-stranded RNA. In two independent transfections we isolated three individual HeLa cell clones (designated kd-COG5#1–3) as antibiotic-resistant cells. Immunoblotting and immunofluorescence analysis (Fig. 2A, supplemental Fig. S1, and see below) showed a dramatic reduction in the steady state levels of Cog5 in all three clones. For example, there was a >95% decrease in Cog5 levels in the kd-COG5#1 clone compared with wild type HeLa cells. Nevertheless, this clone exhibited normal morphology and apparently normal growth at 37 °C.

Characterization of Cog5 Knockdown Cells—Immunoblotting analysis of all three kd-COG5 clones and control HeLa cells (Fig. 2A) showed that a dramatic reduction in Cog5 levels did not substantially alter the steady state levels of Cog1–4 or 8; however, it was accompanied by a marked reduction in the levels of Cog6 and 7. Together with the studies described above, these results are consistent with Cog2–4 and Cog5–7 forming independent subcomplexes in which Cog1 associates (physically or functionally) more directly with the Cog2–4 subcomplex than with the Cog5–7 subcomplex (see Fig. 1F), whereas Cog8 more closely associates with, but is not strictly dependent on, the Cog5–7 subcomplex. Immunofluorescence analysis of clone kd-COG5#1 with antibodies to the lobe A subunits Cog1 and Cog3 (Fig. 2B) and Cog2 (data not shown) established that there was essentially normal Golgi localization of Cog1–3 (and by inference, Cog4) in the kd-COG5#1 cells despite the ~95% reduction in Cog5 and substantial reductions in Cog6 and 7 levels. Because the Cog2–4 subcomplex is not associated with the Golgi in Cog1-deficient (ldlB) cells (Cog3 in Fig. 1C and Cog2 (13, 18), data not shown), it seems likely that Cog1 and/or Cog8 plays a key role in recruiting this subcomplex, and perhaps the entire COG complex, to the Golgi. Cog8 was originally assigned to be a subunit in lobe B (22, 24) and is physically and functionally associated with the Cog5–7 subcomplex (Refs. 22, 24 and this study). However, in the kd-Cog5 cells the steady state levels of Cog8 were similar to that in control HeLa cells. We therefore used a co-immunoprecipitation and immunoblotting approach to test the possibility that Cog8 forms a subcomplex with Cog1–4 relatively independently of the Cog5–7 subcomplex. Cytosols from either control HeLa or kd-COG5#1 cells were harvested and immunoprecipitated with anti-Cog1 or control anti-Bet1 antibodies. Bet1 is a SNARE protein required for endoplasmic reticulum-to-Golgi transport (9, 47, 48). The precipitates were then subjected to SDS-PAGE and immunoblotting with antibodies to the Cog1, 2, 6, 7, and 8 subunits or a control anti-Sec8 (subunit of the octameric exocyst complex) antibody (Fig. 2C). As expected, all five COG subunits examined were immunoprecipitated from control HeLa cell cytosol by the anti-Cog1 antibody, but not by the control anti-Bet1 antibody (compare lanes 3 and 4). The low yield (<5% of input amounts) of the COG subunits, including Cog1 itself, in the immunoprecipitates was presumably because of limitations inherent in the anti-Cog1 monoclonal antibody. As a consequence, the precipitated Cog1 may not necessarily represent the entire population of Cog1 in the cells. The Cog1, 2, and 8 subunits were also efficiently co-immunoprecipitated from kd-COG5#1 cytosol by the anti-Cog1, but not the anti-Bet1 antibody (lanes 5 and 6). There was substantially less co-immunoprecipitation of Cog6 and 7 from the kd-COG5#1 cells than from the control HeLa cells (lanes 4 and 6). These results indicate that Cog8 can form a subcomplex with Cog1 and 2 (and presumably Cog3 and 4) in the absence of normal amounts of Cog5–7. Furthermore, an anti-Cog8 antibody was also able to co-immunoprecipitate Cog1–3 from the cytosol of the kd-COG5#1 cells (data not shown). The control Sec8 was not observed in any of the immunoprecipitates. These results indicate that Cog8 can form a subcomplex with Cog1 and 2 (and presumably Cog3 and 4) in the absence of normal amounts of Cog5–7. Moreover, immunofluorescence microscopy established that Cog8 exhibited Golgi localization in all three Cog5 knockdown clones as well as wild type HeLa cells (Fig. 2D). Thus, it appears that Cog8 can form a subcomplex with Cog1–4 and then associate with the Golgi despite the absence or a substantial reduction in the amounts of the Cog5–7 subunits (see model in Fig. 2E).

We next examined the effects of the dramatic reduction in Cog5 levels on a subset of the COG-dependent Golgi integral membrane proteins called GEARs (GOS-28, GS15, GPP130, CASP, giantin, and golgin-84) and COG-independent controls (GM130, Vtila, Bet1) (43). The steady state levels of GEARs are reduced in Cog1 (ldlB)- and Cog2 (ldlC)-deficient mutant cells, and some GEARs are abnormally degraded by proteasomes (43). Analysis in Fig. 3A (immunoblotting) and 3B (immunofluorescence microscopy)
showed that among the six GEARs and three COG-independent proteins tested, only GS15, a Golgi SNARE protein, exhibited substantially decreased steady state levels in all three Cog5-deficient HeLa cell clones. The increased level of GOS-28 in only one of the clones, kd-COG5#3, was presumably because of clonal variation. The reduction in the kd-COG5 cells of GS15, but not other Golgi-associated proteins (including the control molecules GM130 and Vti1a), was confirmed by immunofluorescence microscopy (Fig. 3B). The decrease of GS15 in the kd-COG5#1 cells was restored by transfection with a cDNA expression plasmid for wild type COG5 cDNA (data not shown). Thus, the influence of COG subunit deficiencies on the GEARs depends on the subunit that is depleted, and presumably on the remaining COG subunits and subcomplexes and their interactions with the Golgi.

The ldlB and ldlC cells were isolated originally because of their low density lipoprotein receptor (LDLR) deficiency (11). This phenotype is a consequence of the instability of LDLRs in these mutants because of the incomplete Golgi-associated processing of the oligosaccharides of the receptors (12, 33). We therefore examined the processing and activity of LDLRs in the Cog5 knockdown cells to compare the effects on Golgi-mediated glycoprotein processing of deficiencies in Cog1, 2, or 5. In wild type cells, LDLRs are initially synthesized as a 120-kDa glycosylated precursors (endoplasmic reticulum form) that are converted by Golgi-associated oligosaccharide processing of their N- and O-linked sugars to a 160-kDa mature forms (49, 50). Immunoblotting studies have shown that at steady state most of the LDLRs in normal mammalian cells are in a mature form on the cell surface with lesser amounts seen in either the biosynthetic precursor (p) form or a degraded (d) form that is slightly smaller than the precursor (Ref. 50, and see Fig. 4A, top, lanes 1 (HeLa) and 5 (CHO)). In ldlB and ldlC cells there is substantially less LDLR present, and the electrophoretic mobility of most of the LDLR is intermediate (indicated by **) between that of the precursor/degraded (p/d) and mature (m) forms (Fig. 4A, top, lanes 6 and 7), due to aberrant glycosylation (12). There was little systematic difference between the LDLRs in wild type (lane 1) and Cog5 knockdown (lanes 2–4) cells, except that the ratio of precursor/degraded to mature forms might have been a bit higher in the knockdown cells. There were no readily detected Cog5-dependent changes in the electrophoretic mobilities of the LDLR forms, indicating that Golgi-associated glycosylation defects in these clones (see below) were far less extensive than those in ldlB and ldlC cells. This conclusion was confirmed by analysis of the amounts and mobilities of the very heavily glycosylated GEAR GPP130. GPP130 is highly unstable in ldlB and ldlC cells (Ref. 43; Fig. 4A, bottom, lanes 6 and 7); however, its steady state levels and electrophoretic mobilities in the Cog5 knockdown clones were similar to those of the wild type HeLa cells (lanes 1–4).

The absence of substantial effects of Cog5 deficiency on the electrophoretic mobilities and steady state expression levels of the LDLR and GPP130 were strikingly different from those of Cog1 (ldlB) and Cog2 (ldlC) deficiencies (12, 43), raising the questions of whether Cog5 deficiency influenced the ultrastructure of the Golgi as does Cog1 and Cog2 deficiency (dilation of a subset of Golgi cisternae, Ref. 24) and whether there were any global glycosylation defects in Cog5-deficient cells, as is the case for ldlB and ldlC cells (12, 13, 18). Fig. 4B shows representative transmission electron microscopic images of the Golgi region of control HeLa (left panel) and kd-COG5#1 (right panel) cells. The most striking difference was the abnormal dilation of some 30–40% of Golgi cisternae in the Cog5-deficient cells. Cisternal dilation also was seen in Cog1- and Cog2-deficient cells (24). There also appeared to be fewer readily detected coated vesicles in the Golgi region of the kd-COG5#1 cells than in the controls; however, additional studies will be required to explore this possible effect of Cog5 depletion. To investigate possible Cog5-dependent global cell surface glycosylation defects, we examined the lectin sensitivities of these cells. We used four lectins: wheat germ agglutinin (binds terminal sialic acid and N-acetylglucosamine residues)
and phytohemagglutinin (binds galactose in β1–4 branches of complex N-linked chains) to which ldlB cells are resistant (12); concanavalin A (binds mannose residues) to which ldlB cells are hypersensitive (12); and peanut agglutinin (PNA, binds terminal galactose residues on O-linked oligosaccharides) to which Cog7-deficient fibroblasts are hypersensitive (44). There were no significant differences in the sensitivities of the control and Cog5-deficient HeLa cells to three of the lectins, wheat germ agglutinin, phytohemagglutinin, and concanavalin A (data not shown). However, Fig. 4C, top, shows that unlike the control HeLa cells, the Cog5-deficient cells strongly stained with PNA, suggesting the Cog5 deficiency probably reduces terminal sialylation of some cell surface glycoconjugates, as is the case for Cog7-deficient human fibroblasts (Fig. 4C, bottom, Ref. 44, and see below).

Because the Cog5 deficiency had little effect on the processing and steady state levels of LDLRs, we were not surprised to find there were no significant differences between the kd-COG5#1 and control HeLa cells in LDLR-mediated binding and degradation of 125I-low density lipoprotein (data not shown). However, immunofluorescence analysis of the intracellular distribution of LDLRs in the Cog5 knockdown and wild type HeLa controls (supplemental Fig. S1) indicated that, relative to the controls, there was a greater intracellular accumulation of LDLRs in all three knockdown clones, especially in perinuclear regions. Thus, even though there was little, if any, effect of Cog5 depletion on posttranslational glycosylation of the LDLR, there appears to have been an effect on its intracellular trafficking.

**Effects of a Mutation in the Human COG7 Gene on the Expression of COG Subunits and GEARs**—Comparison of some of the phenotypes of ldlB and ldlC mutants with those of a subset of cells from CDG patients led us to propose that some forms of CDG might arise because of COG deficiency. Indeed, the first case of such COG-deficient CDG due to mutation in the gene encoding Cog7 was reported recently (44). Sialylation of glycans on the surfaces of Cog7-deficient mutant fibroblasts is reduced (e.g. increased PNA binding, Ref. 44, Fig. 4C, bottom). As was the case for the Cog5-deficient HeLa cells, there was no obvious abnormality in the electrophoretic mobility of the heavily glycosylated GPP130 protein in the Cog7 mutant fibroblasts (Fig. 5, right, and see below). Thus, the glyco phenotypes of Cog5-deficient HeLa cells and Cog7-deficient human fibroblasts appear to be similar and significantly milder than those of the Cog1- or Cog2-deficient CHO cells.

We used immunoblotting of Cog7-deficient and control fibroblasts to compare the steady state levels of five GEARs (GS15, GOS-28, GPP130, CASP, golgin-84) and three COG-independent control proteins (GM130, Vti1a, Bet1) (Fig. 5, right). Only two of these proteins exhibited a reproducible Cog7 dependence, the SNARE GS15 (as was the case in the Cog5-deficient HeLa cells) and the golgin CASP (unlike Cog5-deficient cells), both of which had reduced expression in the Cog7 mutant cells. Once again, the effects of COG subunit deficiency on GEAR expression depended on the COG subunit affected.

**DISCUSSION**

In the current study we have examined the subunit organization and function of the mammalian COG complex in human (HeLa and fibroblasts) and hamster (CHO) cultured cells. Previous biochemical, imaging, and genetic studies had suggested that the eight distinct COG subunits were organized into two subcomplexes, lobe A (Cog1–4) and lobe B (Cog5–8) (24, 45). Analysis of CHO cell mutants with defects in Cog1 (ldlB cells) and Cog2 (ldlC cells) played a key role in defining the influence of mammalian COG on the structure and function of the Golgi apparatus and in helping to formulate the two-lobe model of COG (11–13, 18, 24). In this study, we used a complete set of anti-COG antibodies and additional Cog5- and Cog7-deficient mammalian cells to extend the analysis of COG structure and function.

The Cog5-deficient (≤ ~5% of control) cells were generated by stably expressing a COG5 RNA interference vector in HeLa cells. Cog7-deficient fibroblasts were derived from a patient with lethal CDG, type Ile (44). We examined the effects on the steady state levels of each of the eight COG subunits in the cells with the complete loss of, or dramatic reduction in, the expression of either Cog1, Cog2, Cog5, or Cog7. In each case, there was substantial reduction in the intracellular levels of at least one, and as many as all seven, other subunits in the deficient cells. These results helped identify putative stable subcomplexes. In addition, we used size exclusion chromatography of cytosols from wild type and Cog1- and Cog2-deficient cells to identify putative COG subcomplexes (e.g. Cog2–4, Cog5–7, and the Cog5–8 subcomplex previously identified by fractionation of bovine brain extracts; see Ref. 24). Analysis of the Cog5- and Cog7-deficient cells, including co-immunoprecipitation and immunoblotting, helped confirm the existence of a Cog5–7 subcomplex. Ternary interactions between Cog5–7 were also observed in a previously reported in vitro translation and co-precipitation study (46) and in the recent study of Ungar et al. (52).

Analysis of the Cog5-deficient cells also provided evidence for the presence of a stable subcomplex of Cog1–4 plus Cog8. Immunofluorescence microscopy was used to determine which subunits were involved in the association of some subcomplexes with the Golgi apparatus.
Based on these and earlier (22, 24) data, we propose the model for the intersubunit architecture and Golgi association of the COG complex shown in Fig. 6A. It is important to note that, although this model is based on the current and previously published data, its formulation was informed by our generation of an intersubunit and Golgi/COG interaction map in an independent study that included in vitro co-translation and co-immunoprecipitation identification of subunit interactions (Fig. 6B; Ref. 52) and is fully consistent with the model presented here.

While this work was in progress, Loh and Hong (46) reported the results of their analysis of the subunit connectivity map of mammalian COG based on an in vitro translation and co-precipitation approach. Their model (Fig. 6C) is consistent with the original description of the two-lobed model for COG (Cog1–4 and Cog5–8; Refs. 24, 45) and the formation of a stable Cog5–7 subcomplex; however, it differs significantly from the other two models. Loh and Hong (46) proposed that Cog4 is a central subunit, bridging two ternary complexes (Cog1–3 and Cog5–7) and that Cog8 is incorporated into the COG complex only because of its association with the Cog5–7 subcomplex in a Cog5-dependent fashion. However, the data presented here provide strong support for a direct in vivo interaction of Cog8 with the Cog1–4 subcomplex in the virtual absence of Cog5 as well as reduced levels of Cog6 and 7, in addition to its independent interactions with Cog5–7. Furthermore, previous studies indicated assembly of a complex of Cog8 with Cog5–7 (bovine brain and CHO cytosols, Refs. 24, 46), and the chromatographic studies reported here support the existence of such subcomplexes. Indeed, in vitro studies of Ungar et al. (52) have identified direct interactions of Cog8 with both Cog1 and Cog5–7. Thus, Cog8 probably serves as a bridge between the Cog1–4 and Cog5–7 subcomplexes. It is possible that the differences between the models (Fig. 6, A and B versus C) are because of unidentified experimental artifacts or inappropriate assumptions used to construct them. Nevertheless, it is possible that many of the specific binary interactions between individual subunits and subcomplexes in all these models are correct; however, differences in methodologies may have resulted in the inability of any one approach to identify all binary interactions in the three-dimensional COG complex. The complete agreement of the model in Fig. 6A, which is based on analysis of COG subunit-deficient cells and purification of COG from bovine brain (Ref. 24 and this study), and in Fig. 6B, which is based on in vitro co-translation/co-immunoprecipitation studies (52), provides strong support for these models.

Immunofluorescence analysis of the intracellular distribution of the COG subunits in Cog1-, Cog2-, Cog5-, or Cog7-deficient cells suggests that Cog1/8 plays a key role in mediating the association of the COG complex with the cytoplasmic face of the Golgi apparatus (Fig. 6A and B). We found that the Cog2–4 subcomplex can be recruited to the Golgi apparently independently of the Cog5–7 subcomplex, provided Cog1 is present. Because of the previous localization of Cog8 to lobe B and our new findings that Cog8 forms a Golgi-associated complex with Cog1–4 in Cog5-deficient cells, Cog1 and Cog8 appear to play key roles in forming a bridge between the Cog2–4 and 5–7 subcomplexes. This bridging function for Cog8 apparently depends on the presence of Cog1, which is also required for COG association with the Golgi.

The current study provides additional insight into how individual subunits or subcomplexes of COG influence the structure and activity of the Golgi apparatus. Earlier studies showed that loss of Cog1 or Cog2 in CHO mutant cells resulted in dilation of a subset of Golgi cisternae (24), a dramatic alteration in medial and trans-Golgi-associated glycoprotein conjugation with associated global defects in N- and O-linked protein glycosylation, and lipid-linked glycosylation that lead to altered sensitivities to a variety of lectins (12, 53). These mutants also exhibited abnormalities in LDLR posttranslational processing and the consequent loss of LDLR stability and activity (11–13, 18). The steady state levels of a subset of Golgi-associated membrane proteins, called GEARs (mannosidase II, GOS-28, GS15, GPP130, CASP, giantin, and golgin-84) were also reduced in these mutant cells, which we proposed might be because of defects affecting their retention in and/or retrieval to their appropriate intra-Golgi sites (43). Cog5 deficiency in HeLa cells generated a pattern of dilated Golgi cisternae reminiscent of that in Cog1- and Cog2-deficient cells and resulted in global glycosylation abnormalities (increased PNA binding), providing additional evidence of the importance of COG for Golgi structure and function. Nevertheless, unlike the case for the Cog1- and Cog2-deficient cells, the global glycosylation abnormalities in Cog5-deficient cells were subtle (e.g. there was no evidence from SDS-PAGE analysis of altered glycosylation of the LDLR or the heavily glycosylated Golgi protein GPP130) and, as a consequence, the LDLR activity of Cog5-deficient cells was essentially normal. Furthermore, the steady state level of only one of the six GEARs examined, GS15, was substantially reduced in the Cog5-deficient cells. Thus, assuming that the effects of COG subunit deficiencies are similar in CHO and HeLa cells, it appears that the cisternal dilation observed in Cog1, 2, and 5 deficiency is not dependent on or a direct cause of either profound defects in glycoconjugate synthesis (such as those seen in Cog1- and Cog2-deficient cells) or reduced levels of all six GEARs. It is noteworthy that cisternal dilation and reductions in the levels of GS15 were observed in Cog1, Cog2, and Cog5 deficiencies, raising the possibility...
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that these defects are causally linked and may be due to disruption of lobe B-associated COG functions.

In yeast, deficiencies in the Cog1–4 subunits result in more profoundly abnormal phenotypes (e.g., dramatic growth defects) than deficiencies in Cog5–8 (22). The COG-deficient mammalian cells are similar in that the Cog1 and Cog2 deficiencies generated more severe, but not substantially growth inhibiting, abnormal phenotypes than the Cog5 and Cog7 deficiencies. Indeed, there were similarities in the abnormal phenotypes for the Cog5- and Cog7-deficient cells: mild PNA-sensitive glycosylation defects and reduction in the GEAR GS15 (Ref. 44 and this study), although there were some differences (e.g., normal level of the GEAR CASP in Cog5 deficiency but reduction in Cog7 deficiency and somewhat reduced levels of Cog8 in the Cog7 mutants). Thus, loss-of-function or reduction-of-function analysis continues to show that there are striking functional distinctions between lobe A- and lobe B-associated COG subunits, as well as more subtle differences in the functions of subunits within the same lobe (e.g., Cog5 and Cog7 in lobe B).

The relatively mild abnormal phenotypes of the Cog5-deficient mammalian cells are consistent with those for Cog5 deficiency in D. melanogaster ("four-way stop", Ref. 32) and yeast (22, 23, 28, 29). In Drosophila, loss of Cog5 function causes failure of spermatogenesis and male sterility but no other overt abnormal phenotypes (32). Some cog5 mutants in yeast were isolated because of their defects in mannosylphosphorylation of cell wall mannoproteins (29). In addition, there is abnormal glycosylation of the secreted cell wall glycan exoglucanase Exg1p in the mutants (29). These findings are reminiscent of the incomplete sialylation (increased PNA binding) of cell surface glycans in the Cog5 knockdown HeLa cells.

During the preparation of this report, Zolov and Lupashin (51) reported the phenotypic effects of transient (48–72 h) RNA interference-mediated partial depletion of Cog3 (18% of control) in HeLa cells. Partial loss of Cog3 resulted in partial (2–3-fold) decreases in Cog1, Cog2, and Cog4, with little effect on Cog5–8. These results lend additional support to the proposal that Cog1–4 and 5–8 are organized into subcomplexes. Among the numerous intriguing observations reported by Zolov and Lupashin for the partial Cog3 depletion were the following: 1) the partial fragmentation of the Golgi ribbon, apparently without substantial cisternal dilation or loss of stack formation; however, it is not clear why substantial Golgi fragmentation is observed in cells with partial loss of Cog3 but not in the Cog1-negative (Idlb) and Cog2-negative (Idlc) cells (13, 24); and 2) generation of numerous COG complex-dependent (CCD) vesicles that were located throughout the cytoplasm and contained three GEARs, GS15, GOS-28, GPP130. These three GEARs are the most sensitive GEARs to Cog1 and Cog2 deficiency (43). The GEAR giantin appears to remain with the fragmented Golgi ribbon in a perinuclear distribution rather than redistributing with CCD vesicles. Zolov and Lupashin (51) present evidence that anterograde transport through the Golgi (VSVG protein) was not disrupted by the partial Cog3 depletion, consistent with our earlier report that anterograde transport does not appear to be disrupted in Cog1 and Cog2 mutants (33), but that retrograde trafficking of Shiga toxin was blocked. Furthermore, they presented co-immunoprecipitation data using normal rat liver extracts that support either a direct or indirect physical interaction of COG with Golgi proteins associated with intracellular membrane trafficking in general and retrograde transport in particular (SNAREs, golgins and COP1; Refs. 9, 54–56), it will be important for future investigations to explore potential roles of COG in controlling the selectivity, movement, targeting, and/or docking/fusion steps in membrane trafficking. Such studies will substantially contribute to helping define the precise mechanisms(s) by which COG influences Golgi structure and function.

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Addendum—An independent analysis of the subunit connectivity map of the Saccharomyces cerevisiae COG complex (58) that is in good agreement with the models proposed here and in the accompanying paper (52) appeared after this paper had been reviewed.