Identification and Characterization of GCP16, a Novel Acylated Golgi Protein That Interacts with GCP170*

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GCP170, a member of the golgin family associated with the cytoplasmic face of the Golgi membrane, was found to have a Golgi localization signal at the NH2-terminal region (positions 137–237). Using this domain as bait in the yeast two-hybrid screening system, we identified a novel protein that interacted with GCP170. The 2.0-kilobase mRNA encoding a 137-amino acid protein of 16 kDa designated GCP16 was ubiquitously expressed. Immunofluorescence microscopy showed that GCP16 was co-localized with GCP170 and giantin in the Golgi region. Despite the absence of a hydrophobic domain sufficient for participating in membrane localization, GCP16 was found to be tightly associated with membranes like an integral membrane protein. Labeling experiments with [3H]palmitic acid and mutational analysis demonstrated that GCP16 was acylated at Cys69 and Cys72, accounting for its tight association with the membrane. A mutant without potential acylation sites (C69A/C72A) was no longer localized to the Golgi, indicating that the acylation is prerequisite for the Golgi localization of GCP16. Although the mutant GCP16, even when overexpressed, had no effect on protein transport, overexpression of the wild type GCP16 caused an inhibitory effect on protein transport from the Golgi to the cell surface. Taken together, these results indicate that GCP16 is the acylated membrane protein, associated with GCP170, and possibly involved in vesicular transport from the Golgi to the cell surface.

In mammalian cells, the Golgi complex is composed of a highly ordered parallel array of cisternae that form a stacked structure typically found in the perinuclear region of the cell. The major functions of Golgi complex are thought to be in processing and sorting of lipids and proteins en route from the endoplasmic reticulum to the plasma membrane and other destinations (1). Various enzymes involved in proteolytic and oligosaccharide processing of the transported proteins are localized to subcompartments of the Golgi complex (1–3). The ordered structure of Golgi complex is thought to reflect the requirement for these enzymes and of the protein sorting machinery to be compartmentalized to allow a specific series of post-translational modifications and sorting reactions to be carried out. The conservation of Golgi structure throughout the eukaryotic kingdom implies an essential role in Golgi function (4).

It has been known that the sera of some patients with autoimmune disease react with a cytoplasmic antigen localized in the Golgi complex (5–7). Over the past few years evidence has described several cDNA of Golgi autoantigens that are given the name golgins. A common structural feature of golgins is a cytoplasmic coiled-coil domain (8) similar to that of the myosin family. These coiled-coil proteins localized in the Golgi complex were grouped as the golgin family, for example, giantin/macro-golgin/GCP372/GCP364 (6, 7, 9, 10), golgin-245/p230 (11, 12), p210 (13), golgin-160/GCP170 (14, 15), golgin-97 (16), GM130/golgin-95 (14, 17), golgin-84 (18), and golgin-67 (19).

The best studied of the golgin proteins are GM130 and giantin. Initially identified as a component of the detergent-insoluble Golgi matrix, GM130 was later found to bind to GRASP65, a cis-Golgi membrane protein required for stacking of Golgi cisternae in vitro (20). GRASP65 exists in Golgi membranes as part of a complex with GM130 (21), which acts as the receptor for p115 during the docking of vesicles with Golgi membranes (22). Giantin binds p115, suggesting the presence of a complex that tethers Golgi cisternae containing GM130 to giantin on vesicles via p115 (23). Disruption of GM130-giantin tethering complexes inhibits transport through the Golgi and causes the accumulation of transport vesicles (24, 25). Taken together, these observations suggest that golgins couple Golgi structure to Golgi function.

GCP170 is a peripheral membrane protein with a long coiled-coil domain (15). Recently, GCP170/golgin-160 was shown to be a caspase substrate during apoptosis (26). The cleavage of GCP170 occurred in the NH2-terminal head of GCP170 and is required for efficient disassembly of the Golgi complex during apoptosis. This suggests that cleavage of GCP170 may disrupt protein-protein interactions important for Golgi function. In the present study we have characterized the Golgi targeting of GCP170 as a first step toward understanding its function, demonstrating that the essential domain of GCP170 for Golgi localization is in the NH2-terminal head domain. Using this domain as bait in the yeast two-hybrid screening system, we identified a novel protein (termed GCP16) interacting with GCP170.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening, cDNA Cloning, and Northern Blotting—The NH2-terminal region (positions 137–237) of GCP170 (15) was fused to the LexA DNA-binding domain and used as the bait construct. EGY48 harboring the β-galactosidase reporter plasmid was transformed with the bait plasmid and the HeLa cell cDNA library generated in pB42AD vector (27)/(BD Bioscience Clontech, Palo Alto, CA). Screening was performed according to the manufacturer’s instructions. β-Galactosidase activities expressed by the specific interaction between the bait and prey constructs were measured using o-nitrophenyl-galacto-
side as a substrate (28). Of about 10^6 clones screened, a positive cDNA clone (2.0 kilobase pairs) was obtained and designated D1E2, which was found to contain a complete open reading frame. D1E2 contained the entire coding sequence, and its translation product was designated GCP16. Northern blotting was carried out using human multiple tissue Northern blot (BD Bioscience Clontech) containing poly(A)^+ RNA (2 μg/μl), which was hybridized with [32P]-labeled 1E2 cDNA or human β-actin cDNA as a probe (15).

Preparation of Anti-GCP16—cDNA encoding a chimeric protein of GCP16 fused to the COOH terminus of glutathione S-transferase (GST)^1 was constructed in expression vector pGEX4T-1 (15) and designated GST-GCP16. The recombinant protein was expressed in E. coli and purified from bacteria and injected into rabbits to raise anti-GST-GCP16 antibodies as described previously (15). The anti-GST antibody was removed from the antiserum by passing it through GST-coupled Sepharose 4B beads, and the remaining antibody was used as anti-GCP16, unless otherwise indicated. In some experiments, we used rabbit anti-GCP16/N, which was raised against the NH2-terminal region (positions 1–23) of GCP16 and prepared by essentially the same procedures as described above. In addition, polyclonal antibodies were raised in rabbits against a synthetic polypeptide (CTIYEDRGMSSGR) corresponding to the COOH-terminal region (positions 126–137) of GCP16. The antibodies were purified by chromatography through a GCP16-peptide-Sepharose column.

Construction and Transfection of Expression Plasmids—cDNAs encoding the wild type and deletion mutants (D1, D2, and D3) of GCP170 were inserted into EcoRi-Xhol site of pSG5 expression vector at the downstream of the sequence encoding the Met-FLAG tag so that the products could be recognized by the monoclonal anti-FLAG antibody M2 (29, 30). Deletion mutants derived from the above (D1) were constructed by site-directed mutagenesis using the method of Kunkel (31) with synthetic oligonucleotides 5′-AGGGACAGATCCCACAGGCCG-GTGTTGCGTTCTCCATGACACC-3′ for ΔPR, 5′-GTCCTGGCTGA-GCCCTCTTCAATCAGTCTCCGGCTCCGTT-3′ for ΔH1, 5′-CTATCGAAGAAAAGCCTTCATACAAAGAATCGCGCATGTTT-TTCG-3′ for ΔH2, and 5′-CATCTATGGGAAAGAAAAACTCCGGAGGAG-CCTATGGCATTCTGTCG-3′ for ΔH3. ΔH3 was prepared by substituting the 13 bp deletion of the wild type cDNA for C69A, the following synthetic oligonucleotides were used: 5′-CAGTGGCACACGCGCCCAGTTCCAG-GAGGACAGATCCCACATGGCG-3′ (wild type); 5′-GAGGACAGATCCCACATGGCG-3′ (ΔCys); 5′-GAGGACAGATCCCACATGGCG-3′ (ΔCys/Ala).

Expression vectors for the LexA two-hybrid system (pLexA or pB42AD). For site-directed mutagenesis for the substitution of Cys with Ala in GCP16, the following synthetic oligonucleotides were used: 5′-GTGTTGCGTTCTCCATGACACC-3′ for ΔPR, 5′-GTCCTGGCTGAGCCCTCTTCAATCAGTCTCCGGCTCCGTT-3′ for ΔH1, 5′-CTATCGAAGAAAAGCCTTCATACAAAGAATCGCGCATGTTTTATC-3′ for ΔH2, and 5′-CATCTATGGGAAAGAAAAACTCCGGAGGAGCCTATGGCATTCTGTCG-3′ for ΔH3. ΔH3 was prepared by substituting the 13 bp deletion of the wild type cDNA for C69A, the following synthetic oligonucleotides were used: 5′-CAGTGGCACACGCGCCCAGTTCCAG-GAGGACAGATCCCACATGGCG-3′ (wild type); 5′-GAGGACAGATCCCACATGGCG-3′ (ΔCys); 5′-GAGGACAGATCCCACATGGCG-3′ (ΔCys/Ala).

Metabolic Labeling, Immunoprecipitation, and SDS-PAGE—HeLa cells (4 × 10^6 cells/dish) or COS-1 cells (5 × 10^6 cells/dish) were cultured for 5 or 14 h (29). The cells were fixed with the Zamboni fixative for 2 h and permeabilized by treatment with 0.1% saponin and incubated with anti-FLAG M2 antibody for 2 h, followed by incubation with peroxidase-conjugated secondary antibody for 1 h. After the peroxidase reaction, the cells were washed three times with PBS and solubilized for electron microscopy as described previously (36).

Analysis of Protein Transport—Protein transport in the secretory pathway was examined using the temperature-sensitive glycoprotein of vesicular stomatitis virus (VSV-Gts045) (37). COS-1 cells were transfected with the pSG5 expression vector containing the sDPPIV cDNA fused to green fluorescent protein (GFP-VSV-Gts045) (35). After incubation at 39.5 °C for 20 h, the cells were transferred to the permissive temperature at 32 °C and incubated for an additional 2 h. The cells were fixed and observed for the GFP image. When indicated, FLAG-tagged GCP16 constructs were co-transfected with GFP-VSV-Gts045 plasmid into COS-1 cells. Protein transport was also analyzed by pulse-labeling experiments, for which experiments a mixture of dipeptidyl peptidase IV (dDPPIV) was generated by the substitution of Leu by Ala at position 28 of the wild type DPPIV (37). COS-1 cells were transfected with the pSG5 expression vector containing the sDPPIV cDNA and FLAG-tagged GCP16 cDNA constructs (wild type or mutant). After being cultured for 20 h, the cells (2 × 10^6 cells/35-mm dish) were pulse-labeled for 10 min with [35S]methionine (370 Kbp/dish). At the indicated times of chase, the cell lysates and medium were harvested and subjected to immunoprecipitation of DPPIV (38). The immunoprecipitates were analyzed by SDS-PAGE (7.5% gels) and fluorography. The fluorograms were scanned for quantification of DPPIV by the LAS-1000 Luminescent Image Analyzer (Fuji Photo Film Co., Tokyo, Japan).

Novel Acylated Golgi Protein, GCP16

1 The abbreviations used are: GST, glutathione S-transferase; BFA, brefeldin A; DPPIV, dipeptidyl peptidase IV; ΔDPPIV, secreted form of DPPIV; GFP, green fluorescent protein; PNS, post-nuclear supernatant; VSV-G, G protein of vesicular stomatitis virus; VSV-Gts045, temperature-sensitive VSV-G.
RESULTS

Golgi-targeting Domains of GCP170—It is predicted that GCP170 contains an extremely long α-helical domain that likely forms a coiled-coil following the NH₂-terminal non-coil domain (Fig. 1A, top panel). Such a characteristic secondary structure suggests that the protein is composed of a globular head and a stalk/tail. We first examined the role of the head and coiled-coil domains of GCP170 for the Golgi targeting/localization. When the full-length and three truncated forms (D1, D2, and D3), which were FLAG-tagged, were transfected into HeLa cells, all of the expressed proteins were co-localized with the Golgi marker giantin (Fig. 1A). The result suggests that GCP170 may contain at least two domains for the Golgi targeting: one in the NH₂-terminal region covered by D1 and the other in the COOH-terminal region covered by D2 and D3. Both D2 and D3, however, contain a large part of the coiled-coil domain that may be easily able to form a dimer with the endogenous GCP170 and allow the mutants to reach the Golgi, even if they do not have their own targeting signal. Thus, it is likely that the Golgi-targeting signal is contained in the NH₂-terminal domain D1.

We then examined the Golgi-targeting domain of D1 in more detail (Fig. 1B). When five mutants with narrower deleted domains were constructed from D1 and transfected into cells, only a mutant lacking the H2 domain (positions 137–237) failed to localize to the Golgi region (Fig. 1B, panel g), although all of the other mutants showed essentially the same Golgi localization profile as that of the parental D1. These results indicate that the Golgi-targeting signal of GCP170 is contained in residue positions 137–237 of the NH₂-terminal region, which is a domain similar to that reported by Hicks and Machamer (39).

Isolation of Clone 1E2 Encoding a GCP170-interacting Protein—Because the NH₂-terminal region (137–237) of GCP170 was found to contain the Golgi-targeting domain, we tried to identify a protein that interacts with GCP170. We used the yeast two-hybrid system, in which the NH₂-terminal region (positions 137–237) of GCP170 was inserted into the LexA fusion vector and used as bait. We screened the HeLa cell cDNA library in the pB42AD fusion vector and obtained a 2.0-kilobase pair cDNA clone, 1E2. The 1E2 product was found to interact specifically with the NH₂-terminal region (137–237) of GCP170 (Fig. 2A). 1E2 contained a full-length cDNA includ-
ing the complete open reading frame, which encodes a protein of 137 amino acid residues with a calculated mass of 16 kDa (Fig. 2B). We designated the protein GCP16 (for the Golgi complex-associated protein of 16 kDa). The coiled-coil probability analysis showed that GCP16 contained a short coiled-coil domain (positions 30–60) (Fig. 2C, top panel), whereas the protein did not contain a sequence characteristic for the NH₂-terminal signal sequence nor a hydrophobic domain sufficient to span a membrane in other regions, as shown by a hydropathy analysis (Fig. 2C). The interaction between GCP16 and GCP170 detected by yeast two-hybrid was also confirmed by co-immunoprecipitation of the two proteins from Triton X-100 extracts of HeLa cells. The immunoblot analysis showed that native GCP16 and GCP170 interact in cells.

Ubiquitous Expression of GCP16—The GCP16 mRNA level was examined by Northern blotting of poly(A)/H11001 RNA from various human tissues with 32P-labeled 1E2 cDNA. A single 2.0-kilobase message was detected in all of the tissues examined and was especially abundant in testis, ovary, and spleen, indicating that GCP16 is ubiquitously expressed (Fig. 3A). A similarity search revealed that there are GCP16 homologues in the mouse (Mus musculus), nematode (Caenorhabditis elegans), and fruit fly (Drosophila melanogaster) (Fig. 3B), although these homologues have not been identified at the protein level. No related proteins, however, were found in the yeast (Saccharomyces cerevisiae) and plants.

Expression and Intracellular Distribution of GCP16—For expression of GCP16, the GCP16 cDNA with or without FLAG tag was prepared and transfected into HeLa or COS-1 cells. The expressed GCP16 was detected by immunofluorescence staining with anti-FLAG or anti-GCP16 antibodies. The exogenously introduced protein was localized to a juxtanuclear region corresponding to the Golgi complex, where giantin and GCP170 were co-localized (Fig. 4A). The intracellular localization of GCP16 was examined in more detail by immunoelectron microscopy. As shown in Fig. 4C, immunoperoxidase products were detected on the characteristic stack and related structures of the Golgi complex. We then examined the biosynthesis of GCP16 in cells that were incubated with [35S]methionine. In control cells without transfection, the endogenous GCP16 was detected as a weak band of 16 kDa in SDS-PAGE (Fig. 4B, lanes 1 and 2), whereas much heavily labeled bands of 16 and 17 kDa were detected in cells transfected with the GCP16 and FLAG-GCP16 cDNA, respectively (Fig. 4B, lanes 3 and 4), indicating the overexpression of each protein.

It is well known that the Golgi complex is disrupted by...
treatment with brefeldin A (BFA) and that a differential response to the drug is often observed depending on Golgi components examined (15, 36, 40–42). As previously reported (15), it was confirmed that GCP170 was rapidly released from the Golgi complex, detected as early as 2 min after treatment with BFA (Fig. 5, panel d), and completely dispersed into the cytoplasm within 10 min (Fig. 5, panel f). In contrast, GCP16 remained associated with the Golgi at 2 min after the treatment (Fig. 5, panel c) and then gradually dispersed into the cytoplasm (possibly redistributed into the endoplasmic reticulum). Such a behavior of GCP16 is quite similar to that of giantin and other integral membrane components of the Golgi (15, 41).

Characterization of Membrane Association of GCP16—A postnuclear fraction (PNS) of HeLa cells was subfractionated into cytosol, total membranes, and Golgi-enriched fractions, which were analyzed by Western blotting. Anti-GCP16 antibodies recognized a 16-kDa protein in the total membranes and Golgi fractions as well as in the PNS but not in the cytosol fraction (Fig. 6, lanes 1–4). The protein was never released from the membranes by treatment with 1 M NaCl or 0.1 M Na2CO3 and only partially extracted from the membranes with 1% Triton X-100 (Fig. 6, lanes 5–10). Finally, the protein was completely recovered into the detergent phase when subjected to phase separation with Triton X-114 (Fig. 6, lanes 11 and 12). These results indicate that GCP16 is associated with the membrane as tightly as an integral membrane protein. This is in contrast to the properties of GCP170 (15); GCP170 was also present abundantly in the cytosol, and the membrane-associated protein was easily released from the membrane with 1 M NaCl and 0.1 M Na2CO3, reflecting a typical peripheral membrane protein.

Despite the lack of a transmembrane domain, GCP16 behaved as an integral membrane protein, suggesting the possibility that the protein is modified by acylation with fatty acids. To test this possibility, we carried out metabolic labeling of GCP16 with [3H]palmitic acid in cells that were transfected with GCP16. The labeled fatty acid was efficiently incorporated into the protein (Fig. 6B, lane 1 of upper panel). It is likely that the acylation occurs at cysteine residues, which are contained in GCP16 in four positions: 24, 69, 72, and 81 (Figs. 2B and 3B). To examine which Cys residue is modified by acylation, we prepared GCP16 mutants with substitutions of Cys by Ala. Any single substitution of Cys to Ala did not completely abolish the incorporation of [3H]palmitic acid, although a significant decrease of the labeling was detected in mutants with C69A or

![Fig. 3. Ubiquitous expression and homology of GCP16. A, poly(A)⁺ RNA (2 μg/lane) from different human tissues was separated by SDS-PAGE and probed with ³²P-labeled GCP16 cDNA (about 2 kilobase pairs). β-Actin mRNA levels are also shown as a control. B, alignment of GCP16 with the predicted homologues in mouse (accession number AB041568.1), Drosophila (D.mel; accession number AB041568.1), and nematode (C.ele; accession number Z99281.1). Identical residues are boxed, and gaps introduced into the alignment are indicated by hyphens.](image_url)
Cys72 → Ala (Fig. 6B, lanes 2–5 of upper panel). When the two Cys residues (C69A/C72A) or three residues (C69A/C72A/ C81A) were substituted by Ala, the protein was no longer labeled at all (Fig. 6B, lanes 6 and 7). A labeling experiment with [35S]methionine confirmed that all of the mutants were expressed at the same level as the wild type GCP16 (Fig. 6B, lower panel).

We then examined the intracellular localization of the mutants by immunofluorescence microscopy. HeLa cells transfected with the FLAG-tagged mutants of GCP16 were double-stained with anti-FLAG (panel a) and anti-giantin antibodies (panel b), as described in the legend to Fig. 1. COS-1 cells were incubated with rabbit anti-GCP16 antibody to detect the endogenous GCP16 (panels c and e) and co-incubated with guinea pig anti-giantin antibodies (panel d) or guinea pig anti-GCP170 antibodies (panel f). The cells were then immunostained with Alexa488-conjugated anti-rabbit IgG (panels c and e) or with Alexa594-conjugated anti-guinea pig IgG (panels d and f). B, control HeLa (lane 1) and COS-1 cells (lane 2) and cells transfected with GCP16 (lane 3) or FLAG-tagged GCP16 (lane 4) were labeled with [35S]methionine for 14 h. The cell lysates were prepared and subjected to immunoprecipitation with anti-GCP16 (lanes 1–3) or anti-FLAG antibody (lane 4). The immunoprecipitates were analyzed by SDS-PAGE (15% gel) and fluorography. C, COS-1 cells transfected with the FLAG-tagged GCP16 were fixed with Zamboni’s fixative, permethilized with saponin, and incubated with anti-FLAG. The cells were then incubated with peroxidase-conjugated secondary antibody and processed for electron microscopy. N, nucleus; M, mitochondria; GC, Golgi complex.

![Fig. 4. Expression and intracellular localization of GCP16.](image)

![Fig. 5. Response of GCP16 to BFA treatment.](image)
ment with 0.1 M Na₂CO₃ (Fig. 6D), in contrast to the wild type (Fig. 6A). Taken together, these results indicate that GCP16 is acylated with palmitic acid at the cysteine residues at positions 69 and 72, through which the protein is anchored to the membrane, accounting for its nature as the integral membrane protein.

Interaction of GCP170 with the Acylated Form of GCP16—The acylation of GCP16 was found to be prerequisite for its membrane association and Golgi localization. It is of interest to know whether the interaction with GCP170 is also affected by the acylation of GCP16. The interaction of GCP16 and GCP170 was examined by co-immunoprecipitation of the two proteins from Triton X-100 extracts of cells that had been transfected with FLAG-tagged wild type GCP16 or nonacylated mutant (C69A/C72A). GCP170 was detected in the anti-FLAG immunoprecipitate from the cells expressing the wild type GCP16 but not in the precipitate from the cells expressing the nonacylated form (Fig. 7, lanes 5 and 6). The result suggests that GCP170 interacts with GCP16 tightly associated with the Golgi membrane by acylation but not with the nonacylated form in the cytosol.

Involvement of GCP16 in Protein Transport from the Golgi to the Cell Surface—To address the function of GCP16, we transfected the FLAG-tagged wild type GCP16 or the nonacylated mutant (C69A/C72A) into COS-1 cells and examined the effect of their overexpression on the Golgi structure and protein transport. Protein transport in the secretory pathway was examined using a temperature-sensitive and GFP-tagged VSV-G protein (GFP-VSV-G/ts045). In control cells, the VSV-G protein was retained in the endoplasmic reticulum at the nonpermissive temperature (39.5 °C) and transported to the cell surface via the Golgi complex at the permissive temperature (32 °C), as revealed by the GFP image (Fig. 8A, panels a and b). In cells overexpressing the wild type GCP16 (Fig. 8A, panels c and e), the VSV-G protein was transported to and retained in the Golgi without being transported to the cell surface even at the per-
missive temperature 32 °C (Fig. 8A, panel j). Thus, it is likely that the overexpression of the wild type caused an inhibitory effect on the protein exit from the Golgi and/or transport to the cell surface. This is in contrast to the overexpression of the mutant GCP16 (Fig. 8A, panel i), which allowed the VSV-G protein to be transported to the cell surface (Fig. 8A, panel j). On the other hand, when the wild type and mutant GCP16 were expressed even at a high level, no significant change was observed in the Golgi structure, as monitored by localization of the endogenous giantin and GCP170 (Fig. 8B, panels a–h).

We also examined the effect of overexpression of wild type GCP16 on secretion of sDPPIV (Fig. 9). Pulse-chase experiments showed that in control cells, sDPPIV started to be secreted at 30 min of chase, followed by rapid increase in the medium. No significant change was observed in a time course of secretion from cells expressing the nonacylated mutant GCP16 (C69A/C72A). In cells overexpressing the wild type GCP16, however, the secretion of sDPPIV was significantly retarded and remained at a low level as compared with that of the control and mutant-expressing cells (Fig. 9). When the chase time was prolonged up to 90 min, however, the secretion of sDPPIV in cells transfected with the wild type GCP16 reached 57% of the total, which was almost the same level as that in mock transfected and mutant-transfected cells (58 and 62%, respectively). Taken together, these results (Figs. 8 and 9) suggest that GCP16 is involved in protein transport from the Golgi to the cell surface, although details of the mechanism remain to be determined.

**Differential Domains of GCP170 for GCP16 Binding and Golgi Targeting**—We initially postulated that GCP16 may function as a receptor for binding of GCP170 to the Golgi membrane. As described above, however, the overexpression of either the wild type or the nonacylated mutant GCP16 caused no significant effect on the Golgi localization of GCP170 (Fig. 8). This raised the possibility that GCP170 has another site for the Golgi targeting distinct from the GCP16-binding site. We therefore prepared another set of mutants (Fig. 10A) derived from the mutant D1 that exerted the function in both Golgi targeting and GCP16 binding (Figs. 1B and 2A). Three mutants M1, M2, and M4 were localized to the Golgi, whereas mutant GCP16 (C69<sup>72→A</sup>) was not.
M3 lacking the sequence of positions 170–190 was dispersed into the cytoplasm (Fig. 10B, panel d). When the GCP16 binding activity was examined by the yeast two-hybrid assay, only the M2 with deletion of positions 137–169 exerted no activity for the GCP16 binding (Fig. 10C). The results indicate that GCP170 contains the distinctly different sites for the Golgi localization and the GCP16 binding in consecutive positions.

DISCUSSION

In the present study, using the yeast two-hybrid screening system with the Golgi localization domain of GCP170 as bait, we isolated the novel 16-kDa protein (GCP16) that is localized to the Golgi complex. Data base searches show that GCP16 has 96.6% identity in amino acid sequence to an unannotated gene product of mouse (GenBank™ accession number AB041568.1) and a significant similarity to those of D. melanogaster (accession number AE003756.10) and C. elegans (accession number Z99281.1) with identities of 53.7 and 32.8%, respectively. No homologous sequence, however, is found in yeast and plants, indicating that GCP16 is absent in yeast and plants, as reported for GCP60 and giantin (35). This is unusual because most vesicular transport factors and Golgi components are evolutionarily conserved (43). It is possible that GCP16, GCP60, and giantin are components of a system that might represent one of the additional levels of complexity intrinsic to higher eukaryotic cells.

Although having no hydrophobic domain that participates in membrane localization, GCP16 was tightly associated with the Golgi membrane, not released from the membrane by treatment with 0.1 M Na₂CO₃ as well as 1 M NaCl, indicating its nature as the integral membrane protein. Indeed, the protein was found to be acylated with palmitic acid at positions Cys⁶⁹ and Cys⁷₂. Of four Cys residues contained in GCP16, both residues are well conserved in the other homologues so far reported, although Cys⁶⁹ is substituted by Val in C. elegans. In contrast to GCP170, more than a half of which was present in the cytosol, GCP16 was completely recovered in the membrane fraction by cell fractionation. This fact indicates that all of the molecules of GCP16 are acylated and directly associated with the membrane, suggesting that GCP16 interacts with GCP170 on the membrane, not in the cytosol. The difference in membrane association of the two proteins accounts for the different response to BFA; the peripheral protein GCP170 is rapidly dissociated from the membrane into the cytosol, whereas GCP16 is redistributed to the endoplasmic reticulum in the same manner as other integral membrane proteins along with the disruption of the Golgi (41, 44). The acylation of Golgi and other membrane proteins are thought to be one of the post-translational modifications occurring in the Golgi complex (45). In addition, in a cell-free vesicular transport assay, acyl-CoA esters including palmitoyl-CoA were shown to be essential for either the budding or the fusion process (46). Palmitoyl-CoA also serves as a substrate for the acylation of Golgi proteins including GCP16.

Our data demonstrate that GCP16 is anchored to the Golgi membrane at the middle portion of the molecule, because the Cys⁶⁹ and Cys⁷₂ residues are acylated. Adjacent to the mem-

![Figure 9](image-url)

**Fig. 9. Effect of overexpression of wild type and mutant GCP16 on secretion.** A, COS-1 cells were transfected with plasmids for the following proteins; sDPPIV alone (control); sDPPIV and FLAG-tagged wild type GCP16 (WT); and sDPPIV and FLAG-tagged mutant GCP16 (C⁶⁹, ⁷₂→A). The cells were pulse-labeled with [³⁵S]methionine for 10 min and chased. At the indicated times of chase, the cell lysates and medium were prepared and used for immunoprecipitation of DPPIV. The immunoprecipitates were analyzed by SDS-PAGE (7.5% gels) and fluorography. B, fluorograms shown in A were scanned for quantification of DPPIV as described under “Experimental Procedures.” The values are expressed as percentages of DPPIV secreted into the medium by taking the total (cell and medium) amount of DPPIV as 100% at each time point (means ± S.D. of three separate experiments).
branched-anchoring domain, the protein contains a short coiled-coil domain, which could serve as a partner that interacts with GCP16, as observed in other proteins including GCP60 (35). This is explained by the additional finding that GCP170 contains the subdomain for Golgi localization independent from the presence of nonacylated protein, the wild type GCP16 endogenously present on the membrane functions normally for interacting with GCP170. In contrast, the overexpression of the wild type GCP16 caused the inhibitory effect on the transport of VSV-G protein from the Golgi to the cell surface. This is considered to be a dominant-negative effect of the overexpression of GCP16, as observed in other proteins including GCP60 (35).

Recent morphological observations have shown that the Golgi complex is fragmented during apoptosis (47, 48), although the molecular mechanisms underlying fragmentation are largely unknown. It is of interest to note that in apoptotic cells, the NH2-terminal head domain of GCP170 was cleaved by caspases, whereas a mutant form of GCP170 lacking the caspase-2 cleavage site appeared to delay the Golgi fragmentation (26). In addition, Lane et al. (49) most recently reported that the stacking protein GRASP65 was also cleaved by caspase-3 during apoptosis, resulting in Golgi fragmentation. These observations strongly suggest that GCP170 and GRASP65 of the golgin family are indeed required for the assembly and maintenance of the Golgi structure. Caspase-2 and caspase-3 were shown to cleave GCP170 at the COOH side of Asp at positions 59, 139, and 311 (26), all of which are included in the head domain. In particular, it should be emphasized that the Asp139 is found in the GCP16-binding subdomain, supporting the possibility that GCP16 is involved in maintaining the function and structure of Golgi complex. Taken together, the present study demonstrates that GCP16 is tightly associated with the Golgi membrane by its acylation, interacts with GCP170, and is possibly involved in maintaining the structure and function of the Golgi complex, although further direct evidence is required to establish its involvement in the Golgi structure and function.

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Fig. 10. Subdomains of GCP170 for GCP16 binding and Golgi localization. A, a schematic representation of deletion mutants (M1–M4) derived from D1 (see Fig. 1). Each mutant and D1 were FLAG-tagged. B, HeLa cells expressing the FLAG-tagged mutants of GCP170 were immunostained with anti-FLAG M2 antibody. The cells expressing the mutant M3 (panel d) were co-immunostained with anti-giantin antibody (panel e). C, yeast two-hybrid analysis was performed with four pLexA constructs containing the indicated mutants M1–M4 (used as the bait) and pB42AD-GCP16 (as the prey). β-Galactosidase activity obtained with pLexAM1 is taken as 1, and relative values of enzyme activity with the other bait constructs are shown as the means ± S.D. (n = 3).
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