An Essential Cytoplasmic Domain for the Golgi Localization of Coiled-coil Proteins with a COOH-terminal Membrane Anchor*

Yoshio Misumi, Miwa Sohda, Akiko Tashiro, Hiroshi Sato†, and Yukio Ikehara§

From the Department of Biochemistry, Fukuoka University School of Medicine, Jonan-ku, Fukuoka 814-0180, Japan, and the †Department of Virology and Molecular Oncology, Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Japan

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Proof and Correspondence to:
Dr. Yukio Ikehara
Department of Biochemistry, Fukuoka University School of Medicine, Jonan-ku, Fukuoka 814-0180, Japan
TEL: +81-92-801-1011 Ext. 3250
FAX: +81-92-864-3865
E-mail: yikehara@fukuoka-u.ac.jp

SUMMARY
Giantin is a resident Golgi protein which has an extremely long cytoplasmic domain (about 370 kDa) and is anchored to the Golgi membrane by the COOH-terminal hydrophobic domain (CMD) with no luminal extension. We examined the essential domain of giantin required for the Golgi localization by mutational analysis. The Golgi localization of giantin was not affected by the deletion of its CMD or by substitution with the CMD of syntaxin-2, a plasma membrane protein. The giantin CMD fused to the cytoplasmic domain of syntaxin-2 could not retain the chimera in the Golgi apparatus. Sequential deletion analysis showed that the COOH-terminal sequence (positions 3059-3161) adjacent to the CMD was the essential domain required for the Golgi localization of giantin. We also examined two other Golgi-resident proteins, golgin-84 and syntaxin-5, with a similar membrane topology as giantin. It was confirmed that the cytoplasmic domain of about 100 residues adjacent to the CMD was required for their Golgi localization. Taken together, these results suggest that the COOH-terminally anchored Golgi proteins with long cytoplasmic extensions have the Golgi localization signal(s) in the cytoplasmic sequence adjacent to the CMD. This is in contrast to previous observations that a transmembrane domain is required for the Golgi localization by other Golgi proteins transported from the endoplasmic reticulum.

INTRODUCTION
The Golgi complex plays a crucial role in intracellular transport and modification of exportable proteins and lysozomal proteins. It is organized by three functionally distinct subcompartments, the cis-Golgi network (CGN)\(^1\), the Golgi stack, and the trans-Golgi network (TGN) (1). During transport through the Golgi complex, exportable proteins are sequentially modified by processing of N- and O-linked oligosaccharides (2), proteolytic cleavage (3), sulfation (4) and so on. These modifications are accomplished by enzymes localizing on the lumenal side of the specified subcompartments.

Localization of these Golgi enzymes have been thought to be signal-dependent. Type II membrane proteins with a short cytoplasmic tail, including glycosyltransferases, contain the Golgi retention signals in and around their transmembrane domains (TMDs) (ref. 5 for review). Two models have been proposed for how the TMDs of Golgi enzymes could participate in their Golgi retention; the oligomerization or kin-recognition model (6, 7) and the lipid-sorting or bilayer-thickness model (5, 8). In contrast, the Golgi-localization signals of type I membrane proteins are likely contained in the cytoplasmic tail, as demonstrated for TGN38 (9) and the pro-protein processing enzyme furin (10, 11), both of which are localized to the TGN by tyrosine-containing motifs. These proteins, irrespective of type I or II, have a common feature in the membrane topology that the majority of their protein masses protrude from the membrane into the lumen.

Giantin is an integral Golgi-resident protein (12) which has no cleavable signal sequence at the NH\(_2\) terminus but contains a single hydrophobic sequence at the COOH terminus that could participate in membrane localization (13-15). Thus, it is believed that giantin is cytoplasmically oriented and anchored to the Golgi membrane by the COOH-terminal hydrophobic domain (CMD for COOH-terminal membrane-anchoring domain). Although containing 24 amino acid residues enough for spanning the lipid bilayer of membranes, the CMD is not considered to be a typical TMD, because giantin has no lumenal-side sequence following the CMD. Linstedt \textit{et al}. (16) recently demonstrated that newly synthesized giantin was first inserted into the endoplasmic reticulum (ER) membrane and then transported to the Golgi complex in the same manner as well known for Golgi-associated membrane proteins such as glycosyltransferases. They also suggested that attachment of the CMD to the ER was
essential for the Golgi localization of giantin.

The yeast Sed5p is a t-SNARE which is primarily localized to the Golgi and involved in vesicular transport between the ER and the Golgi complex (17). Syntaxin-2 which is also a t-SNARE, however, is localized to the plasma membrane (18). Both Sed5p and syntaxin-2 are COOH-terminally anchored proteins with the same membrane topology as giantin. The different localization of these proteins, despite the same length of their CMDs, raises the possibility that the Golgi localization signal of giantin and Sed5p is contained in a cytoplasmic domain rather than the CMD. In fact, it was reported that Sed5p requires a cytoplasmic domain in addition to the CMD for targeting to the Golgi (19).

In the present study we carried out mutational analysis of giantin to determine a domain responsible for its targeting and retention to the Golgi, demonstrating that the cytoplasmic domain of about 100 residues adjacent to the CMD is essential for the localization of giantin to the Golgi complex.
Preparation of Species-specific Anti-giantin Antibodies — Giantin contains substantially different amino acid sequences in several domains between rat and human, which include domains R1 (positions 547-617), R2 (1178-1239), and R3 (1668-1764) for rat and H1 (positions 556-625), H2 (1188-1260) and H3 (1689-1784) for human. cDNA constructs encoding these domains were fused with that of glutathione S-transferase (GST). The GST-fusion antigens were expressed in and purified from bacteria, followed by immunization in rabbits as described previously (20). Among the antibodies raised, anti-H1 antibody was found to be specific for giantin of human and monkey, while anti-R2 antibody was specific for the rat antigen without cross-reactivity to the human and monkey antigens, when examined by immunofluorescence microscopy and immunoblotting.

Construction of Expression Plasmids — The cDNA encoding rat giantin/GCP364 (GW) (15) was inserted into the EcoRI site of pSG5 expression vector. The cDNA encoding rat syntaxin-2 (S2W) was obtained by the method of RT-PCR using rat brain mRNA as a template and synthetic oligonucleotides 5’-ATGATATCATGCGGGACCGGCTGCCGGA-3’ and 5’-TACTCGAGGAGGCAAGCACCACGCGCCA-3’ (18). The PCR product was ligated to downstream of the sequence encoding the Met-FLAG” tag for recognition by the monoclonal antibody M2 (21). A mutant of giantin lacking the CMD (GΔM) was generated by introducing the termination codon TGA into the position Arg3163 (CGC). For construction of the chimeric proteins G-SM (the giantin cytoplasmic domain fused with the CMD of syntaxin2) and S2-GM (the syntaxin cytoplasmic domain with the giantin CMD), appropriate restriction endonuclease sites were generated in the giantin cDNA (BbrPI at nucleotide number 9780) and in the syntaxin-2 cDNA (SmaI at nucleotide number 798) by site-directed mutagenesis with synthetic oligonucleotides 5’-TTCCCGGACCACGGCTGGATAATTGCTGCTGTGCTGT-3’ for giantin, and 5’-AGACGGAAAACCGTGGATAATTGCTGCTGCTGTGCT-3’ and 5’-ACGGAACCGGATAATTGCTGCTGCTGCTGTGCT-3’ for syntaxin-2. The mutated cDNAs were digested with BbrPI or SmaI and reciprocally fused with proper orientations. Amino acid residues at joining sites were re-mutated for having the correct sequence. The COOH-terminally deleted mutants D1 to D5 were prepared by introducing the termination codon into appropriate sites of the GW plasmid. The D5-SM chimera was constructed by using G-SM as
a template with a synthetic oligonucleotide 5’-CAGCTGCCTCCAGCCCAGCAACCCG-CTGGATAATTGCTGC-3’. For construction of NH₂-terminally deleted mutants (D6-D9, D6ΔM, D9ΔM, D9-SM, I-SM, II-SM and III-SM), proper restriction endonuclease sites protruding the blunt end were generated in appropriate sites of GW, GΔM and G-SM, and the constructs were digested with the proper restriction endonucleases. The mutants were ligated in frame to the FLAG-tag sequence. All the constructs prepared by site-directed mutagenesis were verified by sequencing (21).

The cDNA encoding golgin-84 was obtained by the method of RT-PCR from HeLa cell mRNA as a template using synthetic oligonucleotides 5’-GAATTCATGTCTTGGTTTGTTGATCTTGC-3’ and 5’-GAATTCATTTGCCATATGGTTGGTCGTGG-3’ (22). The human syntaxin-5 cDNA was obtained by using synthetic oligonucleotides 5’-ATGGATCCATGTC-CTGCCGGGATCGG-3’ and 5’-ATGTAAACAGGCTCAGTGCAACACTGG-3’ (18). Truncated mutants of golgin-84 and syntaxin-5 were constructed by introducing proper restriction endonuclease sites protruding blunt end with similar treatments as described above. All the cDNA constructs were tagged with FLAG and inserted into the expression vector pSG5.

Cell Culture and Transfection — COS-1 cells and HeLa cells were cultured as described previously (23). Each plasmid (10 µg) was transfected into COS-1 cells with the Lipofectin reagent (21). At 20 h after transfection, cells were incubated at 37 ÚC with or without cycloheximide (50 µg/ml) for 5 h and fixed for immunofluorescence microscopy. For immunoblotting analysis, 20 µg of each plasmid was transfected into cells using electroporation apparatus (21) and transfected cells were cultured for 20 h as above.

Immunofluorescence Microscopy and Immunoblotting Immunofluorescence microscopy was carried out as described before (20). Primary antibodies used were as follows; rabbit antiserum for human giantin (anti-H1) and for rat giantin (anti-R2) (dilution factor for each, 1:50), mouse monoclonal anti-FLAG M2 (1:100), and rabbit anti-β-COP IgG (15 µg/ml). Rhodamine-conjugated goat anti-rabbit IgG (1:50) or fluorescent isothio-cyanate-conjugated anti-mouse IgG (1:50) were used as the secondary antibodies. For immunoblotting, proteins were separated by SDS-PAGE (5% or 10% gels) followed by immunoblotting with the
indicated antibodies (at dilution 1:1000 of each antibody). The immunoreactive proteins were visualized using ECL kit (21). In some experiments the immunoblotts were scanned with a GT-8500 scanner (Epson, Inc., Tokyo) and analyzed by Adobe photoshop (Adobe Photosystems, Columbia, MD) and NIH Image software.

Preparation of a Golgi-enriched Fraction — Transfected cells were harvested with a cell scraper, washed twice with Dulbecco’s phosphate-buffered saline, and homogenized by 20 passages through a 25-gauge needle in 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose and a protease inhibitor mixture (21). The homogenate was centrifuged at 600 x g for 5 min, the postnuclear supernatant (PNS) was separated into a membrane fraction and a soluble fraction by centrifugation at 105,000 x g for 1 h. The Golgi fraction was prepared by the method of Balch et al. (24). The PNS fraction of transfected cells was mixed with PNS from HeLa S3 cells (approx. 8 mg protein), adjusted to 1.4 M sucrose and loaded on 0.8 M and 1.2 M sucrose layers. After centrifugation at 73,000 x g for 2.5 h, a turbid band floated at the 0.8 M/1.2 M sucrose interface was harvested as the Golgi-enriched fraction. The Golgi fraction was found to contain UDP-galactosyltransferase (55%), acid phosphatase (0.8%), 5’-nucleotidase (4.4%), calnexin (5.7%) and protein (2%), as compared with those of the PNS fraction (100% each).

Other Analytical Methods — Enzyme assays for UDP-galactosyltransferase (25), acid phosphatase (26) and 5’-nucleotidase (27) were performed by the established methods.
RESULTS

Giantin Cytoplasmic Domain is Sufficient for the Golgi Localization — To examine the role of the cytoplasmic domain and the C-terminal membrane-anchoring domain (CMD) for targeting and localization of giantin to the Golgi apparatus, we prepared cDNA constructs of rat giantin with or without the CMD, syntaxin-2 and their chimeras (Fig. 1A), which were transfected into COS-1 cells. The expressed proteins were analyzed by immunoblotting (Fig. 1B) and immunofluorescence microscopy (Fig. 1C). The expressed proteins were detected by anti-R2 antibody (specific for rat giantin) or anti-FLAG. When cell homogenates were separated into membranes and cytosol, all the expressed proteins including giantin without CMD (GΔM) were recovered in the membrane fraction (Fig. 1B). Immuno-fluorescence microscopy revealed that the mutant GΔM was localized to the Golgi region, as observed for the wild-type giantin GW (Fig. 1C).

Syntaxin-2 is also an integral membrane protein with the same membrane topology as giantin but is localized to the plasma membrane (Fig. 1C, S2W). To confirm the role of cytoplasmic domain of giantin for the Golgi localization, we examined the intracellular distribution of chimeric proteins constructed by giantin and syntaxin-2. A chimera of the giantin cytoplasmic domain with the CMD of syntaxin-2 was localized to the Golgi region (Fig. 1C, G-SM), whereas another chimera of the syntaxin-2 cytoplasmic domain with the giantin CMD was expressed on the cell surface (Fig. 1C, S2-GM).

To reinforce these observations, we isolated a Golgi-enriched fraction from the transfected cells and analyzed the expressed proteins by immunoblotting (Fig. 2A). When compared with those of postnuclear fractions (PNS), the giantin mutants without the CMD or substituted with the syntaxin-2 CMD were recovered in the Golgi-enriched fraction at the same level as the wild-type giantin (Fig. 2B). These were in contrast with the recovery of the wild-type syntaxin-2, although the syntaxin-2 mutant replaced by the giantin CMD was significantly recovered in the Golgi fraction.

It has been suggested that giantin forms a disulfide-linked homodimeric conformation in vivo and associates with other membrane proteins or cytoskeletal proteins (12, 16). This raises the possibility that giantin without the CMD expressed in COS-1 cells may form dimers with
endogenous one and be co-transported to the Golgi apparatus. To test this possibility, cell lysates were subjected to immunoprecipitation with anti-R2 antibody (specific for the exogenously introduced giantin), and the resultant immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-R2 or with anti-H1 (specific for the endogenous giantin). When blotting was probed with anti-R2, the mutants GΔM and G-SM as well as the wild-type GW were detected in the transfected cells (Fig. 2C, lanes 2-4). In contrast, when being probed with anti-H1, no endogenous giantin was detected (Fig. 2C, lanes 6-8). The endogenous giantin was detected with anti-H1 only when it was used for both immunoprecipitation and immunoblotting (Fig. 2C, lane 5). Thus, it is unlikely that the exogenously introduced giantin molecules are localized to the Golgi by association with the endogenous giantin. Taken together, these results suggest that the cytoplasmic domain itself of giantin has a signal for its targeting and localization to the Golgi apparatus.

**Essential Domain of Giantin required for the Golgi Localization** — The cytoplasmic domain of giantin required for the Golgi localization was examined by deletion analysis, for which a series of COOH-terminally-truncated mutants (D1 to D5) were constructed and expressed in COS-1 cells (Fig. 3A). When cell homogenates were separated into membranes and cytosol, the smallest NH2-terminal fragment D1 was recovered exclusively in the cytosolic fraction (Fig. 3B, lanes 1 and 2). The amount of the mutant proteins associated with membranes was increased as they contained longer extensions to the COOH terminus (Fig. 3B, lanes 3-8). The longest mutant D5, lacking a small COOH-terminal segment, was completely recovered in the membrane fraction as observed for D5-SM with the syntaxin-2 CMD (Fig. 3B, lanes 9-12). When cells were examined by immuno-fluorescence microscopy, three mutants D1 to D3 were detected throughout the whole cell including the cell periphery, while the membrane-bound forms D4 and D5 showed a profile lacking the cell periphery staining, clearly different from that of the above three mutants D1 to D3 (Fig. 3C). D4 and D5 appeared to be localized mostly to the endoplasmic reticulum (ER), because the two mutants were co-localized with the ER marker calnexin (data not shown). To exclude the possibility that no localization of D5 to the Golgi is due to the lack of CMD, we examined the cellular distribution of D5-SM which contained the CMD of syntaxin-2 at the COOH
terminus (Fig. 3A, D5-SM), demonstrating that the mutant also showed a typical ER staining pattern (Fig. 3C). These results indicate that the Golgi targeting signal is present in the COOH-terminal segment beyond the amino-acid residue 2803. This was confirmed by analysis of another set of NH2-terminally-truncated mutants with or without the CMD (Fig. 4A). All the expressed proteins were found to be recovered in the membrane fraction (Fig. 4B) and localized to the Golgi region (Fig. 4C), irrespective of the presence or absence of the CMD.

The COOH-terminal domain was further characterized for the Golgi targeting and localization, for which three mutants D9 (2804-3187 amino-acid segment of giantin), D9ΔM (D9 without CMD) and D9-SM (substituted with syntaxin-2 CMD) were constructed (Fig. 5A). Both the expressed D9 and D9-SM were detected in the Golgi region, co-localizing with the endogenous giantin (Fig. 5B). The mutant D9ΔM without the CMD, however, showed a diffuse distribution throughout the cell. This is in contrast with the clear Golgi localization of the longer segment D8ΔM (Fig. 4C). Possible reasons for the different behavior of D8ΔM and D9ΔM will be discussed later.

As shown in Fig. 3C and Fig. 4B, the expressed mutant proteins were localized to the compact spot-like structure in the juxtanuclear region, which is a characteristic feature of the Golgi complex found in COS-1 cells (15, 28). Recently, however, it has been shown that a similar compact structure, the aggresome, is also formed in a juxtanuclear region by misfolded proteins (29, 30). To distinguish true Golgi localization from the formation of aggregates, we carried out a more careful co-localization analysis with endogenous giantin in HeLa cells where the Golgi structure is less compact than that in COS-1 cells. Both the mutants D8M and D9-SM expressed were well co-localized with endogenous giantin in perinuclear regions corresponding to the Golgi (Fig. 6A and B, each a and b). When the cells were incubated with brefeldin A for 30 min, the mutants as well as endogenous giantin were dispersed into the cytoplasm (panels c and d). Further incubation of the cells without the drug allowed each protein to re-localize to the perinuclear regions (panels e and f). Taken together, these results support the conclusion that the expressed mutants are localized to the Golgi, not being clustered into the aggresome.
The COOH-terminal D9 sequence is predicted to contain three coiled-coil domains. We constructed shorter segments which contained one of the coiled-coil domains (I, II and III) and were supplemented with the syntaxin-2 CMD (SM) (Fig. 7A). When these were expressed, only the construct containing the domain III was found to localize to the Golgi (Fig. 7B, III-SM). In addition, the insertion of the domain III into syntaxin-2 prevented transport of syntaxin-2 to the plasma membrane, resulting in its retention in the Golgi (Fig. 7B, S2/III). These results suggest that the domain III with the coiled-coil domain adjacent to the CMD is essential for the Golgi localization of giantin. Shorter fragments of III-SM failed to target them to the Golgi (data not shown).

Common Features of COOH-Terminally-Anchored Golgi Proteins — Syntaxin-5 is a mammalian homologue of Sed5p localized to the Golgi (19, 31), which has the same membrane topology as giantin and contains a coiled-coil domain adjacent to the CMD (Fig. 8A). Similar syntaxin-5-derived constructs as those of giantin were prepared and expressed in COS-1 cells. When observed by immunofluorescence microscopy (Fig. 8B), syntaxin-5 without the CMD (S5M) or substituted with syntaxin-2 CMD (S5-SM) was localized to the Golgi, as observed for those of giantin. However, syntaxin-5 lacking an additional COOH-terminal cytoplasmic domain (S5D1) was detected throughout the cytoplasm. In contrast, the COOH-terminal cytoplasmic fragment of 79 residues, without the CMD (S5D2) or supplemented with syntaxin-2 CMD (S5D2-SM), was localized to the Golgi.

Golgin-84 is a transmembrane protein, consisting of a long cytoplasmic coiled-coil domain, a TMD and a short luminal domain of 16 residues (22) (Fig. 9). Essentially the same results as those of giantin and syntaxin-5 were obtained for golgin-84 by mutational analysis. Golgin-84 without the TMD and lumenal domain (g84T) or substituted with the syntaxin-2 CMD (g84-SM) was localized to the Golgi. However, golgin-84 without COOH-terminal 161 residues (g84D1) was detected throughout the cytoplasm. It was confirmed that the COOH-terminal cytoplasmic domain of 125 residues was required for the Golgi localization of golgin-84 (Fig. 9B). This domain contains a part of coiled-coil domains. Additional deletion of the coiled-coil domain (positions 569-623) resulted in distribution of the mutant throughout the cytoplasm (data not shown).
DISCUSSION

Giantin is an integral membrane protein, of which the single hydrophobic domain CMD at the COOH terminus functions as the anchor to the Golgi membrane (12-15). It was proposed that the localization of giantin to the Golgi involves at least three steps; insertion into the ER membrane, controlled incorporation into transport vesicles, and retention within the Golgi (16). The CMD of giantin was suggested to be essential for the insertion into the ER membrane and transport to the Golgi, because deletion of the CMD caused no attachment of giantin to the ER, resulting in its distribution into the cytoplasm (16). This is in contrast to our results, which demonstrated that giantin lacking the CMD is still associated with the ER and transported to the Golgi, although we agree with the proposal that newly synthesized giantin is initially associated with the ER and then transported to the Golgi, like conventional membrane proteins of secretory pathway (16).

Giantin appears to have another cytoplasmic domain, in addition to the CMD, for the initial association with the ER membrane, because the mutant D5 without a COOH-terminal domain after position 2804 was completely associated with the ER membrane (Fig. 3). Although the mutant D5 was not transported to the Golgi, the mutant D8M (2619-3162) was localized to the Golgi (Fig. 4). In addition, the mutant D9M (2804-3162) was neither associated with the ER nor transported to the Golgi, whereas the counterpart D9 with the CMD was localized to the Golgi (Fig. 5). Taken together, these results suggest that giantin contains domains with the following characteristics; 1) a cytoplasmic domain between positions 2619 and 2804 interacts with the ER membrane but is not sufficient to target the protein to the Golgi; 2) the Golgi targeting domain is contained in the sequence 2804-3162; and 3) the lack of the cytoplasmic domain (2619-2804) for the ER interaction can be compensated by the CMD, and the presence of either domain of the two is prerequisite for the ER association and subsequent transport to the Golgi. It is also clear that the CMD of giantin does not function as the primary determinant for the Golgi localization, as supported by the finding that its deletion or substitution with the CMD of syntaxin-2 causes no effect on the Golgi localization of giantin. This is in contrast to other Golgi-resident type II and III membrane proteins of which the TMD is involved in their...
Golgi localization (5, 6).

Exit from the ER is now considered to be a selective event involving sorting and concentration, in contrast to a constitutive 'bulk flow' process as suggested previously (32). Recent studies have shown that a di-acidic sorting signal is required for efficient ER export (33, 34). This motif, which contains the amino acids Asp or Glu separated by a variable residue (D/E-X-E/D), is found in the cytoplasmic tail of a number of transmembrane proteins and suggested to facilitate interaction with the COPII coat machinery. The di-acidic exit motif is located within or close to the Tyr-based sorting motif Y-X-X-Ø (where Ø is a bulky hydrophobic residue). Thus, it is likely that the ER accumulation of giantin mutants observed here is due to the lack of the ER exit motif. In fact, the motif is not found in the sequence of mutant D5 (1-2803), while it is found in positions 2898-2906 (Y-L-M-A-I-S-D-K-D) and in two other positions 3053-3055 (E-T-E) and 3086-3088 (E-E-E), all or either of which are contained in the competent mutants D6 - D9 and I - III with or without the CMD.

It has been suggested that the TMD of Golgi-resident type II and III membrane proteins is involved in the Golgi retention or localization; the TMD-dependent localization is probably reflects the lipid composition and thickness of target membranes (5, 6, 8). Although not containing any obvious distinguishing sequence motif, the Golgi TMDs are on average five residues shorter than those of plasma membrane proteins. The relevance of this difference in TMD length to retention is supported by mutational analysis; lengthening the TMD of Golgi enzymes results in their transport to the plasma membrane (5-7). It is, however, unlikely that the TMD or CMD of giantin is critically involved in the Golgi localization. Giantin has the same residue number in the CMD as that of the plasma membrane protein syntaxin-2 and replacement of the CMD by that of syntaxin-2 or even the lack of the CMD does not influence the Golgi localization of giantin.

In this study we also examined two other COOH-terminally anchored proteins, golgin-84 and the t-SNARE syntaxin-5, which are localized to the Golgi with the same membrane topology as giantin. As giantin has been shown to tether COPI vesicles to the Golgi membrane (35), golgin-84 was suggested to play a similar role in tethering transport vesicles to a target membrane (22). Our results demonstrated that the three COOH-terminally-anchored proteins
have the common feature for the Golgi localization; each cytoplasmic domain of
approximately 100-amino acid residues close to the CMD or TMD is primarily required for
the targeting and retention to the Golgi. This is essentially the same result obtained for the
yeast Sed5p (17). The cytoplasmic domain of syntaxin-5 required for the Golgi localization is
highly conserved in that of Sed5p (36). The importance of a similar cytoplasmic domain for
the Golgi retention was also demonstrated for the Golgi enzyme Mnt1p (37) and the TGN-
localized t-SNARE syntaxin-6 (38). Syntaxin-6 has a cytoplasmic H2 domain adjacent to the
TMD, which is predicted to form a 63-residue amphipathic α-helical structure and play a
major role for the Golgi localization (38). In contrast, the plasma membrane syntaxin-1 was
suggested to have no signal for the ER attachment or the Golgi localization in the cytoplasmic
domain (39).

In addition to giantin and golgin-84, there are many other coiled-coil proteins which are
peripherally associated with the cytoplasmic face of the Golgi, including GM130/golgin-95
(40), golgin-97 (41), golgin-160/GCP170 (20, 42), and golgin-245/p230 (42, 43). Golgin-
97, golgin-245/p230 and several other proteins are found to contain a conserved COOH-
terminal sequence (about 50 amino acids), which is designated the GRIP domain (44), Rab6-
interacting domain (45), or Golgi-localization domain GLD (46). The domain contains a
consensus sequence including a Tyr-based motif. In fact, the deletion of the domain or
substitution of Tyr by Ala in the Tyr-based motif resulted in failure of the proteins in correct
localization to the Golgi (44-46), suggesting that this domain functions in targeting these
coiled-coil proteins to the Golgi. It is of interest to know whether giantin also contains such a
motif in the corresponding domain. The consensus sequence proposed for the GRIP domain,
however, is not found in the COOH-terminal cytoplasmic domain (domain III) of giantin nor
in those of golgin-84 (g84D2) and syntaxin-5 (S5D2). At present we cannot find a possible
consensus sequence or motif among the domains of giantin, golgin-84 and syntaxin-5, except
for the common structural feature that they contain a coiled-coil domain. This suggests the
possibility that the COOH-terminal coiled-coil domain may play an important role in
interaction of giantin (and the two others) with the Golgi membrane, although details of the
mechanism remains to be solved.
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**FOOTNOTES**
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§ To whom correspondence should be addressed: Department of Biochemistry, Fukuoka University School of Medicine, Jonan-ku, Fukuoka 814-0180, Japan.
Fax: 81-92-864-3865; E-mail: yikehara@fukuoka-u.ac.jp.

1 The abbreviations used are: CGN, cis-Golgi network; CMD, COOH-terminal membrane-anchoring domain; ER, endoplasmic reticulum; GST, glutathione S-transferase; PCR, polymerase chain reaction; PNS, post-nuclear supernatant; RT-PCR, reverse transcription PCR; SNARE, SNAP (soluble N-ethylmaleimide-sensitive fusion protein) receptor; TGN, trans-Golgi network; TMD, transmembrane domain

FIGURE LEGENDS
Fig. 1. Expression of giantin with or without the CMD. A, Expression plasmids were constructed for wild-type giantin (GW), giantin without the CMD (GΔM) or with the CMD of syntaxin-2 (G-SM), wild-type syntaxin-2 (S2W), and syntaxin-2 with the CMD of giantin (S2-GM). The position of FLAG is also shown. B, homogenates prepared from COS-1 cells at 20 h after transfection with the vector alone (Mock) or with the indicated plasmids were fractionated into membranes (P) and cytosol (S) fractions. The samples were analyzed by SDS-PAGE (5% gels for giantin and 10% gels for syntaxin-2) and immunoblotting with anti-R2 antibodies (lanes 1-8) or anti-FLAG antibodies (lanes 9-12). C, at 20 h after transfection with the indicated plasmids, cells were fixed and subjected to immunofluorescence microscopy. The primary antibodies used were anti-R2 antibodies (GW, GΔM and G-SM) or anti-FLAG monoclonal antibody (S2W and S2-GM). Mock transfected cells stained for β+COP are also shown.

Fig. 2. Recovery of expressed proteins in the Golgi fraction. A, PNS fractions were prepared from cells transfected with the indicated plasmids and used for isolation of the Golgi-enriched fraction. Both the PNS and Golgi fractions were analyzed by SDS-PAGE and immunoblotting, as shown in Fig. 1. B, the recovery of each protein in the Golgi fraction was quantified from the data shown in A. The values are expressed as the means ± S.D (n = 4) by taking those in the PNS as 100%. C, experiments were carried out to rule out the possibility that the endogenous giantin is involved in the dimer formation with the exogenous proteins. Lysates prepared from cells transfected with the indicated plasmids were used for immunoprecipitation with anti-R2 (lanes 2-4 and 6-8) or anti-H1 (lanes 1 and 5). The immunoprecipitate was analyzed by SDS-PAGE and immunoblotting with anti-R2 (lanes 1-4) or anti-H1 (lanes 5-8).

Fig. 3. Intracellular distribution of COOH-terminally-deleted mutants. A, shown are giantin mutants with various sizes of COOH-terminal deletion (D1-D5) and D5
with the syntaxin CMD (D5-SM). D1, D2 and D3 were designed to contain one, two and three major coiled-coil domains, respectively, of the four (open boxes in the top schema). B, cells expressing the indicated mutants were fractionated into membranes (P) and cytosol (S) fractions. The samples were analyzed by SDS-PAGE and immunoblotting with antibodies to R1, R2 and R3. C, cells expressing the indicated mutants were observed by immunofluorescence microscopy, for which anti-(R1 + R2 + R3) antibodies were used as the primary antibodies.

Fig. 4. Intracellular distribution of NH2-terminally-deleted mutants.
A, schematic representation of giantin mutants with various sizes of NH2-terminal deletion (D6-D8) and additional deletion of the CMD (D6ΔM-D8ΔM). Each mutant was tagged with the FLAG at the NH2 terminus. The expressed giantin mutants were analyzed by immunoblotting (B) and immunofluorescence microscopy (C) as described Fig. 1, using anti-FLAG antibody, instead of anti-R2.

Fig. 5. Requirement of the CMD for Golgi targeting of D9 with a COOH-terminal domain.
A, mutants of a COOH-terminal domain with (D9) or without the CMD (D9M) or with the CMD of syntaxin-2 (D9-SM) were constructed and tagged with the FLAG at the NH2 terminus. B, the mutants expressed were immunostained with anti-FLAG (upper panels), as shown in Fig. 1. The endogenous giantin was co-immunostained with anti-H1 (B, lower panels).

Fig. 6. The effect of brefeldin A on the Golgi localization of giantin and mutants. HeLa cells expressing D8M (A) or D9-SM (B) were incubated in the presence of brefeldin A (2 µg/ml) for 30 min (c - f) and further incubated for 90 min after the drug was removed from the medium (e and f). The cells, including those before the drug treatment (a and b), were fixed and double-immunostained with anti-FLAG for each mutant (a, c, and e) and with anti-H1 for the endogenous wild-type giantin (b, d, and f).

Fig. 7. The essential domain for Golgi targeting and retention. A, coiled-coil probability of
the mutant D9-SM and schematic illustration of constructs containing each coiled-coil domain (I, II and III) with the syntaxin-2 CMD (SM). A mutant of syntaxin-2 containing the giantin domain III (S2/III) is also shown. Each mutant was tagged with the FLAG at the NH₂ terminus. B, the mutants expressed were analyzed by immuno-fluorescence microscopy with anti-FLAG.

**Fig. 8. The essential domain of syntaxin-5 for the Golgi localization.** A, coiled-coil probability of syntaxin-5 and schematic representation of wild type (S5W) and mutant constructs of syntaxin-5; S5M lacking the CMD (closed box), S5-SM containing the CMD of syntaxin-2 (shaded box) and deletion mutants without (S5D1 and S5D2) or with the CMD of syntaxin-2 (S5D2-SM). Each construct was tagged with the FLAG at the NH₂ terminus. B, the wild type and mutants of syntaxin-5 expressed in COS-1 cells were observed by immunofluorescence microscopy after being stained with anti-FLAG.

**Fig. 9. The essential domain of golgin-84 for the Golgi localization.** A, coiled-coil probability of golgin-84 and schematic representation of wild type (g84W) and mutant constructs of golgin-84; a closed box indicates the TMD of golgin-84 and shaded boxes indicate the CMD of syntaxin-2. Each construct was tagged with the FLAG at the NH₂ terminus. B, the proteins expressed in COS-1 cells were observed by immuno-fluorescence microscopy after being stained with anti-FLAG.
Fig. 7 Misumi et al.
An essential cytoplasmic domain for the Golgi localization of coiled-coil proteins with a COOH-terminal membrane anchor
Yoshio Misumi, Miwa Sohda, Akiko Tashiro, Hiroshi Sato and Yukio Ikehara

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