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Multilayer Interactions Determine the Golgi Localization of GRIP Golgins

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***G*olgin-97, *R*anBP2α, *I*mh1p and *p*230/golgin-245 (GRIP) domain golgins are targeted to the Golgi membrane through their GRIP domains. By analyzing more than 30 mutants of golgin-97 and golgin-245 GRIP domains for their properties of dimerization, interaction with ARF like protein 1 (Arl1)-GTP and Golgi targeting, we found hierarchically organized three-tier interactions governing the Golgi targeting of GRIP domain golgins. GRIP domain self-dimerization is necessary for bivalent interaction with Arl1-GTP. Unexpectedly, however, these two interactions are not sufficient for Golgi targeting, as a third group of residues, including positive-charged arginine between α1 and α2 and hydrophobic residues C-terminal to the GRIP domain, turn out to be essential. Surface plasmon resonance analysis indicates that GRIP domain interacts directly with membrane lipid, partially through the third group of residues such as W744 of golgin-97. This third tier of interaction with the membrane could be mediated by non-specific hydrophobic and electrostatic forces.**

Key words: Arl1, Golgi, golgin, GRIP domain, Shiga toxin B fragment

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The Golgi apparatus of higher eukaryotic cells consists of a series of dynamically stacked cisternae and plays a central role in the secretory pathway. Golgi membrane proteins, including peripheral and transmembrane proteins, contribute to the structure and cellular functions of this organelle. Among various peripheral Golgi proteins, golgins are unique in that, except their N- and C-termini, the majority of these molecules are predicted to adopt a structure of coiled-coils (1,2). Thus, golgins are proposed to be long rod-like molecules. Although the functions of most golgins are still largely unknown, several roles of golgins have been hypothesized: 1) tethering or repelling transport vesicles, 2) connecting adjacent Golgi cisternae, 3) acting as a matrix or meshwork of the Golgi and/or 4) assembling or scaffolding other proteins (1,2). There have been more than a dozen of distinct golgins identified in human cells, and some of them have homologues in yeast. Except for the predicted existence of extensive coiled-coil regions, these golgins share homologies in primary sequences. However, some golgins, including golgin-97, golgin-245/p230/tgolgin-1, GCC88 and GCC185 in mammals and Imh1p in yeast, have a conserved *g*olgin-97, *R*anBP2α, *I*mh1p and *p*230/golgin-245 (GRIP) domain of about 50 residues in their C-termini, and they are collectively referred to as GRIP golgins (3–6). The GRIP golgins are peripherally associated with membrane of *trans*-Golgi/*trans*-Golgi network (TGN), and their GRIP domains are necessary and sufficient for Golgi targeting. The cellular function of GRIP golgins is still not clear. Imh1p (Sys3p) is implicated in vesicular transport between endosomes and the late Golgi in yeast (7). Our recent study indicates that golgin-97 functions in protein trafficking from the early endosome/recycling endosome (EE/RE) to the TGN, probably acting as a tethering factor (8). We and others have shown that golgin-97 and golgin-245 are effectors of small GTPase Arl1 (9–11). Active Arl1 can interact with the GRIP domain and recruit these golgins to the Golgi membrane. Subsequently, we and others have solved the crystal structure of Arl1-GTP/golgin-245 GRIP complex (10,12). In the crystal structure, GRIP domain forms a homodimer in which each subunit separately interacts with one molecule of Arl1-GTP. The two molecules of Arl1-GTP are proposed to anchor the GRIP golgin dimer on the membrane. Our previous mutagenesis analysis has shown that the dimerization of GRIP domain is essential for its Golgi localization. In this study, we analyzed the relationship among dimerization, interaction with Arl1-GTP and Golgi localization of a series of GRIP mutants and found there are three hierarchical tiers of interactions contributing synergistically to Golgi targeting of GRIP golgins. Our experiments unexpectedly revealed an interaction between GRIP domain and phospholipids (third tier of interaction), though weak in nature, is essential to GRIP golgin localization.

Results

Dimerization of GRIP domain is essential for the interaction with Arl1-GTP, which recruits GRIP golgins onto Golgi membrane

Through mutagenesis study of the GRIP domain of golgin-245, we previously showed that GRIP domain can homodimerize, which is essential for its Golgi targeting (12). To examine the relationship between dimerization and Arl1-GTP interaction, a total of 19 site-directed and truncation mutants of golgin-245 GRIP domain (Figure 1A, as indicated by asterisks) were further characterized, including

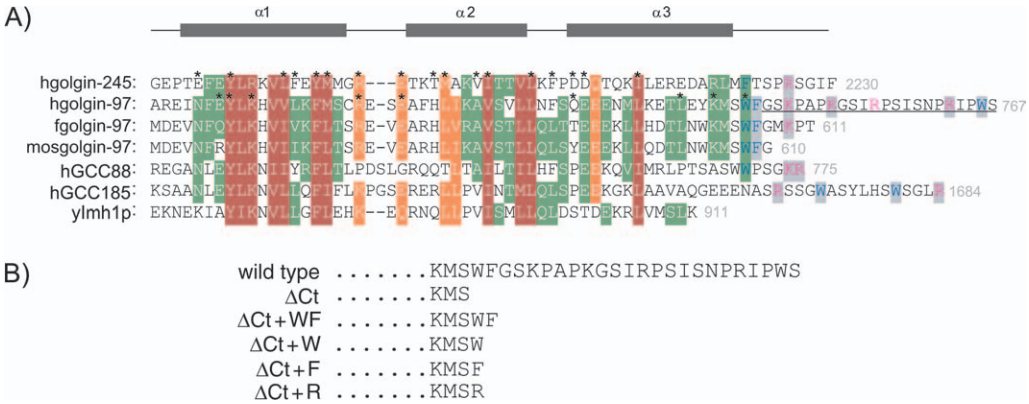

Figure 1: The alignment of GRIP domains and mutagenesis generated in this study. A) Multiple alignment of C-terminal GRIP domains of golgin-97, golgin-245, GCC88, GCC185 and Imh1p. The secondary structure of golgin-245 GRIP domain is represented in the illustration above golgin-245 sequence in correspondent positions. Conserved residues are color shaded. The positive-charged residues at the C-termini are colored pink and the hydrophobic amino acids (W and F) are colored blue. The underline in C-terminus of golgin-97 indicates residues nonessential for its Golgi targeting. Prefix: h, human; f, fly; mos, mosquito and y, yeast. The symbol '*' indicates a point mutation to Ala of this amino acid that is discussed in this study. The Gene Bank accession numbers of GRIP golgins are: NP_002069 (hgolgin-245), AAB81549 (hgolgin-97), NP_610875 (fgolgin-97), XP_321318 (mosgolgin-97), AAH14100 (hGCC88), NP_852118 (hGCC185) and NP_013412 (ylmh1p). The multiple alignment was assembled by DNA Star using Clustal W method and shaded by GeneDoc. B) The tail sequences of golgin-97 GRIP domain deletion mutants.

previous mutants together with new mutants: E2174A, L2182A, R2189A and D2207A. Yeast two-hybrid interaction assays were used to test the interaction of these mutants with Arl1-Q71L (Figure 2A). Combined with our previously obtained data (12), the dimerization, interaction with Arl1-GTP and Golgi localization of each of these 19 mutants are listed in Figure 2C. Mutations of critical residues located at Arl1-GTP/GRIP interface (12), such as Y2177, T2193, M2194 and V2197, abolished the interaction with Arl1-GTP and Golgi targeting, supporting the concept that interaction with Arl1-GTP is necessary for Golgi targeting. Interestingly, the interaction with Arl1-Q71L was also abolished wherever mutation was introduced at critical residue on GRIP–GRIP dimerization interface. These include mutants such as F2183A, Y2185A, I2198A, L2202A, F2204A, I2212A and Δα3 (the deletion of helix 3), which was shown to abolish the dimerization of GRIP domain (12). For example, when F2204 was mutated to Ala, the GRIP domain is unable to form a dimer (12). Concomitantly, the resulting monomeric GRIP domain did not interact with Arl1-GTP, although F2204 is buried inside the GRIP/GRIP dimer interface and is not directly involved in Arl1-GTP–GRIP interaction. Alpha-3 does not form any contact with Arl1-GTP from the structure (12); however, the deletion of α3 (Δα3) abrogated its interaction with Arl1-GTP. Although the GRIP dimerization and Arl1-GTP–GRIP interaction involve nonoverlapping residues of the GRIP domain, the strong correlation between dimerization and Arl1-GTP interaction suggests that dimerization is necessary for interaction with Arl1-GTP.

In addition to mutant GRIP domains of golgin-245, we also created several new point mutations in the GRIP domain of golgin-97. Together with our previously generated mutants (9), these eight site-directed mutants (Figure 1A, as indicated by asterisks) were analyzed for their dimerization (Figure 3A), interaction with Arl1-Q71L (Figure 3B) and Golgi localization (Figure 3C). Mutations in GRIP domain of golgin-97 revealed the same correlation between dimerization and interaction with Arl1-GTP. The results summarized in Figure 3D support that, similar to golgin-245, dimerization of the golgin-97 GRIP domain is critical for interaction with Arl1-GTP.

These results strengthen our conclusion that Arl1 regulates Golgi recruitment of golgin-97 and golgin-245 by interaction with the GRIP domain (9). They also highlight that at least two layers of interactions are involved in Golgi targeting of GRIP domain: the first being dimerization of GRIP domain followed by the second, interaction of dimerized GRIP domain with two molecules of Arl1-GTP. It is most likely that the formation of GRIP dimer configures the GRIP domain to create an interface for the binding of Arl1-GTP.

Identification of residues that are not involved in dimerization and interaction with Arl1-GTP but are necessary for Golgi targeting

We have next addressed the important question as to whether dimerization followed by interaction with Arl1-GTP is sufficient for Golgi targeting of GRIP golgins. By examining the results of these diverse mutants of golgin-245 (Figure 2C) and golgin-97 mutants (Figure 3D), we found some mutants that are very informative. R2189A in golgin-245 (Figure 2B, g–i), and L738A (data not shown) and W744A in golgin-97 (Figure 3C, m–o) are unusual in that they are no longer targeted to the Golgi apparatus, although they are able to dimerize and interact with Arl1-GTP. The positive-charged residue at position 2189 of

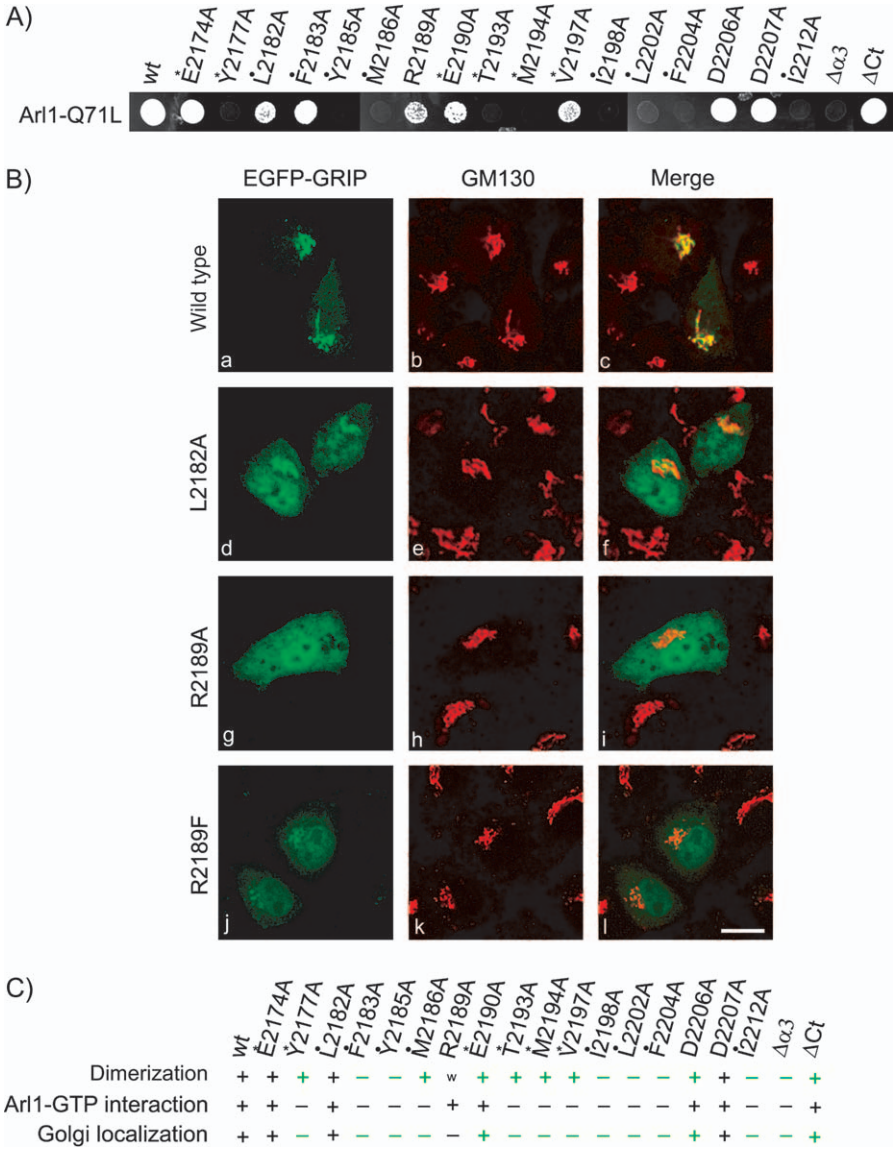

Figure 2: Analysis of golgin-245 GRIP domain mutants in Arl1-GTP interaction and Golgi localization. A) Golgin-245 GRIP domain mutants (Gal4-DNA-AD) were tested for their interaction with Arl1-Q71L (Gal4-DNA-BD) using yeast two-hybrid assays. The growth of the yeast patch indicates a positive interaction. B) The immunofluorescence microscopy showing the cellular distribution of GFP-tagged wild type (a), L2182A (d), R2189A (g) and R2189F (j) in NRK cells. A Golgi marker, GM130, was co-stained as red by Texas red (b, e, h and k). Bar, 10 μm. C) Summary of dimerization, interaction with Arl1-GTP and Golgi localization of Goglin-245 GRIP mutants. The symbols '+' and '−' indicate positive and negative interaction or localization, respectively, 'w' indicates weak but clear interaction. '*' and '.' indicate residues located in Arl1/GRIP and GRIP/GRIP structural interfaces, respectively. Black color indicates the results obtained from this study. Data adopted from a previous study (12) are colored green. GFP, green fluorescent protein. EGFP, enhanced green fluorescent protein.

golgin-245 is conserved in other GRIP golgins as well. When the equivalent residue of golgin-97, R709, was mutated to Ala, the resulting GRIP domain lost its Golgi localization in the majority of transfected NRK cells (Figure 3C, d–f), supporting the importance of this positively charged residue. W744A mutation was earlier reported to greatly reduce Golgi localization of the GRIP domain (3), but we found that W744A GRIP domain was undetectable on the Golgi apparatus in our experimental conditions (Figure 3C, m–o). Importantly, in the full length context of golgin-97, W744A mutant also has greatly reduced Golgi localization, with the majority of the expressed protein being distributed in the cytoplasm (Figure 4, g–i). The results obtained with the systematic analysis of these mutants imply that there could be a third tier of interaction involving residues, such as R2189 of golgin-245, and R709, L738 and W744 of golgin-97, in addition to dimerization (first tier) and Arl1-GTP interaction (second tier).

Alignment of golgin-97 from different species revealed that W744 and the following F745 residues are highly conserved (Figure 1A). Golgin-97 from vertebrates, such as human and mouse, has extra 20 amino acids after the GRIP domain, as compared with fly and mosquito golgin-97s (Figure 1A). As the C-terminus of mosquito golgin-97 ends with only two residues (F and G) after the conserved W, it suggests that the long stretch of C-terminal region after W744F745 in human golgin-97 may not be essential for Golgi targeting. Supporting this possibility, deleting residues after F745 of human golgin-97 GRIP domain (ΔCt + WF) (Figure 1B) did not affect Golgi targeting (Figure 5, d–f). However, a further deletion including the W744 and F745 (ΔCt) (Figure 1B) abolished the Golgi targeting of GRIP domain (Figure 5, a–c) and the full-length golgin-97 (Figure 4, d–f). A deletion till F745 (ΔCt + W) (Figure 1B) rendered extremely weak Golgi localization (Figure 5, g–i), implying that F745 is important for Golgi

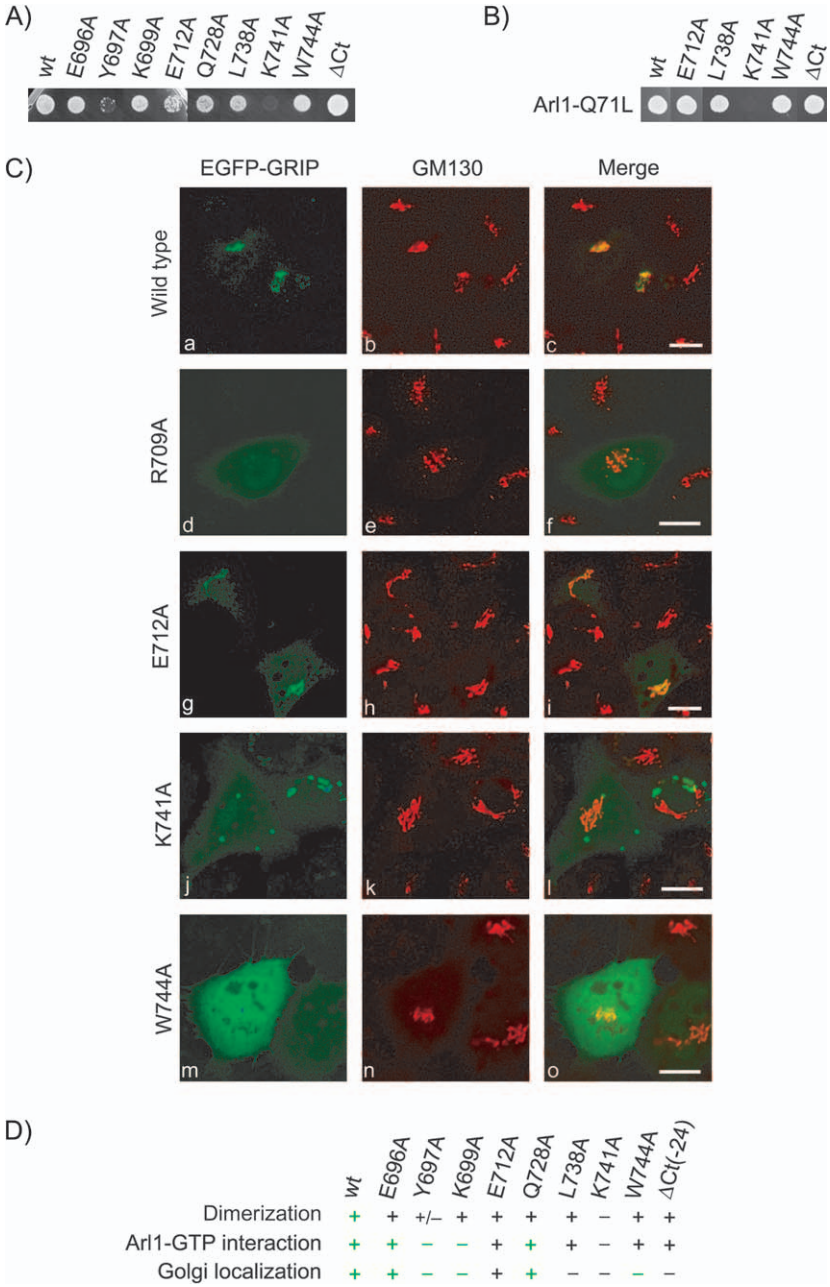

Figure 3: Analysis of golgin-97 GRIP domain mutants in self-dimerization, interaction with Arl1-GTP and Golgi localization. A) Various golgin-97 GRIP domain mutants in Gal4-DNA-BD were tested for interaction with their correspondent Gal4-DNA-AD fusions in yeast two-hybrid assays. B) Golgin-97 GRIP domain mutants (Gal4-DNA-AD) were tested for their interaction with Arl1-Q71L (Gal4-DNA-BD) using yeast two-hybrid assays. C) The localization of GFP-tagged wild type (a), mutant (d, g, j and m) golgin-97 GRIP domains together with GM130 (Texas red, b, e, h, k and n) in NRK cells. Bars, 10 μm. D) Summary of the dimerization, interaction with Arl1-GTP and Golgi localization of Goglin-97 GRIP mutants. The symbols '+' and '–' indicate positive and negative interaction or localization, respectively. Black color indicates the results obtained from this study. Data adopted from previous studies (3,9) are colored green. EGFP, enhanced green fluorescent protein.

targeting. Thus, the C-terminal stretch of 22 amino acids (underlined in Figure 1A) of human golgin-97 is not essential for its Golgi localization though it could contribute further stabilization for Golgi anchoring, whereas the two preceding hydrophobic residues W744F745 are critical, although they are not important for either dimerization or interaction with Arl1-GTP.

The third tier of interaction could be GRIP domain and lipid interaction

As our previous data showed that endosome-targeted sortin nexin 3 (SNX3)-Arl1-Q71L can translocate endogenous golgin-97 and golgin-245 to endosomal membrane (9), Arl1-GTP could be the only protein determining the membrane targeting of the GRIP domain. It is plausible to speculate that the third tier of interactions may involve binding of a third group of residues with membrane lipids. To explore this possibility, we tested the association of recombinant glutathione S-transferase (GST)-GRIP fusion proteins with liposome-coated L1 chip and monitored the resulting surface plasmon resonance (SPR) signal in BIACORE2000 (BIACORE, Uppsala, Sweden). Various concentrations of GST-golgin-97 GRIP wild type, W774A and GST proteins were injected on phosphatidylcholine

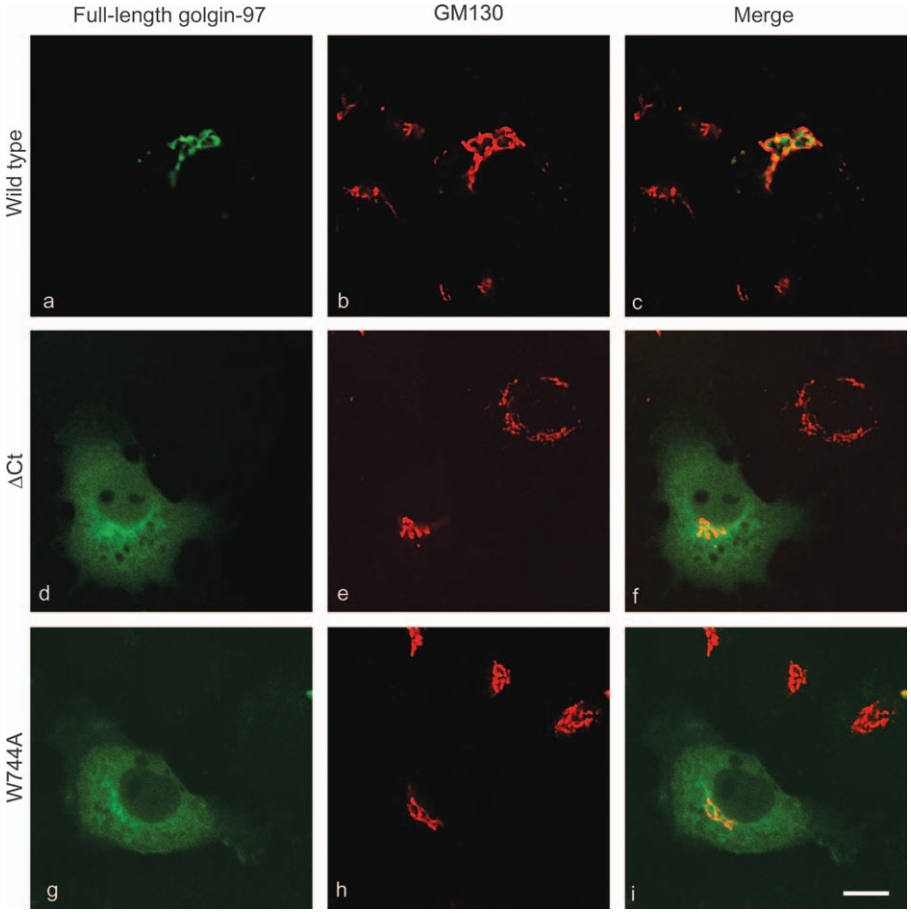

Figure 4: ΔCt and W744A mutation abolish Golgi targeting of full-length golgin-97 in NRK cells. GFP-tagged wild-type golgin-97 (a), golgin-97 ΔCt (d) and golgin-97 W744A (g) are green. GM130 as Golgi marker is red (b, e and h). The merged images are also shown. Bar, 10 μm. EGFP, enhanced green fluorescent protein.

(PC)/phosphatidylethanolamine (PE)/phosphatidylserine (PS) liposome (normal liposome) and typical response curves of 55 nM and 27.5 nM concentration of the recombinant proteins are shown in Figure 6. Both wild type and W774A showed dynamic binding with fast association and dissociation. At 55 nM concentration of each protein, the wild-type GRIP domain had much higher response [270 response units (RU)] than GST (64 RU) (Figure 6). GST-fused golgin-245 GRIP domain also gave similar response (data not shown). However, under the same condition, 55 nM GST-SNX3 generated about 2000 RU in 4% phosphatidylinositol-3-phosphate (PI3P)-containing liposome relative to normal liposome (data not shown), suggesting that GRIP domain has a weak interaction with lipid. W744A mutation significantly lowered the response to 225 RU. The same conclusion can also be reached from 27.5 nM response curves (Figure 6). Among different liposomes we have tested, including normal and phosphatidylinositol-, PI3P- or phosphatidylinositol-4-phospate (PI4P)-containing liposomes, it seems that the GRIP domain shows no preference towards the composition of liposome (data not shown), suggesting that the affinity towards lipid could arise from non-specific hydrophobic and electrostatic interaction. This hypothesis would predict that an affinity towards phospholipids could be conserved when a hydrophobic interaction is changed to an electrostatic interaction or vice versa. Supporting it, the addition of hydrophobic Phe (ΔCt + F) or positive-charged Arg (ΔCt + R) (Figure 1B) made golgin-97 GRIPΔCt associate clearly, despite weakly, with the Golgi (Figure 5, j–o). In golgin-245, changing of the positively charged Arg at 2189 to hydrophobic residue Phe still confers significant Golgi localization (Figure 2B, j–l). Therefore, these data suggest that the third group of residues, including non-polar L738, W744 and F745 in golgin-97 and/or the positively charged R2189 of golgin-245 and R709 of golgin-97, could contribute to hydrophobic and electrostatic interaction. From the Arl1-GTP/GRIP crystal structure, these residues are predicted to be in close proximity to the Golgi membrane, suggesting that they can thus help to anchor the Arl1-GTP/GRIP complex by interacting with membrane lipids.

However, without the membrane anchorage of Arl1, the third tier of interaction alone is not sufficient for the Golgi targeting of GRIP domain. Thus, our data suggest that there are three hierarchical tiers of sequential interactions determining the Golgi targeting of GRIP golgins: the first, dimerization of GRIP domain; the second, interaction of GRIP dimer with two molecules of Arl1-GTP and the third, stabilization on the membrane of the GRIP/Arl1-GTP

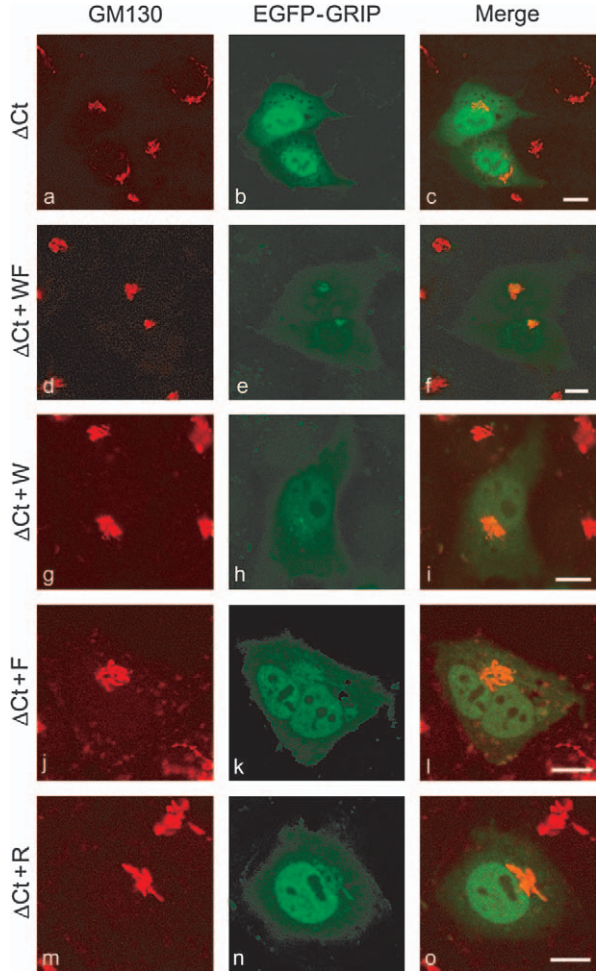

Figure 5: The affinity of golgin-97 C-terminal residues with membrane is mediated by a non-specific hydrophobic and electrostatic interaction. Images show the localization of GFP-tagged golgin-97 C-terminal deletion mutants (b, e, h, k and n) in NRK cells with GM130 as Golgi marker (Texas red, a, d, g, j and m). The merged images are also shown. Bars, 10 μm. EGFP, enhanced green fluorescent protein.

complex by these additional residues interacting with membrane lipids. The Golgi targeting of GRIP golgins depends on all three tiers of interactions from their extended GRIP domains.

The W744 involved in the third tier interaction is functionally important

We have recently shown that golgin-97 may function as a tethering factor in protein trafficking from EE/RE to the TGN using an *in vitro* EE/RE–TGN transport assay, which measures sulfation of modified Shiga toxin B fragment (STxB) (8). In that assay, GST-GRIP domain of golgin-97 strongly inhibited the EE/RE–TGN transport of STxB *in vitro*, and the inhibition was dependent on its interaction with Arl1-GTP. We have thus investigated whether the third group of residues of golgin-97 is functionally important. Recombinant GST-GRIP/W744A was prepared and applied to the transport assay in parallel with wild-type GST-GRIP as control. As shown in Figure 7, 133 μg/mL of GST-GRIP inhibited the transport of STxB to about 30% levels (lane 4) of standard assay (lane 1). The introduction of W744A relieved this inhibition (lane 3), and the transport was almost returned to the level of standard assay (lane 1). We think that golgin-97 GRIP domain incapable of the third tier interaction is unable to compete against endogenous golgin-97 for Arl1-GTP on the membrane, thus suggesting the functional importance of the third group of residues in golgin-97 GRIP domain. The importance of these residues in targeting full-length golgin-97 to the Golgi apparatus (Figure 4) suggests similarly that the third tier interaction is biologically relevant.

Discussion

By analyzing more than 30 GRIP domain mutants of golgin-97 and golgin-245 in terms of dimerization, interaction with Arl1-GTP and Golgi localization, we have found that Golgi targeting mediated by the GRIP domain is a complex process involving three layers of interactions, in a hierarchical (and likely sequential) manner. These three layers of interactions are contributed from three groups of residues in and outside the GRIP domain, which are located at three nonoverlapping interfaces (Figure 8A).

The first tier of interaction is the dimerization of GRIP domain and involves residues within the GRIP/GRIP interface. Our data demonstrate that dimerization is critical for GRIP domain to subsequently bind Arl1-GTP, which is the second tier of interaction. Residues involved in second tier of interaction are located between Arl1-GTP and GRIP interface of the GRIP domain. The homodimerization of GRIP domain could arrange its array of three helices to generate surface for interaction with Arl1-GTP. Furthermore, by bivalently and simultaneously associating with two molecules of Arl1-GTP, which is inserted into the membrane through its myristoyl group, the homodimeric GRIP domain could have much higher affinity towards the membrane. A similar mechanism is utilized by early endosome antigen 1 (EEA1), which is a golgin-like molecule on the early endosome. The two FYVE domains of homodimeric EEA1 bind two molecules of PI3P and thus make membrane association of EEA1 more stable (13).

Unexpectedly, our analysis of these mutants indicates that dimerization and bivalent interaction with Arl1-GTPs are not sufficient to anchor GRIP domain dyad on the membrane *in vivo*. There exists a third tier of interaction that is essential to complete the Golgi targeting of GRIP domain. Through SPR analysis, we demonstrated a direct and dynamic interaction between recombinant GRIP domain and liposome. This interaction utilizes membrane proximal residues, which are on one side of GRIP dimer, and is non-specific, hydrophobic and electrostatic in

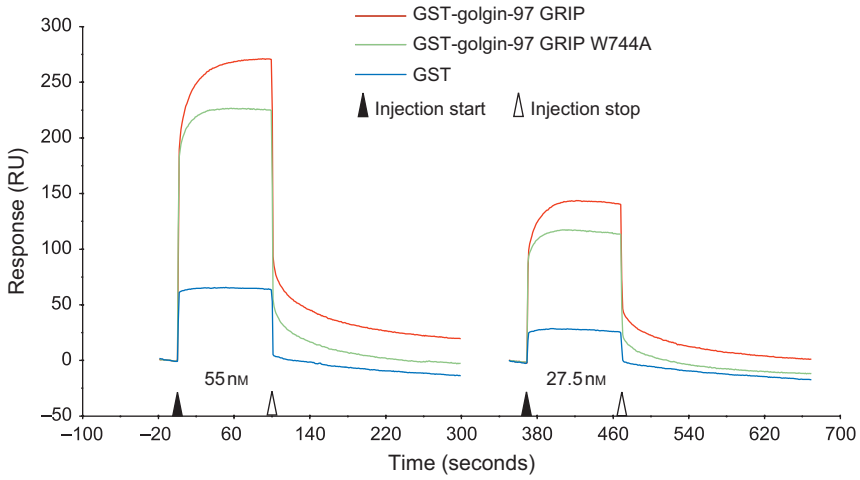

Figure 6: GRIP domain has affinity towards liposome. SPR analysis of GST-golgin-97 GRIP domain wild type (red), W744A (green) and GST (blue) at 55 nM and 27.5 nM concentration. The fusion protein was injected on PC-/PE-/PS-liposome-coated L1 chip (the time-point is indicated by solid black triangle) for association. After the flowing of 50 μL fusion protein, the injection was stopped (indicated by empty triangle) for dissociation of bound fusion protein.

nature. The L738, W744 and F745 residues following the GRIP domain of golgin-97 are likely involved in hydrophobic interaction with the interior of membrane bilayer, while R2189 of golgin-245 and R709 of golgin-97 could be involved in electrostatic interaction with the negatively charged head groups of membrane lipids (Figure 8B).

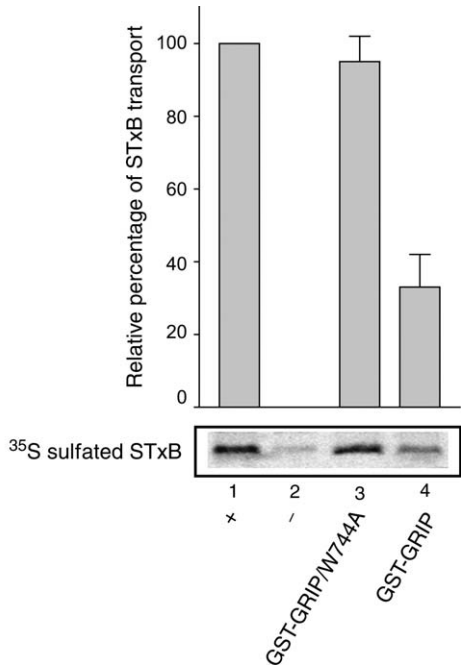

Figure 7: The third tier interacting residue W744 is essential for the function of golgin-97 GRIP domain – the introduction of the W7744A mutation abolished the ability of GST-GRIP to inhibit *in vitro* STxB transport. Relative efficiencies of STxB transport to the TGN as measured by the extents of its sulfation are indicated. Lane 1 (complete reaction without GRIP domain) and lane 2 (reaction without cytosol) are positive and negative controls, and their transports were referenced as 100% and 0%, respectively. In lane 3 and lane 4, the reactions contain 133 μg/mL of GST-golgin-97 GRIP/W744A or the wild-type GST-GRIP, respectively.

Supporting that view, mutation of W744 to Ala significantly reduced the liposome binding of GRIP domain in SPR assays. Although we have not found the essential hydrophobic resides in golgin-245 GRIP domain involved in third tier of interaction, mutation of W2223 at the C-terminal tail of a golgin-245 isoform (the C-terminal terminates with SWLRSSS instead of FTSPRSGIF used in this study) was reported to reduce its Golgi targeting (10). An essential residue for the third tier of interaction, R2189 of golgin-245, which is also conserved as R709 in golgin-97, could have electrostatic interaction with phospholipids as well. Other non-polar and positively-charged residues at the tail region of golgin-97 and golgin-245 could also potentially contribute to the membrane interaction. Although the third group of residues do not participate in the first and second tier of interactions, they are essential not only for Golgi targeting but also for a GRIP golgin's function, as W774A mutation abolished function of golgin-97 GRIP domain in STxB-based EE/RE–TGN transport assay.

The three tiers of interactions suggest a multivalent membrane targeting for GRIP golgins, that is, a GRIP golgin dyad utilizes both its membrane-facing residues and two associated Arl1-GTPs to engage with the membrane. Our finding of the three layers of interactions provides additional insight into the mechanism and molecular basis governing the process of Golgi targeting mediated by evolutionarily conserved GRIP domains.

Materials and Methods

Cloning

Cloning of Arl1-Q71L in pGBKT7 was as previously described (9). Wild-type golgin-245 GRIP domain and its mutants in pGBKT7, pGADT7 and pEGFP-C2 vectors (Clontech, Mountain View, CA, USA): Y2177A, F2183A, Y2185A, M2186A, E2190A, T2193A, M2194A, V2197A, I2198A, L2202A, F2204A, D2206A, I2212A, Δα3 and ΔCt were described as in our previous study (12). Wild-type golgin-97 GRIP domain and its mutants in pGBKT7, pGADT7 and pEGFP-C2 vectors: E696A, Y697A, K699A and Q728A were

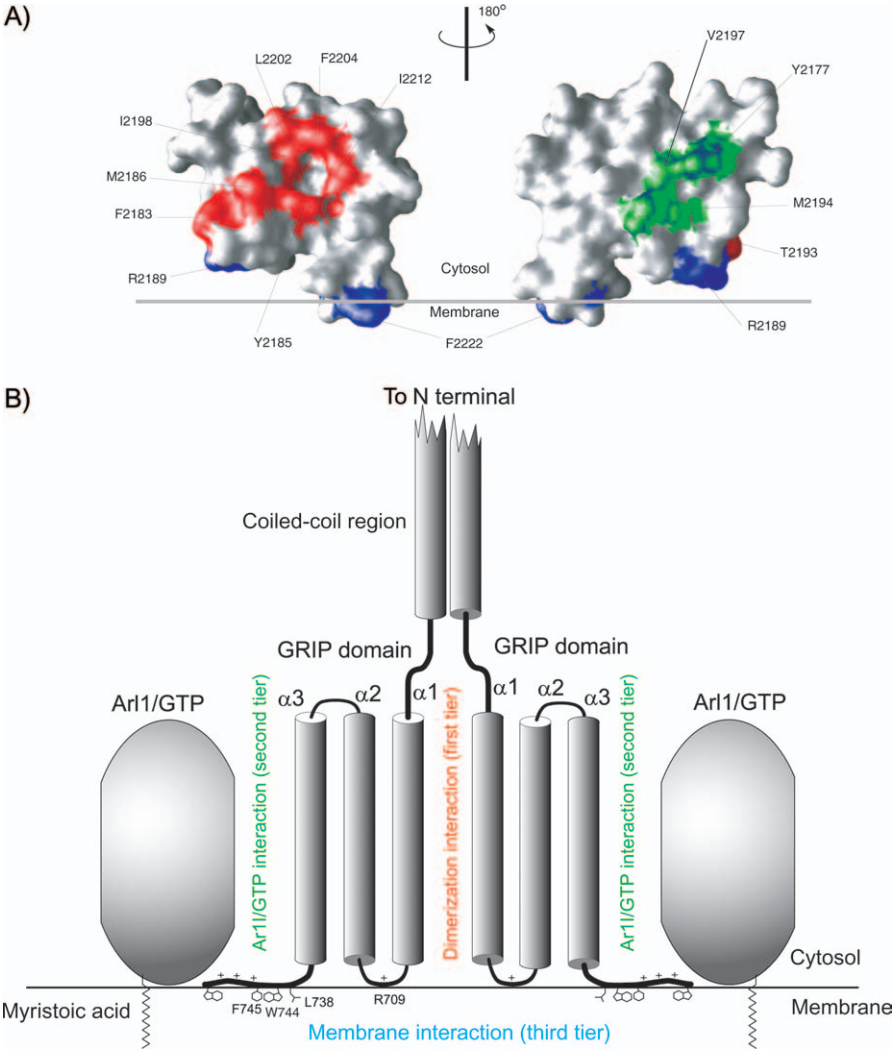

Figure 8: The model of Golgi localization of GRIP domain contributed by 3 groups of residues. A) The distribution of three groups of residues on GRIP domain. The golgin-245 GRIP domain was extracted from Arl1-GTP/GRIP crystal structure (PDB: 1R4A) (12). The residues responsible for the three tiers of interactions: dimerization interaction (first tier), Arl1-GTP interaction (second tier) and membrane interaction (third tier) are colored as red, green and blue, respectively. The corresponding residues are labeled. Note that M2186 is colored as red although its mutation does not abolish GRIP self-interaction (12). B) A schematic model showing the interaction of the GRIP domain with the membrane. Two molecules of Arl1-GTPs, which are membrane attached through their myristoyl groups, recruit the GRIP golgin homodimer by interacting with two GRIP domains. In the GRIP domain, there are three layers of interactions that are essential for this targeting process. GRIP domain folds into an array of three α helices: α1, α2 and α3, which are represented by cylinders. Critical residues involved in the third tier of interaction, including R709, L738, W744, F745 of golgin-97, are labeled. The C-terminal positively-charged amino acids (+) and Trp probably contribute the third tier of interaction as well. Arl1-GTP/GRIP golgin complex is thus anchored on the membrane through both myristoyl groups and the third tier of interaction.

described previously in (9). New mutations for golgin-245 GRIP domain, including E2174A, L2182A, R2189A, R2189F and D2207A, and golgin-97 GRIP domain, including R709A, E712A, L738A, K741A, W744A, ΔCt, ΔCt + W, ΔCt + F, ΔCt + R and ΔCt + WF, were generated by polymerase chain reaction (PCR) mutagenesis as described in (9). Each was subsequently cloned into pGBKT7, pGADT7 and pEGFP-C2 vectors. Golgin-97 GRIP domain and its W744A mutant were also cloned into the pGEB vector (9) to obtain the N-terminal GST-tagged recombinant fusion proteins for transport assay. Golgin-97 full length in pET28a (Merck, San Diego, CA, USA) was generously provided by Fritzler MJ and Chan EK. To generate the green-fluorescent-protein-tagged, wild-type, full-length golgin-97, its coding sequence was extracted by *Eco*RI and *Not*I (end blunted) and ligated with *Eco*RI and *Bam*HI (end blunted) pEGFP-C2 vector (Clontech). The W744A and ΔCt mutations in the context of full length of golgin-97 were produced by PCR mutagenesis (9) subsequently.

Yeast two-hybrid assays

Constructs in pGBKT7 (DNA-BD) and pGADT7 (DNA-AD) were used to transform AH109 and Y187 yeast cells (Clontech), respectively, by LiAc method. The resulting transformed yeast cells were mated as specified in the text. Diploid yeast cells containing both DNA-BD and DNA-AD fusion constructs were selected on SD (synthetic drop out medium)/−Trp/−Leu medium, and then the selected cells were streaked on the SD/−Trp/−Leu/−His/−Ade plate to test the interaction.

Cell culture and immunofluorescence microscopy

NRK cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO_2 environment. NRK cells grown on glass coverslips were transiently transfected with constructs in pEGFP-C2 using Effectene transfection reagent (QIAGEN, Hilden, Germany) according to manufacturer's protocol. The cells were fixed by 4% paraformaldehyde after about 20 h of incubation. Indirect immunofluorescence microscopy was performed as described previously (14). Mouse monoclonal antibody against GM130 was from BD Bioscience (Franklin Lakes, NJ, USA) and served as a Golgi marker in this study.

SPR analysis of GST-GRIP fusion protein and liposome

The procedure was similar to (15) with modifications. Briefly, 640 μg PC, 160 μg PE and 200 μg of PS (Sigma, St. Louis, MO, USA) were dissolved in chloroform. Four per cent of phosphoinositides, such as PI3P and PI4P (Echelon Biosciences, Salt Lake City, UT, USA) can be added to the lipid mixture at the expense of PC. Subsequently, the chloroform was

evaporated in vacuum. The lipid film was resuspended in 10 mM HEPES, 100 mM NaCl, pH 7.2, by bath sonication and extruded through 0.1 μm polycarbonate membrane (Avanti Polar Lipids, Alabaster, AL, USA) to obtain liposome suspension. The surface of L1 chip (BIACORE) was primed by 50 μL 40 mM CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) at 5 μL/min and 10 μL 100 mM NaOH at 5 μL/min to remove previously bound liposomes and proteins. The liposome suspension was injected on L1 chip in BIACORE2000 (BIACORE) at 2 μL/min for 20 μL, resulting about an increase of 5000–6000 RU. The surface of liposome-coated L1 chip was washed by 10 μL 100 mM NaOH at 5 μL/min followed by 60 μL of 0.2 mg/mL BSA (Sigma) at 30 μL/min. BSA served as a blocking reagent to block non-specific binding. To analyze protein lipid binding, 50 μL purified GST-GRIP protein solution was passed over liposome-coated chip at 30 μL/min and the SPR signal was recorded. The bound protein was stripped by passing 10 μL of 100 mM NaOH followed by 60 μL of 0.2 mg/mL BSA at 30 μL/min before testing the next protein. All experiments were conducted at 25°C in 10 mM HEPES, 100 mM NaCl, pH 7.2 buffer. GST-SNX3 and GST-golgin-245 GRIP domain fusion constructs were described in (16) and (12), respectively. The purification of GST fusion proteins is as follows.

STxB EE/RE–TGN transport assay

Golgin-97 GRIP domain and W744A mutant in pGEB were transformed into DH5α *Escherichia coli*. For purification of GST fusion protein, bacteria cultures were grown to an OD600 of 0.5–1 before overnight induction with 0.25 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) at room temperature. Bacterial cells were then pelleted and resuspended in lysis buffer: 50 mM Tris–HCl, pH 8; 0.1% Triton-X-100; 0.5 mM $MgCl_2$; 1 mg/mL lysozyme; 5 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride and Complete Protease Inhibitors (Roche, Basel, Switzerland). Cell lysis was promoted by sonication. The resulting lysates were clarified by centrifugation and applied to a Glutathione Sepharose 4B column (Amersham, Little Chalfont, Buckinghamshire, UK). After washing with buffer containing 50 mM Tris–HCl, pH 8; 0.1% Triton-X-100 and 0.5 mM $MgCl_2$, the bound GST fusion protein was eluted with 10 mM reduced Glutathione (Sigma) in 50 mM Tris–HCl, pH 8 and 0.5 mM $MgCl_2$. The elute was concentrated and subsequently dialyzed extensively in PBS at 4°C. The transport assay was conducted as in our previous studies (8,17).

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