Direct targeting of cis-Golgi matrix proteins to the Golgi apparatus

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SUMMARY

The targeting route of newly synthesized GM130 and GRASP65 to the Golgi apparatus was investigated by three different approaches. First, localization of pulse labeled GM130 and GRASP65 in normal rat kidney (NRK) cells was traced by subcellular fractionation followed by immunoprecipitation. Immediately after the pulse labeling, GM130 and GRASP65 were found in the Golgi but not in the endoplasmic reticulum (ER) membrane fractions, whereas a control Golgi membrane protein was still found in the ER membrane fractions. Second, epitope tagged GM130 and GRASP65 were expressed in NRK cells by plasmid microinjection into the nuclei and their localization was analyzed by immunofluorescence. When ER to Golgi transport was inhibited by prior microinjection of a GTP-restricted mutant of Sar1 protein into the cytosol, the expressed GM130 and GRASP65 showed clear Golgi localization. Last, binding of GM130 and GRASP65 to the membranes was analyzed in vitro. In vitro synthesized GM130 and GRASP65 specifically bound to purified Golgi membranes but not to microsomal membranes. The bound GM130 and GRASP65 were found to form a complex with pre-existing counterparts on the Golgi membrane. These results strongly suggested that GM130 and GRASP65 are directly targeted to the Golgi membrane without initial assembly on the ER and subsequent vesicular transport to the Golgi apparatus.

Key words: Golgi, Membrane binding, Vesicular transport, Subcellular fractionation, Microinjection, Mutant Sar1p

INTRODUCTION

The Golgi apparatus is an organelle situated at the center of the secretory pathway. It receives de novo synthesized secretory and membrane proteins from the endoplasmic reticulum (ER), processes, sorts and sends them out according to their final destinations (Mellman and Simons, 1992). The proteins are transported through the Golgi apparatus by vesicular transport without disturbing their membrane topology (Palade, 1975). Extensive studies during the past decade have revealed the basic molecular mechanisms of vesicle production, targeting and fusion acting from the ER to the Golgi apparatus (Rothman, 1994; Schekman and Orci, 1996). In mammalian cells, the Golgi apparatus is generally observed as a single juxtanuclear ribbon-like structure accumulated at the centriolar region. This structure is further resolved as an assembly of vesicles and stacks of flattened cisternae under the electron microscope (Ladinsky et al., 1999; Mollenhauer and Morré, 1994; Rambourg and Clermont, 1990). The Golgi stack is polarized receiving material from the ER on one side (the cis face) and dispatching cargo to various destinations on the other side (the trans face) (Farquhar and Palade, 1998).

The structure of the Golgi stack is thought to be relatively stable because stacked cisternal membranes are easily purified by gentle disruption of the cells (Slusarewicz et al., 1994; Hui et al., 1998). Therefore, it is reasonable to believe that some molecular organization, either stable or dynamic, does exist on and around Golgi cisternae to support the stacked structure of the Golgi apparatus. The Golgi cisternae might have a membrane domain or a membrane scaffold structure similar to the nuclear lamina, erythrocyte membrane skeleton, caveolae, tight junction or synaptic junction (Bredt, 1998; De Matteis and Morrow, 2000; Schlegel and Lisanti, 2001; Stuurman et al., 1998; Tsukita et al., 2001). It is now well accepted that peripheral membrane proteins can play a pivotal role in organizing and controlling these structures through their binding to integral membrane proteins and membrane lipids. Several protein-protein and protein-lipid interaction domains serving the specific targeting of peripheral membrane proteins have already been identified (Hurley and Misra, 2000; Ponting et al., 1997; Yaffe and Elia, 2001). Another large group of proteins are acylated for membrane targeting and, in many cases, the acylation of the proteins has the effect of increasing its affinity for the membrane, although additional information seems to be needed for the targeting of proteins to the specific membrane (Resh, 1999).

To date, many candidate proteins that fulfill potential structural roles have been identified on Golgi membranes. One group of these proteins localized on Golgi membranes are subtypes of ankyrin and spectrin that are important for the organization of actin-based membrane skeleton (Bennett and Chen, 2001; De Matteis and Morrow, 2000). Another group
are golgins, a large family of coiled-coil proteins (e.g. GM130) that are peripherally or integrally associated with Golgi membranes (Barr and Warren, 1996; Bascom et al., 1999; Griffith et al., 1997; Jakymiw et al., 2000). Additionally, a GM130 binding protein (GRASP65) and a related protein (GRASP55) are implicated in the organization of Golgi cisternae into stacks (Barr et al., 1997; Shorter et al., 1999).

GM130 is a peripheral membrane protein tightly bound to the Golgi membrane on the cytoplasmic side (Nakamura et al., 1995). It was originally identified from a detergent- and salt-resistant Golgi matrix that specifically binds Golgi enzymes and is thought to contain molecules that maintain the Golgi structure (Slusarewicz et al., 1994). GM130 is predicted to adopt a coiled-coil structure similar to myosin heavy chain and intermediate filament proteins with short non-coil N- and C-terminal regions (Nakamura et al., 1995). The N-terminal region binds to p115/TAP and this binding is suggested to be required for the membrane fusion step of intercisternal transport in the Golgi stack (Barroso et al., 1995; Sapperstein et al., 1995; Nakamura et al., 1997). The C-terminal region is required for Golgi localization and binds to GRASP65 (Barr et al., 1998; Nakamura et al., 1997). The N-terminal p115-binding region of GM130 is phosphorylated during mitosis, and this is suggested to trigger the Golgi disassembly (Levine et al., 1996; Nakamura et al., 1997; Lowe et al., 1998; Lowe et al., 2000). Furthermore, GM130 is implicated in function in the ER to Golgi transport (Alvarez et al., 2001; Seemann et al., 2000b). It was first thought that GM130 and p115 function with giantin, an integral membrane protein localized to the Golgi membrane, to tether the COPI vesicles to the Golgi cisternae, because the inhibition of ternary giantin-p115-GM130 complex formation prohibited the docking of the COPI vesicles to Golgi membranes (Sönntichsen et al., 1998). Now, it seems likely that GM130 and giantin function in vesicle tethering together with p115. However, recent reports suggested that giantin works independently from GM130 (Alvarez et al., 2001; Linstedt et al., 2000). The reason for this discrepancy is unclear.

GRASP65 was identified as a N-ethyl-maleimide (NEM) sensitive factor required for the stacking of the Golgi cisternae in in vitro Golgi reassembly assay (Barr et al., 1997). It is myristoylated at the N terminus and has two PDZ-like domains. The second PDZ-like domain is important for binding to GM130 and also for the Golgi targeting (Barr et al., 1998). Together with GM130, GRASP65 is heavily phosphorylated during mitosis, and this is thought to play a major role in Golgi disassembly (Barr et al., 1997). The fact that the PDZ domain is found in proteins that function in organizing special membrane domains, including cell adhesion devices and postsynaptic density, by binding to the integral membrane proteins implies that GRASP65 serves a similar function (Ponting et al., 1997).

GM130 and GRASP65 both localize to the cis face of the Golgi apparatus. Quantitative immunoelectron microscopy revealed that ~70% of GM130 is associated with Golgi stacks, with the remainder on membranes with tubular reticular profiles. ~80% of the stack-associated GM130 was shown to be on the cis face of the Golgi (Nakamura et al., 1995). Similar results were obtained for GRASP65 (Shorter et al., 1999). The cis localization of GM130/GRASP65 is of particular interest when considering the vesicle tethering function of GM130. In interphase cells, GM130/GRASP65 might function as a landmark of the cis-Golgi membrane for anterograde vesicles traveling from the ER or for retrograde vesicles traveling from downstream, more-trans Golgi compartments. If so, targeting and active recycling of GM130/GRASP65 through other compartments of the vesicular transport pathway such as the ER would be unfavorable, because it would cause mistargeting of cis-Golgi-destined vesicles. Therefore, it is essential to first clarify this issue by determining the targeting route of GRASP65 and GM130.

Until now, only the regulated binding of coat molecules such as ARF and coatomer, which dynamically cycle between cytosol and membrane, has been analyzed in detail, and their direct binding to Golgi membranes is now well accepted (Rothman and Wieland, 1996). By contrast, there is no report analyzing the targeting route taken by the peripheral membrane proteins that are tightly associate with Golgi membranes. For all the integral membrane proteins of the Golgi apparatus analyzed to date, the newly synthesized proteins are inserted into the membrane of the ER and transported to the Golgi apparatus. Therefore, it is also possible that newly synthesized peripheral Golgi membrane proteins are first assembled on the ER membrane and then transported to the Golgi apparatus. In fact, there are examples that peripheral membrane proteins assemble on the membrane at different compartments of the secretory pathway. Lck, a src family tyrosine kinase is assembled with CD4 at the intracellular membrane, probably at the Golgi apparatus, and transported to the plasma membrane (Bijlmakers and Marsh, 1999; Bijlmakers et al., 1997). Similarly, PSD95, a protein localized at the post-synaptic density, is associated with the membrane at the endosome and transported to the specialized domain of plasma membrane (El-Husseini et al., 2000).

For the above reasons, we decided to analyze the targeting route of newly synthesized GM130 and GRASP65. Here, we show that GM130 and GRASP65 are directly assembled on the Golgi membrane without prior targeting to the ER by in vivo experiments using cultured cells and in vitro cell-free binding experiments.

MATERIALS AND METHODS

Plasmid construction and protein purification

pCNG2 plasmid harboring a fusion of N-acetylc glucosaminyl-transferase I (NAGT-I) and green fluorescent fusion protein (NAGFP) was kindly provided by D. Shima (Imperial Cancer Research Fund, UK). GM130 C-terminal mutant (ΔC983, M984A) and GRASP65 Golgi-binding mutant (G2A, G196A) were produced as described (Barr et al., 1998). FLAG-GM130 was produced by fusing a FLAG epitope at proline-rich hinge region (E440 was replaced by the sequence DYKDDDDK), and GRASP65-HA was produced by fusing an HA epitope at its C terminus. These constructs were generated by PCR mutagenesis using appropriate primers and cloned in pcDNA3.1. For in vitro transcription-translation, wild-type or mutant GM130 and GRASP65 were subcloned into pSP64YS, a pSP64-based vector that has the 5′ untranslated region of the Xenopus β-globin gene. Briefly, pSP64YS was generated by cutting pSPBP4 with NcoI and BamHI (for GM130) or NcoI and EcoRI (for GRASP65) to remove preprolactin coding sequence and the 3′ untranslated region of the Xenopus β-globin gene (Siegel and Walter, 1988). The fragments coding for GM130,
GRASP65 and their mutants with appropriate restriction sites were generated by PCR and inserted between those sites. pET-11 plasmid encoding Sar1p H79G mutant (mSar1p) was kindly provided by W. E. Balch (The Scripps Research Institute, USA) and the protein was purified as described (Rowe and Balch, 1995).

Cell culture and a stable cell line expressing NAGFP
NRK cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Equitec-Bio, Ingram, TX). An NRK cell line expressing NAGFP was cloned and maintained with 400 μg ml⁻¹ G418 (Gibco BRL Products, Rockville, MD) in the medium.

Microinjection and immunofluorescence microscopy
Microinjection was performed using a semi-automated microinjection system (InjectMan, Olympus Optical, Tokyo, Japan). mSar1p (2 mg ml⁻¹ in purification buffer; 25 mM HEPES (pH 7.4), 125 mM KOAc, 1 mM MgCl₂, 50 mM EGTA, 10 μM GDP, 1 mM glutathione) or the buffer only was mixed with 5 mg ml⁻¹ of cascade-blue-conjugated BSA (Molecular Probes, Eugene, OR), injected into the cytoplasm of NRK cells spread on a CELLocate (Eppendorf, Hamburg, Germany) and the locations were marked. The cells were incubated for 15 minutes at 37°C, then plasmids encoding NAGFP (200 μg ml⁻¹) and FLAG-GM130 (5 μg ml⁻¹) or GRASP65-HA (10 μg ml⁻¹) were co-injected into the nuclei of the mSar1p-injected cells. The cells were then incubated for 45 minutes followed by 15 minutes incubation in the presence of 100 μg ml⁻¹ cycloheximide to inhibit further protein synthesis. The cells were then fixed, permeabilized and processed for immunofluorescence as described (Nakamura et al., 1995). Rabbit antiserum for giantin (Toki et al., 1997) and monoclonal antibodies for the FLAG epitope (anti-FLAG M2; Sigma Chemical, St Louis, MO) or HA epitope (16B2; BAbCO, Richmond, CA) were used for triple labeling the cells. Cy3-conjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse antibodies (Jackson Immuno Research Laboratory, West Grove, PA) were used as secondary antibodies. The stained cells were observed by a confocal microscope (LSM510; Carl Zeiss, Jena, Germany).

Pulse-chase labeling, subcelluar fractionation and immunoprecipitation
Subcellular fractionations with sedimentation and flotation method were performed as described previously with slight modifications (Füllekrug et al., 1997; Lee and Linsteadt, 1999). NRK cells stably expressing NAGFP were grown to confluence in a 145 cm² dish, incubated in methionine/cysteine-free DMEM for 60 minutes, labeled with 7.4 MBq ml⁻¹ [³⁵S]-Express Protein Labeling Mix (NEN Life Science Products, Boston, MA) for 5 minutes, washed and chased with DMEM supplemented with serum for indicated time. The cells were washed and collected with ice-cold PBS, then further washed with homogenization buffer (130 mM KCl, 5 mM MgCl₂, 25 mM Tris-HCl (pH 7.3)). The cells were then homogenized in the homogenization buffer (250 μl for flotation and 500 μl for the other experiments) containing protease inhibitors cocktail (1 mM 4-(2-aminoethyl) benzenesulfonfyl fluoride hydrochloride, 20 μM leupeptin, 15 μM pepstatin and 15 μM chymostatin) by passing through a 27-gauge needle 20 times. The homogenate was centrifuged twice for 5 minutes at 1000 g. The resulting post-nuclear supernatant (PNS) was centrifuged for 30 minutes at 100,000 g to pellet total membranes. In a sedimentation experiment, the PNS was adjusted to 500 μl with homogenization buffer and layered on top of a step density gradient of 500 μl 40%, 1 ml 25%, 1 ml 20%, 1 ml 17.5% and 1 ml 15% (w/v) Nycodenz (Nyncomed, Oslo, Norway) in homogenization buffer. After centrifugation in a SW55Ti rotor (Beckman Instrument, Palo Alto, CA) for 60 minutes at 100,000 g, ten fractions (500 μl) were collected from top to bottom. 125 μl of 5× detergent buffer (5.0% NP-40, 2.5% deoxycholate, 0.5% SDS, 250 mM Tris-HCl (pH 8.0)) was added to each fraction, and sequentially immunoprecipitated with anti-GM130 (Nakamura et al., 1995), anti-GFP (Shima et al., 1997) and anti-Calnexin CT (Stressgen, Victoria, Canada) antibodies.

Fig. 1. Newly synthesized GM130 and GRASP65 are quickly localized to the membrane. NRK cells stably expressing NAGFP were labeled with [³⁵S]-methionine/cysteine for 5 minutes and chased for 0 minutes, 3 minutes, 7 minutes, 15 minutes and 30 minutes. Cells were homogenized and post-nuclear supernatant (PNS) was recovered. PNS (S) was further separated into total membrane (M) and cytosol (C) fractions. (A) GM130, GRASP65, calnexin and NAGFP were immunoprecipitated from each fraction and analyzed by SDS-PAGE and autoradiography. The immature form (im) and the mature form (m) of NAGFP are indicated. An asterisk indicates a non-specific precipitate. The picture shown is a representative of two experiments with similar results. (B) The percentage of GM130 (closed squares), GRASP65 (closed triangles) NAGFP (open circles) and calnexin (open diamonds) recovered in each membrane was plotted for the chase time. The plots are the average of two experiments and vertical bars indicate the ranges. (C) PNS was subjected to flotation as described in Materials and Methods and separated into cytosolic (C) and membrane (M) fractions. GM130 and GRASP65 were immunoprecipitated from each fraction and analyzed by SDS-PAGE and autoradiography. The picture shown is a representative of three experiments with similar results.
Immunoprecipitates were washed once with RIPA buffer (1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl), twice with high salt buffer (0.2% SDS, 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 500 mM NaCl) and once with TEN (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl). Samples were analyzed by SDS-PAGE and autoradiography. In a flotation experiment, the PNS (125 μl) was adjusted to 1.6 M sucrose, 500 μl in homogenization buffer and overlaid with 340 μl 1.4 M and 160 μl 0.8 M sucrose layers. This was centrifuged in SR55S rotor (Hitachi Instruments, Tokyo, Japan) for 180 minutes at 100,000 g. Membrane (400 μl) and cytosolic (600 μl) fractions were collected from top to bottom. >90% of membranes and <10% of the cytosol floated up, as judged by the distribution of total membrane (NAGFP and calnexin) and cytosolic (glyceraldehyde-3-phosphate dehydrogenase) protein markers. Each fraction was solubilized with 5× detergent buffer and immunoprecipitated same as the sedimentation experiment. In Fig. 1B, bands were densitometrically quantified and the percentage binding to the membrane was calculated by using the following equation:

\[
\text{Binding} (\%) = \frac{D_{\text{ppt}}}{(D_{\text{sup}} + D_{\text{ppt}})} \times 100
\]

where \(D_{\text{ppt}}\) is the density of GM130 or GRASP65 precipitated with membranes and \(D_{\text{sup}}\) is the density in the cytosolic fraction.

### In vitro binding assay

Rat liver Golgi and mitochondrial membranes were purified as previously described (Hui et al., 1998; Sakaguchi et al., 1992). Microsomal membranes were purified from rat liver as follows with a modification of the previously described method (Walter and Blobel, 1983). Rat liver was homogenized and subjected to a step sucrose density gradient centrifugation as described in Golgi membrane preparation (Hui et al., 1998). The layer below the interface of 0.86 M and 1.3 M sucrose but above the cell debris was collected, diluted to 0.25 M sucrose concentration by phosphate buffer (0.1 M potassium phosphate (pH 6.7), 5 mM MgCl₂), and centrifuged at 10,000 g for 10 minutes. Supernatant was collected and further centrifuged through a 1.3 M sucrose cushion at 140,000 g for 2.5 hours. The pellet at the bottom of the tube was suspended with the 0.25 M sucrose in phosphate buffer, homogenized by Dounce homogenizer, aliquoted, snap frozen and stored at −80°C. The membranes were fully competent for the membrane translocation assay (Walter and Blobel, 1983). The concentration of protein in the membranes was determined by BCA protein assay reagent (Pierce Chemical, Rockford, IL). GM130, GRASP65 and their mutants cloned in pSP64YS were in vitro transcribed with SP6 RNA polymerase and translated using rabbit reticulocyte lysate as previously described (Sakaguchi et al., 1992) and the translation products were centrifuged at 100,000 g for 5 minutes to remove aggregates. 8 μl of the cleared translation product was incubated with 2 μl of membrane suspension adjusted to the indicated protein concentration at 30°C for 60 minutes. Sample (10 μl) was diluted with 40 μl of translation buffer (150 mM KOAc, 5 mM Mg(OAc)₂, 30 mM HEPES-KOH (pH 7.4)), layered on 100 μl of 0.5 M sucrose in translation buffer and centrifuged for 5 minutes at 100,000 g. Supernatant was removed and precipitated membranes were collected with SDS-PAGE sample buffer. The sample was analyzed by SDS-PAGE and autoradiography. The density of each band was quantified and the binding of GM130 or GRASP65 to the membrane (%) was calculated by using the following equation:

\[
\text{Binding} (\%) = \left(\frac{D_{\text{mem}} - D_{\text{moc}}}{D_{\text{input}} - D_{\text{moc}}}\right) \times 100
\]

where \(D_{\text{input}}\) is the density of input GM130 or GRASP65, \(D_{\text{mem}}\) is the density of GM130 or GRASP65 precipitated with membranes and \(D_{\text{moc}}\) is the density of GM130 or GRASP65 precipitated without membranes.

For immunoprecipitation, the membranes were recovered after the in vitro binding reaction, lysed in 250 μl of IP buffer (20 mM HEPES-KOH (pH 7.3), 200 mM KCl, 0.5% w/v Triton X-100) and centrifuged at 10,000 g for 5 minutes. The supernatant was immunoprecipitated with anti-GM130 or anti-GRASP65 antibody, or preimmune serum using protein A-Sepharose (Amersham Pharmacia Biotech UK, Little Chalfont, UK), washed three times with IP buffer and finally subjected to SDS-PAGE followed by autoradiography.

**Fig. 2.** Newly synthesized GM130 and GRASP65 are localized to Golgi membranes. (A) NRK cells stably expressing NAGFP were labeled with [³⁵S]-methionine/cysteine for 5 minutes and homogenized immediately (upper gallery) or after 30 minutes chase (lower gallery). Post-nuclear supernatant was obtained and analyzed by Nycodenz density gradient as described in Materials and Methods. To the left of the panels (lane 1) is the top (lightest) fraction and the right (lane 10) is the bottom (heaviest) fraction. GM130, GRASP65, calnexin and NAGFP were immunoprecipitated from each fraction and precipitated materials were analyzed by SDS-PAGE and autoradiography (indicated by arrows). Asterisks indicate nonspecific precipitates. The picture shown is a representative of three experiments with similar results. (B) Bands in (A) were quantified by densitometry and percentages of the total sum of the precipitated material in all fractions were calculated and plotted for each fraction.
RESULTS

Newly synthesized GM130 and GRASP65 are quickly localized to the Golgi membranes

To determine the targeting route of GM130 and GRASP65, we first tried to follow the localization of newly synthesized proteins in living cells by biochemical procedures. NRK cells stably expressing NAGFP as a Golgi resident marker protein were pulse-labeled with \( ^{35} \)S-methionine/cysteine for 5 minutes. The cells were then homogenized either immediately or after a 30 minutes chase with cold methionine/cysteine. A post-nuclear supernatant (PNS) was recovered by low speed centrifugation of the homogenate then further separated into cytosolic and membrane fractions by high-speed centrifugation. GM130, GRASP65, NAGFP (a Golgi marker) and calnexin (an ER marker) were then serially immunoprecipitated from each fraction and analyzed by SDS-PAGE followed by autoradiography (Fig. 1A). Most of the labeled NAGFP, calnexin and GRASP65 were recovered in the membrane fraction immediately after the pulse labeling. It should be realized that NAGFP started to show mobility.
shifts after 15 minutes chase, and this was almost complete after 30 minutes chase (Fig. 1A). Western blotting of the cell lysate with anti-GFP antibody showed that most of NAGFP maintained as this mature form in the steady state of the cells (data not shown). The mobility shift of NAGFP is probably caused by \( O \)-linked glycosylation of the NAGT-I moiety because native NAGT-I was reported to receive \( O \)-linked glycosylation in the Golgi apparatus, showing a similar mobility shift (Hoe et al., 1995). Much of the labeled GM130 was also recovered in the membrane fraction, although a small but significant amount was reproducibly found in the cytosolic fraction immediately after the pulse labeling (Fig. 1B). The labeled GM130 gradually disappeared from the cytosolic fraction and was mostly found in the membrane fraction after the 30 minutes of chase (Fig. 1A,B).

It is possible that labeled proteins were pelleted because they were in some heavy cytosolic complexes and not on membranes. To show that the labeled proteins were bound to the membrane, we performed a membrane flotation experiment. The cells were pulse-labeled, chased and a PNS prepared as above. After adjusting the density, the PNS was layered at the bottom of a step density gradient and then membranes were floated up by centrifugation. As shown in Fig. 1C, most of the labeled GM130 floated up with membranes and little remained in the cytosolic fraction either immediately after the labeling or after the chase. Much of GRASP65 was also floated up with the membranes, although slightly more GRASP65 remained in the cytosolic fraction. These results suggested that most of the newly synthesized GM130 and GRASP65 were bound to the membranes.

We then investigated the distributions of the newly synthesized proteins by further subcellular fractionation of the membranes. NRK cells were pulse-labeled, chased and PNS was prepared again as above. This time, PNS was fractionated by sedimentation through a Nycodenz step gradient. Fractions were recovered from top to bottom. Then, GM130, GRASP65, NAGFP and calnexin were sequentially immunoprecipitated from each fraction and analyzed by SDS-PAGE followed by autoradiography. Distribution of total Golgi and total ER membranes was analyzed by western blotting marker proteins (NAGFP and calnexin; data not shown) and found to be the same as the metabolically labeled marker proteins after 30 minutes chase (Fig. 2A,B). Labeled calnexin showed a broad distribution with a peak in the heavier fractions (Fig. 2A,B, fractions 7) and this was not changed after the chase. Immediately after the pulse, labeled NAGFP was distributed similarly to calnexin, with a second peak at the lighter fractions (fractions 2-4). This strongly suggested that most of the newly synthesized NAGFP had translocated into, but was not yet exported from, the ER at this time point. The second peak most probably reflected the small part of NAGFP already transported to the ERGIC/intermediate compartment because total p58/ERGIC53 showed similar peak at the lighter fractions (data not shown). After the chase, the labeled NAGFP exclusively distributed to the lighter fractions as a mature form, indicating that transport to the Golgi was completed at this time point. By contrast, labeled GM130 already appeared exclusively in the lighter fractions immediately after the pulse labeling and remained there throughout the chase. A similar result was obtained for GRASP65. These results clearly showed that newly synthesized GM130 and GRASP65 are already localized to the Golgi membrane at a time when newly synthesized NAGFP is still in the ER.

Fig. 4. GM130 and GRASP65 are directly targeted to Golgi apparatus in vitro. (A) \(^{35}\text{S}\)-labeled GM130 were synthesized in vitro using reticulocyte lysate and incubated with increasing amount of purified Golgi, mitochondrial or microsomal membranes as indicated. Membranes were recovered by centrifugation, the bound material was analyzed by SDS-PAGE and autoradiography. The picture shown is a representative of three experiments with similar results (top gallery). Amounts of membrane-bound GM130 and GRASP65 were quantified as described in Materials and Methods and plotted against the amounts of final membrane concentrations. The results shown were the average of three experiments and the vertical bars indicate standard deviations (bottom). (B) Experiments were carried out for GRASP65 as described in (A).
Taken together, the above results strongly suggested that GM130 and GRASP65 are quickly targeted to the Golgi membrane after synthesis in the cytosol, without prior assembly on the ER and subsequent transport to the Golgi apparatus.

**GM130 and GRASP65 are targeted to the Golgi apparatus in the absence of ER-to-Golgi transport**

We next tried to confirm the direct targeting of newly synthesized GM130 and GRASP65 to the Golgi apparatus morphologically. For this purpose, we first microinjected purified recombinant GTP-restricted mutant Sar1 protein (mSar1p) into the cytosol of NRK cells to inhibit ER-to-Golgi transport (Shima et al., 1998). FLAG-tagged GM130 or HA-tagged GRASP65 was then co-expressed with NAGFP by microinjecting two expression plasmids coding for those proteins together into the nuclei of the mSar1p injected cells. The cells were then fixed and processed for indirect immunofluorescence in order simultaneously to visualize newly synthesized FLAG-GM130 or GRASP65-HA, NAGFP and the endogenous Golgi marker giantin. As shown in Fig. 3A,B, NAGFP showed a nuclear envelope and cytoplasmic reticular pattern of fluorescence typical of the ER in the cells microinjected with mSar1p (top-right panels), whereas NAGFP showed a clear Golgi localization in mock injected cells (bottom-right panels). These results clearly showed that injection of mSar1p inhibited ER-to-Golgi transport and blocked the exit of NAGFP from the ER, as reported previously (Shima et al., 1998). By contrast, in the same mSar1p injected cells, FLAG-GM130 (Fig. 3A) and GRASP65-HA (Fig. 3B) clearly localized to the Golgi apparatus, showing typical juxtanuclear ribbon-like staining patterns, co-localizing with the endogenous Golgi marker giantin (compare left and middle panels).

We did notice that longer incubation of cells with mSar1p caused the gradual dispersal of the Golgi markers. Mannosidase II, a widely used Golgi stack marker protein, tended to be dispersed more rapidly than giantin (data not shown), suggesting that it might be recycled and accumulated back in the ER after the longer incubation. However, as shown in Fig. 3C, most of the mannosidase II showed a typical Golgi pattern, co-localizing with giantin and endogenous GM130, after 100 minutes of incubation with the microinjected mSar1p. Therefore, the Golgi apparatus remained intact during the time period used for our experiments.

These results suggested that GM130 and GRASP65 could localize directly to the Golgi apparatus without functional ER-to-Golgi transport mediated by COPII.

**In vitro binding of GM130 and GRASP65 to Golgi membranes**

The experimental results so far indicated that GM130 and GRASP65 could directly target to the Golgi apparatus. To confirm the specific binding of GM130 and GRASP65 to Golgi but not to ER membranes, we developed an in vitro binding assay by modifying an in vitro microsomal membrane translocation system (Sakaguchi et al., 1992). [35S]-labeled GM130 or GRASP65 were synthesized in vitro and used for Golgi membrane binding assay as described in Fig. 4 (top). Amounts of membrane-bound GM130 were quantified as described in Materials and Methods (bottom). (B) Same as (A) except that wild-type (Wt) or mutant (G2A and G196A) forms of GRASP65 were used for the experiments.

![Diagram](image.png)
and subjected to immunoprecipitation with anti-GM130 or anti-
recovered by centrifugation. The membranes were then extracted
bound to the Golgi membranes and the membranes were
and vice versa. In vitro synthesized GM130 or GRASP65 were
a complex with pre-existing GRASP65 on the Golgi membrane
GRASP65 (F.A.B., unpublished).
binds to GM130 with the same efficiency as wild-type
GRASP65 to the Golgi membrane, because the G2A mutant
to be involved in GM130 binding, it was not enough to localize
GRASP65. Although the second PDZ-like domain was shown
Golgi membranes and the importance of myristoylation and the
results again confirmed the specific binding of GRASP65 to
membranes in the in vitro binding assay (Fig. 5B). These
Correspondingly, these mutants did not bind to Golgi
membranes (Fig. 5A). These results confirmed the specific binding of
GM130 to the Golgi membrane and further implied that
GM130 is binding to the GRASP65 on the Golgi membrane.
It was also shown previously that mutation of GRASP65 at the
myristoylation site (G2A) or in the second PDZ-like domain
(G196A) reduced the Golgi localization (Barr et al.,
Correspondingly, these mutants did not bind to Golgi
membranes in the in vitro binding assay (Fig. 5B). These
results again confirmed the specific binding of GRASP65 to
GRASP65 and the importance of myristoylation and the second
PDZ-like domain for the Golgi membrane binding of
GRASP65. Although the second PDZ-like domain was shown to
be involved in GM130 binding, it was not enough to localize
GRASP65 to the Golgi membrane, because the G2A mutant
binds to GM130 with the same efficiency as wild-type
GRASP65 (F.A.B., unpublished).
Finally, we tried to show whether in vitro bound GM130 made
a complex with pre-existing GRASP65 on the Golgi membrane
and vice versa. In vitro synthesized GM130 or GRASP65 were
bound to the Golgi membranes and the membranes were
recovered by centrifugation. The membranes were then extracted
and subjected to immunoprecipitation with anti-GM130 or anti-
GRASP65 antibodies. The bound [35S]-GM130 was specifically
and quantitatively co-immunoprecipitated by the anti-GRASP65
antibody, suggesting that most of the bound GM130 was in a
complex with pre-existing GRASP65 on the Golgi membrane
(Fig. 6A). The bound [35S]-GRASP65 was also specifically co-
precipitated by anti-GM130 antibody, although the part of it was
not precipitated (Fig. 6B). This suggested that only part of the
bound GRASP65 was in a complex with pre-existing GM130 on
the Golgi membrane.

DISCUSSION

We have shown by three different approaches that newly
synthesized GM130 and GRASP65 are directly targeted to the
Golgi membrane without prior assembly on the ER and
subsequent vesicular transport to the Golgi apparatus. First, the
pulse-chase subcellular fractionation experiments showed that
GM130 and GRASP65 are quickly targeted to the Golgi
membrane without prior assembly on the ER (Fig. 1; Fig. 2).
Second, the morphological analyses showed that newly
synthesized GM130 and GRASP65 are targeted to the Golgi
apparatus under the condition where functional ER-to-Golgi
transport is inhibited by the presence of mSar1p (Fig. 3).
Finally, in vitro cell-free binding experiments showed that
GM130 and GRASP65 are specifically bound to the purified
Golgi membrane but not to microsomal membranes (Fig. 4;
Fig. 5). The direct targeting further suggests that the supply of
de novo synthesized integral membrane proteins is not
necessary for the precise targeting of newly synthesized
GM130 and GRASP65. In other words, sufficient target protein
and/or lipid are already located at the Golgi apparatus. If so,
what are the targets for GM130 and GRASP65?

Previous experiments have shown that GRASP65
specifically binds GM130 and might function as a GM130
receptor on the Golgi membrane (Barr et al., 1998; Barr et al.,
1997). Indeed, it was confirmed that C-terminal GM130
mutants (ΔC983 and M984A) that are defective for
GRASP65 binding (Barr et al., 1998) do not bind to purified rat liver
GM130 receptors (Barr et al., 1998; Barr et al.,
1997). For GRASP65, we have shown that N-
terminal myristoylation and the intact second PDZ-like
domain are necessary for its targeting to the Golgi membrane,
and that GM130 binding alone is not sufficient (Fig. 5B).
Furthermore, our data suggested that only part of the in vitro
bound GRASP65 is forming a complex with
pre-existing GRASP65 on the Golgi membrane (Fig. 6).
Therefore, GM130 probably binds to the free pre-existing
GRASP65 on the Golgi membrane in vitro and also under
physiological conditions in vivo. However, our results cannot
exclude the possibility that there are as-yet-unknown
alternative GM130 receptor(s). In fact, it is known that
GRASP55, which is a close homolog of GRASP65, can also
bind to GM130 but with a much lower affinity than GRASP65
(Shorter et al., 1999). For GRASP65, we have shown that N-
terminal myristoylation and the intact second PDZ-like
domain are necessary for its targeting to the Golgi membrane,
and that GM130 binding alone is not sufficient (Fig. 5B).
Furthermore, our data suggested that only part of the in vitro
bound GRASP65 is associated with pre-existing GM130 and
another part is associated with some other target(s) on the
Golgi membrane (Fig. 6). If GM130 only binds to GRASP65,
GRASP65 has to bind some target(s) on the Golgi membrane
other than GM130 to make the binding of the GM130-
GRASP65 complex to the Golgi membrane possible.
Therefore, it is probable that interactions with other proteins
and/or lipids are playing an important role in targeting
GRASP65 to the Golgi membranes. We are trying to evaluate
these possibilities.

One could argue that GM130 and GRASP65 are targeted to
the ER and then transported to the Golgi stack by unidentified
COPII-independent pathway because mSar1p merely stops the
COPII-dependent ER-to-Golgi transport pathway. However,
we do not think that this is the case for several reasons. First,
targeting to the Golgi membrane as shown by pulse-chase
experiments (Fig. 1; Fig. 2) is too rapid to support the idea of
an indirect pathway. Second, GM130 and GRASP65 can in fact
only bind to the Golgi and not to the microsomal (ER)
membrane in vitro binding experiments (Fig. 4). Third, the
existence of such a COPII-independent cargo transport
pathway has not yet been proved.
The morphological analyses and cell fractionation analyses suggested that the bulk of GM130 and GRASP65 are directly targeted to the Golgi stack. Nevertheless, the possibility cannot be excluded that some of them are targeted to the ERGIC/cis-Golgi compartment and then reaches the cis-Golgi cisternae quickly by a COPII-independent pathway. In fact, an earlier study using immunoelectron microscopy showed that significant amount of GM130 is found in tubular reticular profiles, which are thought to be the ERGIC/cis-Golgi compartment (Nakamura et al., 1995).

Currently, it is controversial to what extent or with what rate Golgi-resident proteins cycle between the ER and the Golgi. In a series of elegant microscopic studies, the continuous recycling of Golgi-resident marker proteins back to the ER in interphase and mitotic cells has been demonstrated (Cole et al., 1998; Cole et al., 1996; Zaal et al., 1999). However, other Golgi-resident proteins were found to reside in discrete Golgi-derived vesicles and do not recycle significantly back to the ER in interphase and mitotic cells (Shima et al., 1998; Shima et al., 1997). There is also some supporting biochemical evidence that Golgi-derived vesicles stay separate from the ER during mitosis (Jesch and Linstedt, 1998). One explanation for these discrepancies is that the recycling rate is different for each Golgi-resident protein, some proteins actively recycling back to the ER while others are sequestered for long periods in the Golgi apparatus. A hybrid model in which some proteins are retrieved back to the ER during mitosis while others remain in Golgi-derived vesicles could explain these discrepancies. In fact, we have observed that mannosidase II tended to be dispersed more rapidly than giantin by longer incubation with mSar1p (S.-I.Y. et al., unpublished). Importantly, in the same condition, GM130 and GRASP65 remained in discrete punctate cytoplasmic structures and not absorbed into the ER (S.-I.Y. et al., unpublished). Similarly, it was recently reported that GM130 and GRASP65 remained in a distinct cytoplasmic compartment under conditions where most of the Golgi resident proteins are forced to return back to the ER (Sönnichsen et al., 1998). Thus, GM130 and GRASP65 might stay at the cis-Golgi and function as a landmark for incoming vesicles through vesicle tethering.

In the cisternal maturation model, COPI vesicles only serve to retrieve retrograde cargo and thus drive Golgi stack maturation in a cis-to-trans direction. Golgi-resident proteins have to be recycled actively back to proper Golgi cisternae, otherwise they will be shipped away by the maturing cisternae. Supporting evidence for this model was recently reported: Golgi-resident membrane proteins can be efficiently incorporated into the transport vesicles produced from purified Golgi membranes in the presence of GTP instead of GTPγS (Lanoix et al., 1999). Whether GM130 and GRASP65 can dissociate from the membrane and transfer to other membranes, there is no need for them to be packaged into retrograde vesicles. GM130 might dissociate from the maturing cisternae and re-associate with new cis cisternae to remain as a landmark for the cis-Golgi membrane.

The in vivo and in vitro experimental systems developed here will be of importance in future analysis of the dynamics of GM130 and GRASP65 and the evaluation of the models for the maintenance of the Golgi structure.

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