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Dynamics of Golgi Matrix Proteins after the Blockage of ER to Golgi Transport

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When the ER to Golgi transport is blocked by a GTP-restricted mutant of Sar1p (H79G) in NRK-52E cells, most Golgi resident proteins are transported back into the ER. In contrast, the *cis*-Golgi matrix proteins GM130 and GRASP65 are retained in punctate cytoplasmic structures, namely Golgi remnants. Significant amounts of the *medial*-Golgi matrix proteins golgin-45, GRASP55 and giantin are retained in the Golgi remnants, but a fraction of these proteins relocates to the ER. Golgin-97, a candidate *trans*-Golgi network matrix protein, is retained in Golgi remnant-like structures, but mostly separated from GM130 and GRASP65. Interestingly, most Sec13p, a COPII component, congregates into larger cytoplasmic clusters soon after the microinjection of Sar1p(H79G), and these move to accumulate around the Golgi apparatus. Sec13p clusters remain associated with Golgi remnants after prolonged incubation. Electron microscopic analysis revealed that Golgi remnants are clusters of larger vesicles with smaller vesicles, many of which are coated. GM130 is mainly associated with larger vesicles and Sec13p with smaller coated vesicles. The Sec13p clusters disperse when p115 binding to the Golgi apparatus is inhibited. These results suggest that *cis*-Golgi matrix proteins resist retrograde transport flow and stay as true residents in Golgi remnants after the inhibition of ER to Golgi transport.

Key words: Golgi remnants, membrane traffic, recycling, tethering, Sar1p, Sec13p.

Abbreviations: CGN, *cis*-Golgi network; EM, electron microscopy; ER, endoplasmic reticulum; IC, intermediate compartment; NAGFP, *N*-acetylglucosaminyltransferase I–green fluorescent fusion protein; TGN, *trans*-Golgi network; VTC, vesicular tubular clusters.

Newly synthesized proteins, oligosaccharides and lipids are transported to the Golgi apparatus from the endoplasmic reticulum (ER), and there they are processed, sorted and then sent out to their final destinations (*1*, *2*). Electron microscopic studies have revealed that the Golgi apparatus has a characteristic stacked cisternal structure, especially obvious in higher eukaryotes including mammalian and plant cells (*2*, *3*). In mammalian cells, many stacked Golgi cisternae are gathered around the perinuclear region or centrosome and connected by tubules to form an elaborate ribbon-like structure visible under the light microscope (*2*).

Exocytic material is transported through the Golgi apparatus by vesicles shuttling within the Golgi and between the Golgi and other exocytic organelles including the ER, endosomes, lysosomes and the plasma membrane (*1*). The transport vesicles are categorized into two classes. The first class are regularly shaped small vesicles (70–100 nm in diameter) produced by distinct cytoplasmic coats (COPI, COPII and clathrin coat), and the molecular mechanisms of their formation have been extensively described (*4*, *5*). The second class are large pleomorphic vesicles observed as tubular or reticular profiles under the electron microscope. The molecular mechanisms for the production of this latter class of vesicles remain obscure, but are proposed to involve the fission of larger vesicles or the fusion of uncoated smaller vesicles (*1*, *2*). Golgi cisternae can also be considered as vesicles of the latter class in the cisternal maturation model where exocytic material is transported by maturing cisternae (*6*, *7*). Which type of vesicles forms the major vehicle for exocytic material, or whether COPI vesicles carry any exocytic cargo at all, is still a matter of debate (*2*, *6*, *7*). There is also a third model that predicts that transient contact of vesicles or cisternae enables the transport of exocytic materials (*8*). Despite these outstanding issues, it is now widely accepted that some Golgi resident proteins must recycle or be retrogradely transported back to

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upstream exocytic compartments to counter the flow of exocytic material, which actively, in the cisternal maturation model, or passively, in the vesicular transport model, transport Golgi resident proteins (*9–11*).

In spite of this extensive anterograde and retrograde vesicular flow, the identity and structure of the Golgi apparatus are apparently maintained. Therefore, it is quite reasonable to assume the existence of structural proteins that help to maintain the identity and structure of the Golgi apparatus. In fact, the mammalian Golgi apparatus has been shown to have a protein scaffold or membrane skeleton on the cytoplasmic side of the cisternal membranes (*12–14*). Many candidate proteins that fulfill potential structural roles have been identified, including (i) golgins, a family of proteins with extensive coiled-coil domains including GM130, golgin-45, golgin-97, and giantin (*15–17*), (ii) golgin binding proteins including GRASP65, GRASP55 and GCP60 (*18–20*), and (iii) subtypes of ankyrin and spectrin that function in the organization of the actin-based membrane skeleton (*21–23*) (for reviews, see Refs. *2*, *24*, and *25*).

The best characterized are the *cis*-Golgi matrix proteins, GM130 and GRASP65. GRASP65 is an NEM sensitive protein that functions in Golgi stacking. It is N-terminally myristoylated and binds GM130 *via* a PDZ-like domain (*18*, *26*). GM130 is involved in cisternal reassembly together with p115 after the mitotic fragmentation of the Golgi apparatus *in vitro* (*27*). GM130 and GRASP65 have been shown to be enriched at the *cis*-cisternae of the Golgi apparatus, but the mechanism of this enrichment remains obscure (*15*, *18*, *19*). p115 localizes at *cis*-Golgi cisternae and the *cis*-Golgi network (CGN)/intermediate compartment (IC) (*28*, *29*) and is reported to function in ER to Golgi transport, intra-Golgi transport and transcytosis (*28*, *30–32*). It has also been reported that p115 binds GM130 and giantin to build a tethering complex that facilitats the docking and fusion of COPI vesicles to cisternal Golgi membranes (*33*). Giantin is a type II transmembrane protein with a huge coiled-coil rich cytoplasmic domain that is compatible with the proposed tethering function (*17*, *34*, *35*). Recently, it was also shown that golgin-45 together with GRASP55 functions in the organization and maintenance of the stacked Golgi structure. GRASP55 is a close homologue of GRASP65 and localizes throughout the Golgi with slight concentration at the medial cisternae (*19*). Golgin-45 was found as a GRASP55 interacting protein and co-localizes with GRASP55 (*36*).

We have recently shown that GM130 and GRASP65 are directly incorporated into the pre-existing Golgi matrix (*37*). It was also recently shown that GRASP65 and GRASP55 can bind to the cytoplasmic tail of p24 family proteins and this might contribute to the specific Golgi membrane localization of GRASP proteins (*38*). However, the precise mechanisms of targeting and organization of Golgi matrix proteins are still obscure. To elucidate the molecular mechanisms of Golgi matrix organization, we aimed to describe the dynamics of Golgi matrix proteins. For this analysis, we took advantage of the dominant negative Sar1p mutant, which inhibits ER to Golgi transport. Here we show that Golgi matrix proteins resist retrograde transport flow and are retained in Golgi remnant vesicular structures under conditions where ER to Golgi transport is inhibited.

MATERIALS AND METHODS

Cell Culture and Production of a Stable Cell Line—NRK-52E cells (*39*) were obtained from Health Science Research Resources Bank (Osaka). NRK cells and NRK-52E cells were grown as described before (*37*). NRK-52E cells stably expressing *N*-acetylglucosaminyltransferase I–green fluorescent fusion protein (NAGFP) were cloned after transfection of pCNG2 (*40*) and maintained in medium containing 1 mg/ml G418 (Sigma-Aldrich).

Production and Infection of Recombinant Adenovirus—A DNA fragment coding a VSV-G (ts045)-EGFP fusion protein was excised from pcDNA3-VSVG-EGFP (*41*, *42*) and cloned into cosmid pAxCAwt using an Adenovirus Expression Vector Kit (Takara Bio, Kusatsu). A DNA fragment coding Sar1p(H79G) or Sar1p(T39N) mutant proteins with a FLAG epitope at the N-terminus [FLAG-Sar1p(H79G) and FLAG-Sar1p(T39N)] was constructed by PCR using pET-11 Sar1p mutants as templates (*43*) and cloned into cosmid pAxCALNLw using an Adenovirus Cre/loxP Kit (Takara Bio) (*44*). Recombinant viruses were produced and cells were infected according to the manufacturer's instructions. Regularly, 3×10^5 NRK-52E cells on a 3 cm dish were used for immunofluorescence and 3×10^6 cells on a 9 cm dish for cell fractionation. Multiplicity of infection (MOI) was 500 for the VSV-G (ts045)-EGFP expressing virus, 1,000 for the FLAG-Sar1p(H79G) or FLAG-Sar1p(T39N) expressing virus, and 100 for the Cre recombinase expressing virus. The same MOI was used for double and triple infection. The cells were cultured for 24 h under standard conditions and then used for experiments unless otherwise stated.

Microinjection of Proteins and Plasmids—Microinjections of proteins and plasmids were performed as previously described (*27*, *37*). The plasmid coding ΔC436-GFP, ΔC237-GFP, and pEGFP (0.3 mg/ml plasmid, 5 mg/ml cascade blue-BSA) was microinjected into the nuclei of the cells. A routine microinjection required 15 min (about 300 cells were injected) and the cells were incubated for the indicated times. For shorter time courses, microinjection was performed for 2 min (about 40 cells were injected).

Antibodies—The following primary antibodies were purchased: mouse monoclonal anti-GM130, mouse monoclonal anti-EEA1 (BD Biosciences, Franklin Lakes, NJ, USA), mouse monoclonal anti-TGN38 (Affinity BioReagents, Golden, CO, USA). The following antibodies were kindly provided: rabbit anti-β-COP (EAGE) (Rainer Duden, University of Cambridge, Cambridge, UK) (*45*), rabbit anti-mannosidase II antibody (Kelly Moremen, University of Georgia, Athens, GA, USA) (*46*). Rabbit anti-giantin (*35*), rabbit anti-GM130 (*15*), mouse monoclonal anti-GRASP65 (7E10) (*19*), affinity purified sheep anti-GRASP55 (FBA34) (*19*), and goat anti-golgin-45 (*36*) were previously described. Rabbit polyclonal anti-p115 was raised against a purified recombinant His-tagged rat p115 fragment (C-terminal 354 to 959 amino acid residues). Rabbit anti-Sec13p was raised against a purified recombinant GST-human Sec13p fusion protein and affinity purified using purified recombinant His-tagged

human Sec13p. Rabbit anti-golgin-97 was raised against purified recombinant His-tagged rat golgin-97 and affinity purified using the same antigen. The following secondary antibodies were purchased and used in appropriate combinations: Alexa488-conjugated goat anti-mouse, goat anti-rabbit, donkey anti-goat antibodies (Molecular Probes, Eugene, OR, USA), Cy3-conjugated goat anti-mouse, donkey anti-mouse, goat anti-rabbit, donkey anti-sheep antibodies, Cy5-conjugated goat anti-mouse antibodies, horse radish peroxydase (HRP) -conjugated donkey anti-sheep, donkey anti-goat, goat anti-rabbit antibodies (Jackson Immuno Research Laboratories, West Grove, PA, USA).

Indirect Immunofluorescence and Confocal Microscopy—Indirect fluorescence was performed by modified methods of Heggeness *et al.* and Bonifacino *et al.* (*47*, *48*). Cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH7.4) for 20 min at room temperature and rinsed thoroughly with phosphate buffered saline (PBS). The cells were then permeabilized with 0.1% Triton X-100 in PBS for 4 min and incubated with 0.2% (w/v) fish skin gelatin (Sigma-Aldrich) in PBS (gelatin-PBS) for 5 min. The cells were incubated for 1 h with primary antibodies diluted in gelatin-PBS and rinsed thoroughly with gelatin-PBS after incubation. The cells were then treated with secondary antibodies in the same way. For TGN38 staining, Triton X-100 treatment was omitted and the incubations with primary and secondary antibodies were performed in gelatin-PBS containing 0.1% saponin. The cells were post-fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 20 min at room temperature. The stained cells were observed under an LSM510 confocal microscope (Carl Zeiss, Jena, Germany).

Subcellular Fractionation and Western Blotting—Subcellular fractionation was performed as previously described except the membranes were centrifuged for 2 h at 100,000 ×*g* in the membrane sedimentation experiments (*37*). In the membrane flotation experiment, 250 μl of post nuclear supernatant was added with 1 ml of HB containing 2.5 M sucrose (final 2 M, 1.25 ml) and layered on the bottom of a 5 ml tube. The sample was then overlaid serially with 1.6 , 1.4, and 0.8 M sucrose layers (1.25 ml, containing HB) and centrifuged in an SW50Ti rotor (Beckman) for 3 h at 50,000 ×*g*. The top 3ml was collected as the membrane fraction and rest of the sample was collected as the cytosolic fraction. Protein was precipitated with trichloroacetic acid (*49*) and solubilized in SDS-PAGE sample buffer. Western blotting was performed as described before (*15*) but using Immobilon-P (Millipore Corp., Bedford, MA, USA). HRP was visualized by ECL western blotting reagents (Amersham Biosceinces, Piscataway, NJ, USA), and images were digitally analyzed and quantified by LAS 1000 plus (FUJIFILM, Tokyo).

Electron Microscopy—The position of the cells of interest was located on an embedded block by referring to the grid pattern of the CELLocate coverslip, and then sections were prepared from this position. Conventional electron microscopy (EM) was performed as described previously (*50*), except 2.5% glutaraldehyde was used for fixation. For immuno-EM, the pre-embedding silver enhancement immunogold method was performed as previously described (*50*) with a slight modification. Cells were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h and washed three times in 0.1 M phosphate buffer (pH 7.4) (PB). The sample was then dipped in PB containing 15% glycerol and 35% sucrose, frozen in liquid nitrogen and thawed, and then incubated in PB containing 0.005% saponin, 10% BSA, 10% normal goat serum, and 0.1% cold water fish skin gelatin for 30 min for blocking. Cells were then treated overnight with rabbit IgG against Sec13 or GM130 in the blocking solution. The cells were then processed, and ultra-thin sections were prepared and observed under an H7000 electron microscope (Hitachi, Tokyo) as described before.

RESULTS

Expression of Sar1p Mutants Using the Adenovirus Expression System—We have previously shown that ER to Golgi transport is rapidly inhibited when NRK cells are microinjected with Sar1p(H79G), and that most of the Golgi resident proteins remain in the Golgi apparatus for up to 60 min (*37*) (see Fig. 5C). We noticed that most of the Golgi resident proteins are then retrieved into the ER after longer incubation times (240 min) (Fig. 5C and unpublished observations). Interestingly, the bulk of the *cis*-Golgi matrix proteins GM130 and GRASP65 relocate to punctate cytoplasmic structures distinct from the ER (Figs. 5C and 7B). Similar results were reported by others while our work was in progress (*51–53*). However, in these studies it could not be determined whether a small amount, if any, of GM130 or GRASP65 relocated to the ER because of the limitations of the morphological analyses used. We, therefore, decided to analyze the effect of Sar1p on Golgi resident proteins in more detail, using a biochemical approach combined with morphological analysis. For this purpose, we produced recombinant adenovirus expressing Sar1p mutants for efficient, controlled, and even expression of the protein. Because Sar1p mutants are toxic to virus packaging cells, the mutants were designed to express only in target cells upon double infection with Cre recombinase expression virus as described in "MATERIALS AND METHODS" (*44*). We only present data using NRK-52E cells (*39*) in this report because they are highly susceptible to virus infection and their cytoplasm is more widely spread and well suited for morphological observations. Similar results were obtained using parental NRK and HeLa cells.

After 12 h of infection, Sar1p mutants started to express and the amount continued to rise until 24 h (Fig. 1A). To analyze the effect on ER to Golgi transport, the transport of VSV-G (ts045)-GFP was monitored. At 39.5°C, VSV-G (ts045)-GFP cannot exit the ER and accumulates in the ER. After shifting the temperature to 32.5°C, most of the accumulated protein exits the ER and is synchronously transported to the Golgi and then on to the plasma membrane (*11*). The cells were singly infected with VSV-G (ts045)-GFP virus or triply infected with Sar1p mutant virus, Cre recombinase virus and VSV-G (ts045)-GFP virus and incubated for 24 h at 39.5°C to allow the accumulation of VSV-G (ts045)-GFP in the ER (Fig. 1B, top left gallery). The cells were then incubated at 32.5°C for 120 min. VSV-G (ts045)-GFP reached the plasma membrane with residual Golgi localization in

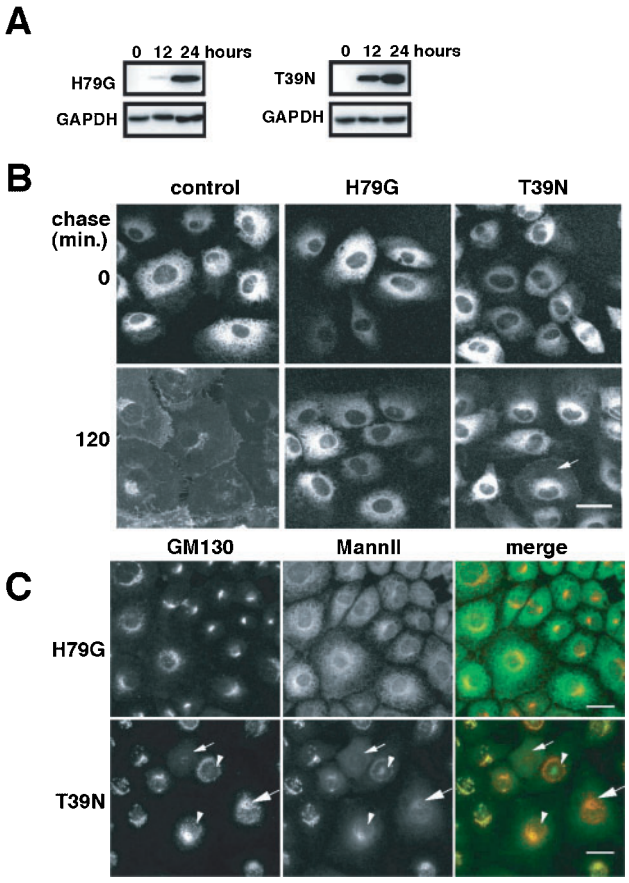

Fig. 1. **ER to Golgi transport is inhibited by infection of recombinant adenovirus expressing Sar1p mutants.** (A) NRK-52E cells were co-infected with recombinant Sar1p(H79G) or Sar1p(T39N) expressing adenovirus and cre recombinase expressing virus. After 0, 12, and 24 h, the expression of Sar1p(H79) was analyzed by western blotting using anit-Flag antibody. The amount of glyceroaldehyde 2-phosphate dehydrogenease (GAPDH) is shown as a control. (B) Cells were infected only with VSV-G (ts045)-GFP virus (left gallery) or triply together with Sar1p(H79G) virus (middle gallery) or Sar1p(T39N) virus (right gallery) and cre recombinase virus. The cells were incubated at 39.5°C for 24 h to express proteins but accumulate VSV-G (ts045)-GFP in the ER (top gallery). The cells were further incubated at 32°C for 120 min to monitor the transport of VSV-G (ts045)-GFP (bottom gallery). (C) NRK-52E cells were co-infected with recombinant Sar1p(H79G) or Sar1p(T39N) expressing adenovirus and cre recombinase expressing virus and incubated at 37°C for 24 h. The cells were then processed for double labeling with anti-GM130 (red, middle gallery) and anti-mannosidase II (Mann II; green, left gallery) antibodies and observed by epifluorescence microscopy. Bar = 20 μm.

control cells (Fig. 1B, bottom left panel). In contrast, VSV-G (ts045)-GFP still accumulated in the ER in virtually all cells after the expression of Sar1p(H79G) (Fig. 1B, bottom middle panel). These results clearly show that ER to Golgi transport is completely blocked by Sar1p(H79G). As was reported before (*51*), mannosidase II, which is mostly enriched in the medial/trans cisternae (*54*), relocated completely to the ER after the expression of Sar1p(H79G) (Fig. 1C, upper gallery). Similar results were obtained for stably expressing *N*-acetylglucosaminyltransferase I–green fluorescent fusion protein (NAGFP), which was used as a Golgi marker in the biochemical experiments described below (unpublished observation). In contrast, a *cis*-Golgi matrix protein, GM130 did not relocate to the ER, but was retained in punctate cytoplasmic structures after the expression of Sar1p(H79G) (Fig. 1C, top left panel), recapitulating the results of microinjection.

In contrast, a GDP restricted mutant, Sar1p(T39N) did not affect the structure of the Golgi apparatus in microinjection experiments even at a protein concentration ten times higher than that at which Sar1p(H79G) showed clear effects (Fig. 5 and unpublished observation). We then tried to express Sar1p(T39N) in the adenovirus expression system. We could express Sar1p(T39N) in moderate levels (Fig. 1A, right gallery), although higher protein expression was hampered by the toxicity of the virus infection. Under the best conditions achieved, the anterograde transport monitored by VSV-G transport was greatly reduced (Fig. 1B). However, significant numbers of cells (~10%) showed plasma membrane staining (Fig. 1B, arrow in bottom right panel) indicating that the transport block was not complete. Under these conditions, mannosidase II was seen to relocate to the ER in about 5% of cells. The relocalization to the ER was incomplete and many cells still showed juxtanuclear Golgi like staining, although the staining intensity at the Golgi area was clearly reduced (Fig. 1C, bottom gallery, arrowheads). Importantly, GM130 was still seen in punctate cytoplasmic structures in cells where most of the mannosidase II was relocated to the ER (large arrowhead), similar to the results in Sar1p(H79G) expressing cells. In a very small number of cells (fewer than 0.1%), diffuse cytoplasmic staining was observed for GM130, similar to previous reports (Fig. 1C, bottom gallery, small arrow) (*52*, *53*). Because of this incomplete effect of Sar1p(T39N) on the Golgi apparatus, we only used Sar1p(H79G) for further study. The difference in the effects of Sar1p(H79G) and Sar1p(T39N) will be discussed later.

Morphological Analysis of the Relocation of Golgi Resident Proteins after the Expression of Sar1p (GTP)—GM130 and GRASP65 completely co-localized in the punctate structures after the expression of Sar1p(H79G), indicating that the *cis*-Golgi matrix proteins relocate to the same structures (Fig. 2A). Hereafter, we call these cytoplasmic punctate structures Golgi remnants.

p115, which localizes to *cis*-Golgi cisternae and the CGN/intermediate compartment (IC) (*28*, *29*), showed a high degree of co-localization with GM130 and GRASP65 in the Golgi area in control cells. Interestingly, this co-localization was preserved after the expression of Sar1p(H79G), suggesting that p115 remaines bound to the Golgi remnants (Fig. 2B). This was confirmed because p115 was efficiently removed from the Golgi remnants by the expression of N-terminal GM130 fragments, which inhibit the binding of p115 to the Golgi membrane (discussed below).

In contrast, β-COP a component of the COPI coat that also localizes at the CGN/IC and *cis*-Golgi cisternae (*45*, *55*), was found to give a diffuse cytoplasmic pattern in the presence of Sar1p(H79G). β-COP was still seen at the Golgi apparatus 1 h after the microinjection of Sar1p(H79G), but staining on the Golgi remnants was greatly reduced after 4 h incubation (unpublished observation). This was in close temporal correlation with the

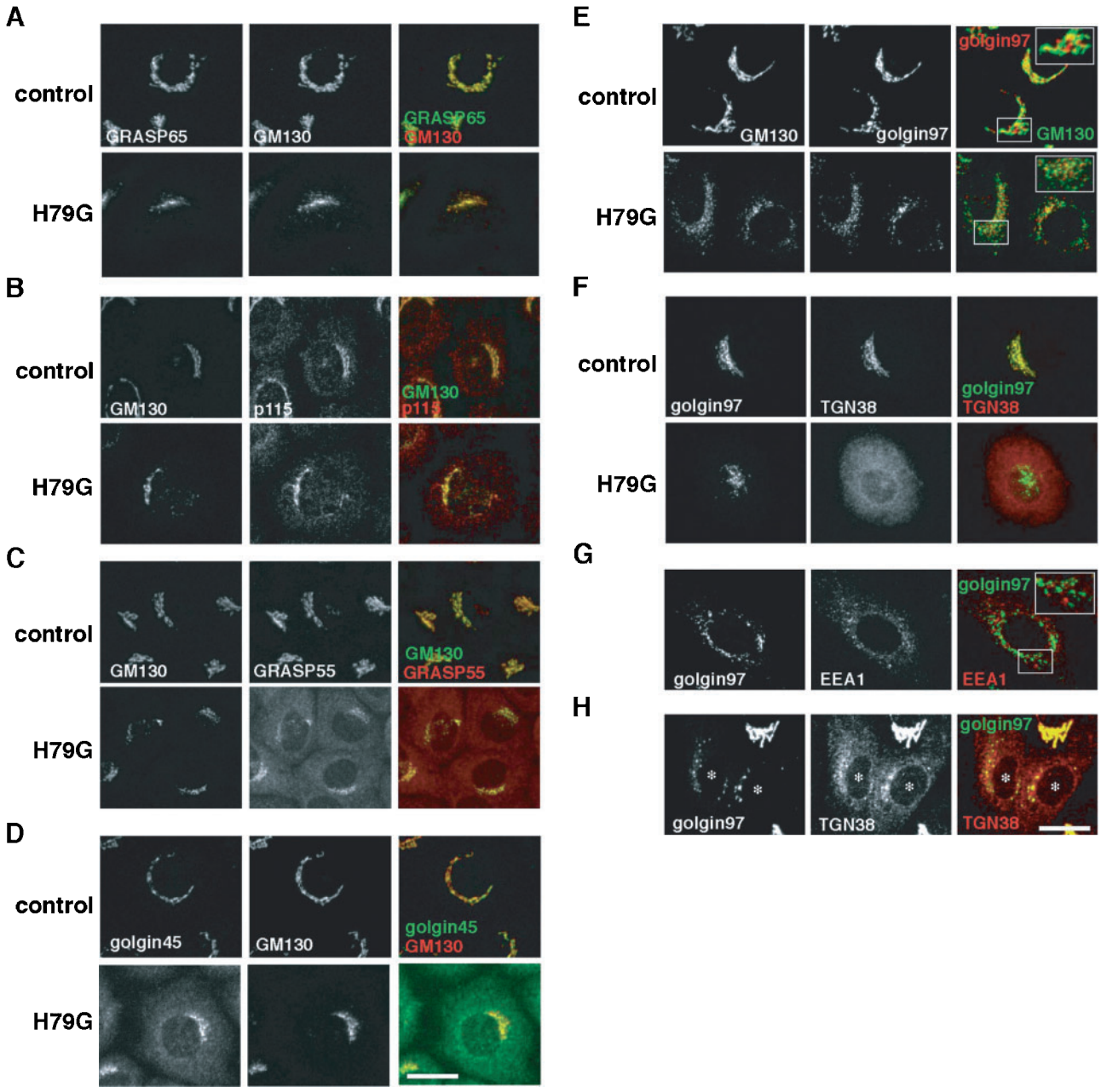

Fig. 2. **Relocalization of Golgi resident proteins after the introduction of Sar1p(H79G).** (A–G) NRK-52E cells were left uninfected (control) or co-infected with recombinant Sar1p(H79G) expressing adenovirus and Cre recombinase expressing virus (H79G) and incubated for 24 h. The cells were then processed for double labeling with antibodies and observed by confocal microscopy. Two-dimensional projections of the confocal images are shown. (A) GRASP65 and GM130. (B) GM130 and p115. (C) GRASP55 and GM130. (D) Golgin-45 and GM130. (E) GM130 and golgin-97. (F) Golgin-97 and TGN38. (G) Golgin-97 and EEA1. (H) Purified recombinant Sar1p(H79G) was microinjected into the cells and the cells were incubated for 4 h. The cells were then processed for double labeling with antibodies to golgin-97 and TGN38. Merged images are shown on the right (left and middle gallery are colored as green and red, respectively). Bar = 20 μm.

relocalization of mannosidase II to the ER. β-COP appeared to relocate to the cytoplasm, although it was not possible to exclude the possibility that there was some β-COP associated with membrane structures such as the ER.

GRASP55 and golgin-45, Golgi matrix proteins that localize mainly to medial cisternae (*19*, *36*), showed overlapping but slightly different staining patterns compared with GM130, as typically found when *cis*- and *medial*-/*trans*-Golgi cisternal proteins are compared (Fig. 2C and D, control) (*15*). After the expression of Sar1p(H79G), some GRASP55 and golgin-45 localized adjacent to GM130 positive structures, but significant amounts of these two proteins were present in a cytoplasmic reticular structure, most probably the ER (Fig. 2C and D, H79G). Giantin, which localizes in all parts of the stacked Golgi cisternae but is enriched at dilated rims (*17*, *34*), showed similar pattern of relocalization (unpub-

lished observation). It is possible that the accumulation of these proteins in the ER might be caused by the accumulation of newly synthesized proteins in the ER (discussed later). However, we still believe that some portion of pre-existing proteins did relocalize in the ER because similar ER-like localization was observed when Sar1p(H79G) was introduced by microinjection into cells incubated for 4 h in the presence of cycloheximide to block new protein synthesis (unpublished observation).

Golgin-97 is a peripheral membrane protein of the golgin family and localizes in the *trans*-Golgi network (TGN) (*16*). It has a GRIP domain at the C-terminus, which is essential for its TGN localization (*56–58*). In control cells, golgin-97 localized in structures very close to, but apparently distinct from, GM130 (Fig. 2E, control). After the expression of Sar1p(H79G), golgin-97 relocated to punctate structures spread throughout the cytoplasm, similar to the behavior of GM130 (Fig. 2E, H79G). However, golgin-97 localized within these structures was discrete from GM130, closely reflecting the difference in the localization of these proteins in control cells (see insets in Fig. 2E). It is known that there is a recycling pathway between the TGN and endosomes, and these two compartments collapse into one after brefeldin A treatment (*59–61*). However, the golgin-97-positive structures that appeared after the expression of Sar1p(H79G) did not co-localize with an early endosomal maker, EEA1 (Fig. 2G), a late endosomal marker, Rab7, or a lysosomal marker, lgp110 (unpublished observations), suggesting that golgin-97 was in distinct TGN remnant structures.

Golgin-97 did co-localize with TGN38, a well-described transmembrane protein of the TGN (*62*), in control cells (Fig. 2F, control). After the expression of Sar1p(H79G) by adenovirus, TGN38 was found not in the golgin-97 positive structure, but in the ER (Fig. 2F, H79G). Because TGN38 has a high rate of turnover (*62*) and newly synthesized protein contributes significantly to this ER localization (discussed later), we next examined whether pre-existing TGN38 relocates to the ER by microinjecting cells with Sar1p(H79G) in the presence of cycloheximide. After 4 h of incubation, much TGN38 was found in the ER, although a significant amount still co-localized with golgin-97 (Fig. 2H). Therefore, the ER localization of TGN38 after the expression of Sar1p(H79G) is partly accounted for by the relocation of pre-existing TGN38 into the ER.

Biochemical Analysis of the Relocation of Golgi Resident Proteins after the Expression of Sar1p (GTP)—We then analyzed the redistribution of these Golgi resident proteins by subcellular fractionation (Fig. 3, A and B). We used NRK-52E cells stably expressing NAGFP as a *medial-/trans*-Golgi cisternal marker because mannosidase II could not be detected by western blotting with available antibodies.

The ER marker calnexin distributed in heavier membrane fractions with a peak in fraction 9 in control cells. This did not change after Sar1p(H79G) expression. In contrast, the Golgi marker NAGFP distributed in lighter fractions with a peak in fraction 7 in control cells, and, after Sar1p(H79G) expression, clearly relocated to the ER fractions with a peak in fraction 9, the same as calnexin. Similar to NAGFP, GM130 and GRASP65 distributed in lighter membrane fractions with a peak in fraction 8 in control cells. Interestingly, GRASP65 showed a second peak in much lighter fractions (fractions 2–3), implying that there was subpopulation of membranes in which only GRASP65 existed. After the expression of Sar1p(H79G), the distribution of GM130 shifted to lighter membrane fractions with a peak in fractions 3 and 4 (Fig. 3, A and B, GRASP65). The distribution of GRASP65 also shifted to a lighter fraction with a peak in fractions 3 and 4, similar to GM130, and the peak in fraction 8 clearly disappeared. The clear differences in the distributions of GM130 and GRASP65 compared to calnexin and NAGFP indicate that GM130 and GRASP65 were not retrieved back to the ER but stayed on Golgi remnant membranes after the expression of Sar1p(H79G).

Golgin-45 distribution, with a peak in fraction 7, was similar to those of GM130 and NAGFP in control cells. In contrast to GM130 and GRASP65, golgin-45 distribution shifted to heavier membrane fractions with a peak in fraction 9 after the expression of Sar1p(H79G), indicating its redistribution into the ER. On the other hand, a significant amount remained in lighter fractions together with GM130 (fractions 2–5), indicating that some of golgin-45 remained in Golgi remnant membranes. GRASP55 co-distributed with golgin-45 with a peak in fraction 7 in control cells. However, like GRASP65, there was also a peak in lighter membrane fractions where less golgin-45 was seen (fractions 2–4), implying that there was subpopulation of membranes in which only GRASP55 existed. After the expression of Sar1p(H79G), the peak in fraction 7 shifted to fraction 9 indicating the redistribution of GRASP55 into the ER. Again, a significant amount of GRASP55 remained in lighter membrane fractions indicating a portion of GRASP55 redistributed to the Golgi remnant membranes. Giantin distributed similarly to GRASP55 with peaks in heavier (fraction 7) and lighter (fraction 2) membrane fractions. Once again, the peak in fraction 7 shifted to fraction 9, indicating its relocalization to the ER upon the expression of Sar1p(H79G). A significant amount of giantin remained in the lighter membrane fractions indicating that a portion of giantin also redistributed to the Golgi remnant membranes.

As reported previously (*62*), TGN38 appeared as two discrete bands (~90 and ~55 kDa) in control cells (Fig. 3A). The upper band (~90 kDa) was solely distributed in Golgi membrane fractions with a peak in fraction 7 and was thought to be a mature form localized in the TGN. The lower band (~55 kDa) occasionally appeared as a doublet or triplet; part of this was present in the ER fractions (fractions 8–10) while the remainder was in lighter fractions (fractions 1–2). The former population was thought to be a newly synthesized immature form of the protein retained in the ER, and the latter population was thought to be an immature form *en route* to the TGN. After Sar1p(H79G) expression, the upper band disappeared almost completely. In contrast, the amount of the lower band increased greatly in the ER fractions (fractions 8–10). This is a strong indication that newly synthesized immature TGN38 accumulated in the ER after Sar1p(H79G) expression. We did not observe a relocalization of the upper band to the ER. This may be caused by

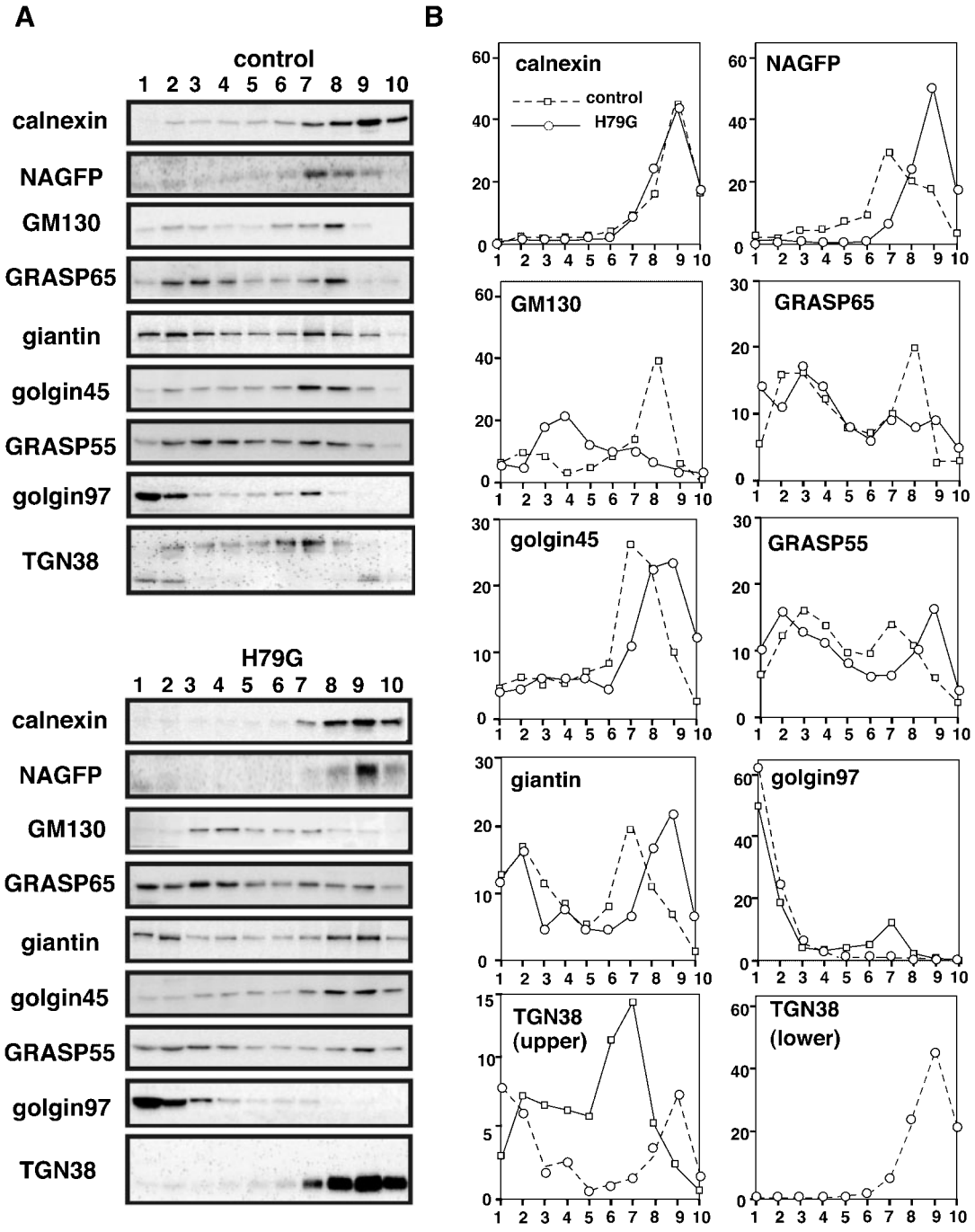

Fig. 3. **Subcellular fractionation analysis of the effect of Sar1p(H79G).** (A) NRK-52E stably expressing NAGFP was infected with only the Sar1p(H79G) virus (control; top gallery) or co-infected with Sar1p(H79G) and cre recombinase adenovirus (H79G; bottom gallery) and incubated for 24 h. Cells were then homogenized and the post-nuclear supernatant was fractionated on a Nycodenz step density gradient. The fractions were analyzed by western blotting for calnexin, NAGFP, GM130, GRASP65, golgin-45, GRASP55, giantin, golgin-97 and TGN38. The left (lane 1) is the top fraction and the right (lane 10) is the bottom fraction. (B) The bands in (A) were quantified, and the relative amount of protein in each fraction (%) for total protein in all fractions were calculated and plotted. Fraction number is indicated at the bottom of each panel.

the degradation of the mature form due to either transport to lysosomes or an ER associated degradation system (*63*).

Much of the golgin-97 was recovered in lighter membrane fractions, although some was co-distributed with TGN38 (a peak in fraction 7). After the expression of Sar1p(H79G), golgin-97 disappeared from the heavier membrane fractions and was found mostly in the top fraction. These results suggest the possibility that golgin-97 dissociated from the membranes during the fractionation procedure. Therefore, we performed a membrane flotation experiment. As shown in Fig. 4, more than 90% of golgin-97 floated in control cells indicating that most golgin-97 was associated with membranes. This result

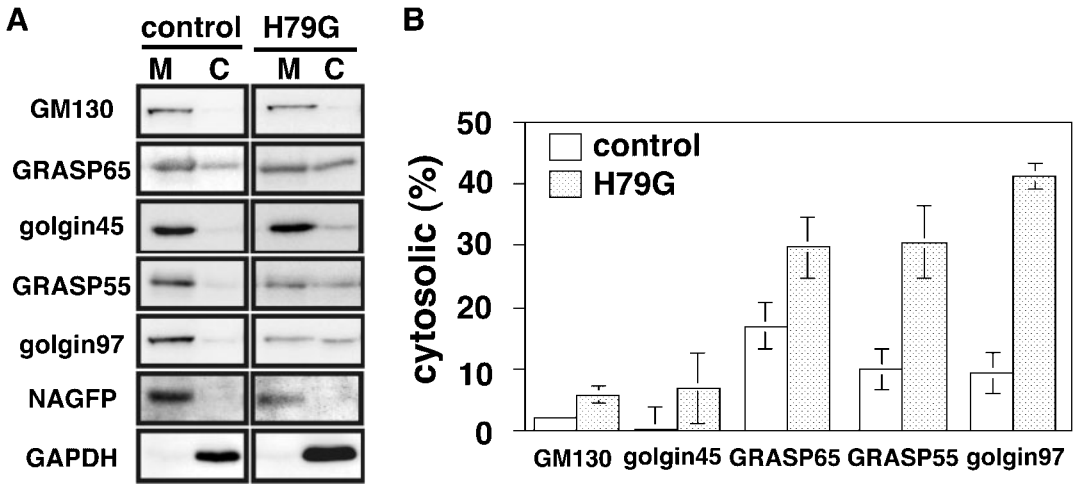

Fig. 4. **Association of Golgi matrix proteins on the membrane.** (A) Control cells (left gallery) and Sar1p(H79G) expressing cells (right gallery) were prepared as described in the legend to Fig. 3. Cells were then homogenized and post-nuclear supernatant was obtained. Membranes (M) were floated up through a sucrose step density gradient and separated from the cytosol (C) as described in "MATERIALS AND METHODS." The samples were analyzed by western blotting for GM130, GRASP65, golgin-45, GRASP55, golgin-97, NAGFP, and GAPDH. (B) The bands in (A) were quantified and % recovery in the cytosolic fraction was calculated as follows: (% cytosolic) = [(% in cytosolic fraction of the protein) – (% in cytosolic fraction of NAGFP)]/[(% in cytosolic fraction of GAPDH) – (% in cytosolic fraction of NAGFP)].

suggests that most golgin-97 associates with a membrane domain distinct from TGN38. About 60% of golgin-97 floated with membranes after the expression of Sar1p(H79G), suggesting that much of the golgin-97 remained bound to the membrane, possibly TGN remnants.

On the other hand, about 40% of golgin-97 was recovered in the cytoplasmic fraction by the expression of Sar1p(H79G). Interestingly, the amounts of GRASP65 and GRASP55 in the cytoplasmic fraction also increased significantly (12 and 21%, respectively). Therefore, some of the binding sites for these proteins may be lost because of the bulk redistribution of the Golgi membrane to the ER. In contrast, more than 90% of GM130 and golgin-45 were recovered in both the membrane fraction in the presence and absence of Sar1p(H79G). Therefore, most GM130 and golgin-45 remain associated with membranes even in the presence of Sar1p(H79G).

Because newly synthesized protein could partially account for the apparent ER relocalization of TGN38, we were concerned that the relocalization of other proteins to the ER might also be caused by the accumulation of newly synthesized proteins. Sar1p(H79G) started to accumulate within 12 h. Thus, newly synthesized proteins are predicted to accumulate for about 12 h under these conditions. The estimated half-lives of GM130 and TGN38 are 20 h (unpublished observation) and 5.8 h based on (*62*), respectively, in NRK cells. The half-lives of golgin-45, GRASP55, giantin have to be in this range because a significantly faster reduction of the staining of these proteins after cycloheximide treatment in comparison with GM130 is not observed. Therefore, the theoretical contributions of newly synthesized proteins are estimated to be between 34% (GM130) and 76% (TGN38) after 12 h. The recoveries of proteins in the ER fractions (fractions 8–10) were 59, 30, 44, and 90% for golgin-45, GRASP55, giantin and TGN38, respectively. Thus, we believe that the accumulation of newly synthesized proteins in the ER contributed significantly in our biochemical analysis, especially for TGN38, which is a transmembrane protein and has a shorter half-life. It is possible that the accumulation of these proteins in the ER is solely accounted for by the accumulation of newly synthesized proteins. If this is the case, pre-existing golgin-45, GRASP55, giantin, and TGN38 might not relocate to the ER. However, we believe some of these proteins relocate to the ER because the relocation of these proteins to the cytoplasmic reticular structure was observed in Sa1p(H79G) microinjected cells when *de novo* protein synthesis was blocked by the presence of cycloheximide (Figs. 2G, 5C, D, and unpublished observation).

The key point is that GM130 and GRASP65 did not appear in the ER, despite the accumulation of other newly synthesized Golgi proteins in the ER. It also has to be emphasized that significant amounts of golgin-45, GRASP55 and giantin remained in the lighter Golgi remnant membrane fractions. Therefore, this is a good indication that *cis*-Golgi matrix proteins and some *medial-/trans*-matrix proteins were retained in Golgi remnants when ER to Golgi transport was blocked, while other *medial-/trans*-cisternal proteins were retrieved back to the ER by retrograde transport.

COPII-Like Vesicles Accumulate and Are Tethered to Golgi Remnant Membranes in the Presence of Sar1p(H79G)— Next, we examined an ER exit site marker, Sec13p. Sec13p is a component of COPII vesicles and is recruited to and accumulated at the ER exit site membrane by Sar1p (*64*, *65*). In control cells, Sec13p was observed as small punctate structures scattered throughout the cytoplasm. Surprisingly, Sec13p accumulated in the Golgi area in close proximity to GM130 after the expression of Sar1p(H79G) by adenovirus (see Fig. 7C). Ward *et al.* recently reported similar results using NRK cells expressing YFP-tagged Sec13p (*53*). They interpreted

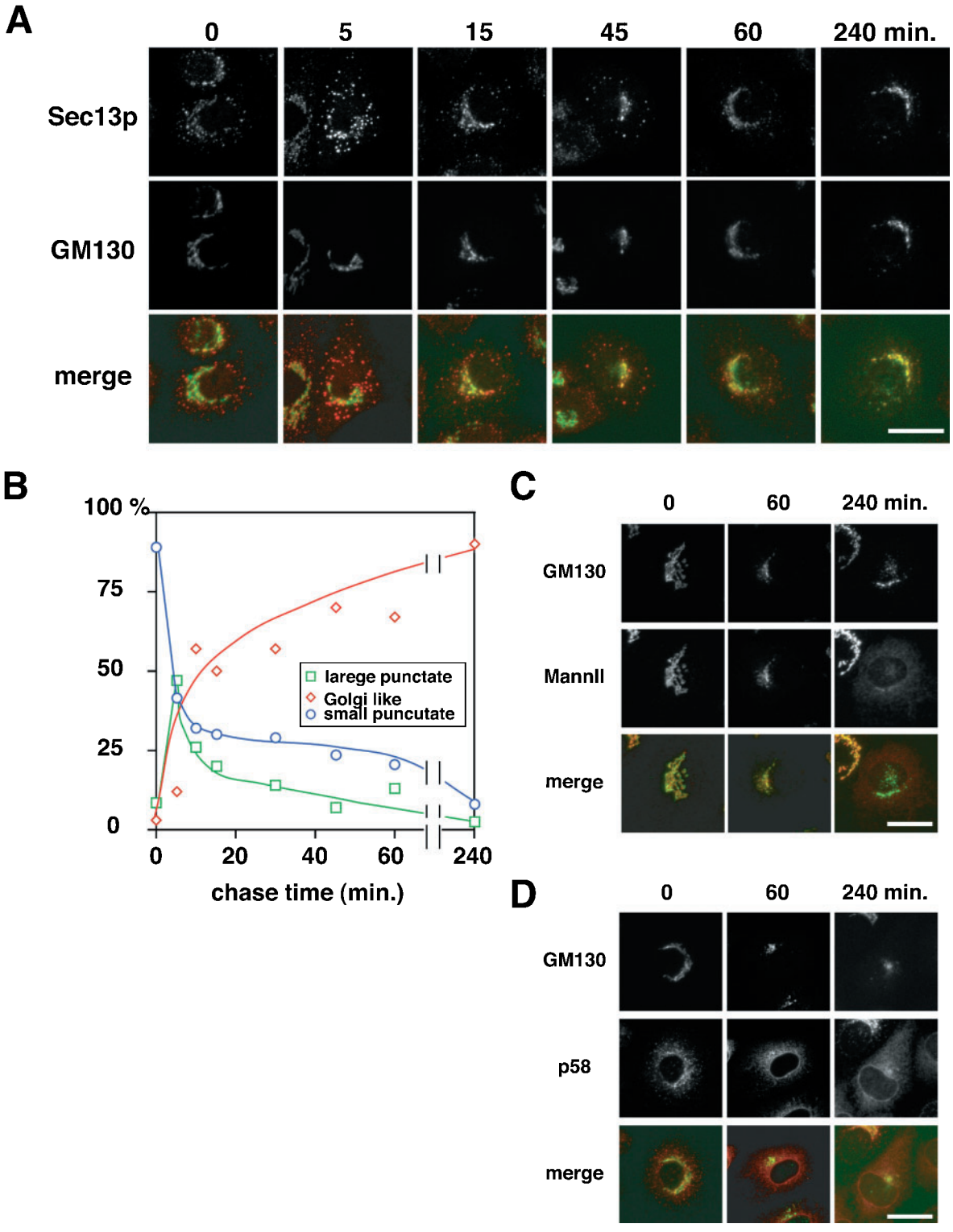

Fig. 5. **Relocalization of Sec13p in the presence of Sar1p(H79G).** (A) Purified recombinant Sar1p(H79G) was microinjected into cells over 2 min and incubated for the indicated times. The cells were processed for double labeling with antibodies for GM130 (green; middle gallery) and Sec13p (red; upper gallery). Cells showing the most representative staining pattern are presented. Merged pictures are shown at the bottom. (B) The morphology of representative Sec13p positive structures in the injected cells shown in (A) were classified into three groups: smaller clusters as seen in noninjected cells (open circles, the pattern shown at 0 min), larger clusters in the periphery of the cytoplasm (open squares, the pattern shown at 5 min), clusters accumulated in the Golgi area (open diamonds, the pattern shown at 15–240 min). The number of cells in each class was counted and the relative number (%) for all the injected cells was calculated and plotted for each time point. (C and D) Purified recombinant Sar1p(H79G) was microinjected into the cells and incubated for the indicated times. The cells were processed for double labeling with antibodies for (C) GM130 (green; middle gallery) and mannosidase II (red; upper gallery), (D) GM130 (green; middle gallery) and p58/ERGIC53 (red; upper gallery). Merged pictures are shown at the bottom. Bar = 20 μm.

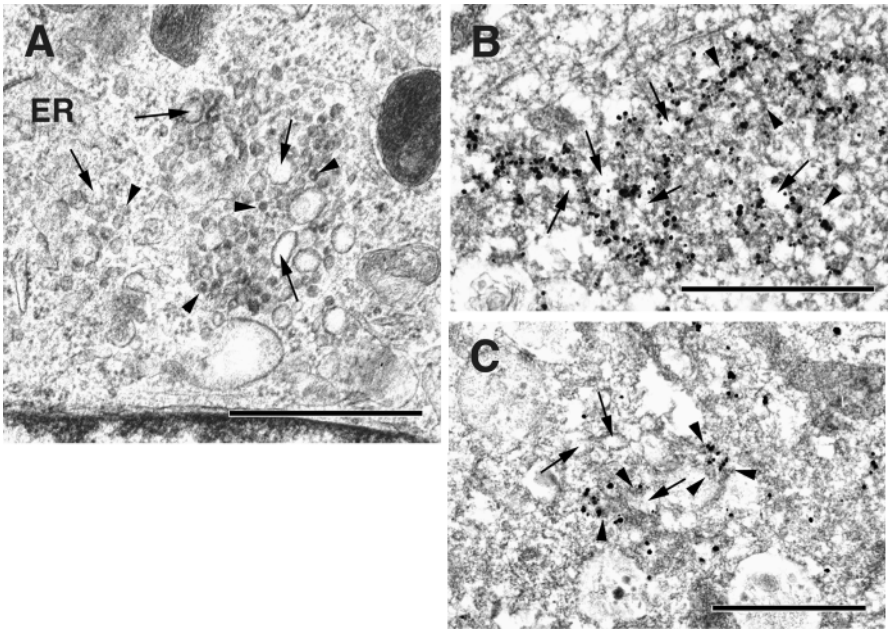

Fig. 6. **Electron microscopic analysis of Sar1p(H79G) injected cells.** NRK cells were microinjected with Sar1p(H79G) and incubated for 100 min. The cells were processed for conventional EM (A) or immuno-EM using affinity purified anti-GM130 antibody (B) or affinity purified anti-Sec13p (C). Arrows indicate larger vesicles and arrowheads indicate smaller coated vesicles. Bar = 1 μm.

this phenomenon as the relocalization of GM130 to ER exit sites. To verify this possibility, we carried out fine time course observations after the microinjection of Sar1p(H79G). As shown in Fig. 5A, Sec13p started to congregate in larger clusters at the periphery of the cytoplasm, then started to accumulate in the Golgi area after 15 min, and this accumulation was complete in 60 min. At this point, Sec13p clusters were very closely associated with GM130 (Fig. 5A), and this persisted up to 240 min when most of the mannosidase II was retrieved back into the ER (Fig. 5C). We then performed a semi-quantitative morphological analysis of Sec13p distribution. The Sec13p clusters in the injected cells were categorized into three groups: (*1*) smaller clusters as seen in non-injected cells, (*2*) larger clusters in the periphery of the cytoplasm, and (*3*) clusters accumulated in the Golgi area. The number of cells showing each pattern was counted and the number of cells showing each pattern in relation to all injected cells (expressed as a %) was plotted for each incubation time (Fig. 5B). The number of cells showing smaller clusters quickly decreased after 5 min of injection, while the number of cells showing larger clusters increased in the 5 min after injection and then decreased concomitantly with the increase in the number of cells showing clusters accumulated in the Golgi area. Because the accumulation of Sec13p in the Golgi area was complete after 60 min, while most mannosidase II and GM130 still remained in the Golgi apparatus (Fig. 5C), it is likely that Sec13p detached from ER exit sites to form larger clusters and moved toward the Golgi apparatus. As reported by Girod *et al*. (*66*), p58/ERGIC53 was quickly relocated to the ER when most of the Golgi resident proteins were still retained in the Golgi apparatus (Fig. 5D, 60 min). However, p58/ERGIC53 did not accumulate in any punctate or clustered structures after extended incubations of up to 240 min (Fig. 5D), indicating that cargo accumulation in the ER exit sites was already blocked after 60 min of incubation. Therefore, it is unlikely that GM130 was rapidly retrieved back to the ER then recycled and accumulated at ER exit sites.

To confirm the accumulation of Sec13p in the Golgi area, Sar1p(H79G) microinjected cells were incubated for 100 min and the injected cells were selectively processed and observed by EM. As shown in Fig. 6A, the Golgi stack was completely disassembled and numerous clusters consisting of larger and smaller vesicles, many of which were coated, were found at the perinuclear region. When the cells were subjected to immuno-labeling with an anti-GM130 antibody using the silver enhancement method, silver grains were mainly associated with larger vesicles (Fig. 6B). In contrast, with the anti-Sec13p antibody, silver grains were mainly associated with smaller coated vesicles (Fig. 6C). This was confirmed by counting the number of silver grains associated with larger and smaller vesicles (Table 1). In the case of Sec13p, 88% of silver grains were associated with smaller vesicles. On the other hand, 89% of silver grains were associated with larger vesicles in the case of GM130. These results suggest that larger vesicles are the vesicular remnants of the Golgi apparatus and the smaller coated vesicles are COPII-like coated vesicles tethered to the Golgi remnants. COPII-like coated vesicles accumulate to form clusters because they are trapped by Sar1p(H79G) and, therefore, cannot uncoat.

Table 1. **Number of gold particles on vesicles.**

	Larger vesicles	Smaller vesicles
Sec13p	23 (12%)	161 (88%)
GM130	436 (89%)	54 (11%)

NRK-52E cells were injected with Sar1p (H79G) and subjected to immuno-EM using anti-Sec13p and anti-GM130 antibodies as described in "MATERIALS AND METHODS." The vesicles in clusters were classified into large and small vesicles. 100 vesicles of each class were randomly selected and the numbers of gold particles associated with all the vesicles were counted.

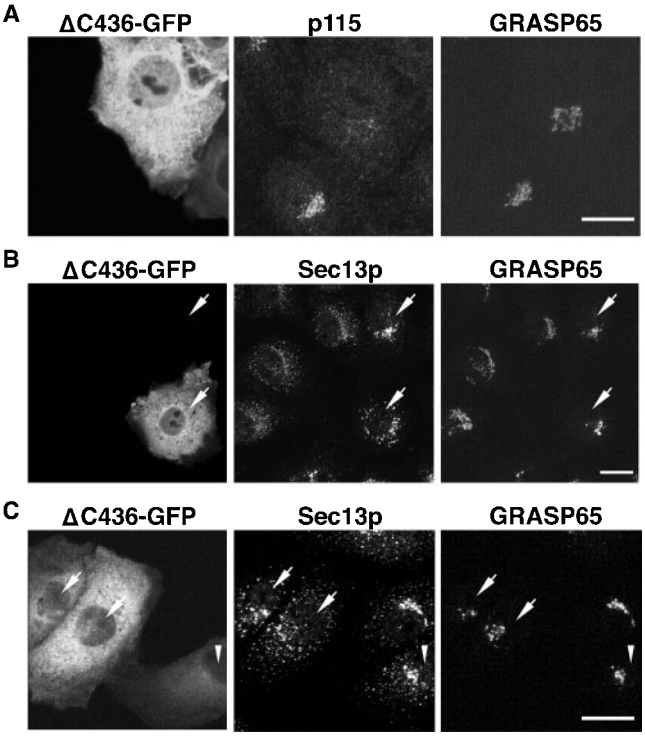

Fig. 7. **Accumulation of Sec13p in the Golgi apparatus is mediated by p115.** (A) NRK-52E cells were microinjected with a plasmid encoding ΔC436-GFP and incubated for 4 h. (B) The cells were then microinjected with the purified recombinant Sar1p(H79G) and further incubated for 60 min. Arrows indicate the Sar1p(H79G) injected cells. The cells were processed for triple labeling with the indicated proteins (middle gallery) and GRASP65 (right gallery) as described in Fig. 2. GFP fluorescence is shown on the left. (C) NRK-52E cells were infected with Sar1p(H79G) expression virus and incubated for 24 h. The cells were then microinjected with a plasmid encoding ΔC436-GFP and incubated for 4 h. Cells were then triple labeled as above. Arrows and arrowheads indicate cells expressing ΔC436-GFP at high and low levels, respectively. Bar = 20 μm.

COPII-Like Vesicles Clusters Are Tethered to the Golgi Remnant Vesicles by p115—It is known that p115 is recruited to vesicular tubular clusters (VTC) or budding COPII vesicles and functions in ER to Golgi transport (*30*, *31*, *67*). It has also been reported that p115 binds GM130 and giantin to build a tethering complex that facilitates the docking and fusion of COPI vesicles to Golgi cisternae (*27*, *33*). We, therefore, examined whether the accumulation of Sec13p clusters to the Golgi remnants is mediated by p115. For this purpose, we took advantage of the observation that a C-terminally truncated fragment of GM130 (ΔC436) containing the p115 binding domain specifically removes p115 from the Golgi apparatus when over-expressed in cells (*27*). Here, we used ΔC436 fused with GFP (ΔC436-GFP) to detect fragment expression more easily. When cells were microinjected with a ΔC436-GFP expression plasmid and allowed to over-express the protein for 4 h, most of the p115 was removed from the Golgi apparatus (Fig. 7A), consistent with previous work (*27*).

When the ΔC436-GFP over-expressing cells were microinjected with purified recombinant Sar1p(H79G) and incubated for 60min, larger clusters of Sec13p formed but did not accumulate in the Golgi area (Fig. 7B). This was confirmed by counting the number of cells showing Sec13p clusters accumulated in the Golgi area in microinjected cells (Table 2). About 75% of cells into which only Sar1p(H79G) was injected showed Sec13p accumulation in the Golgi area (Fig. 8A). On the other hand, fewer than 25% of cells showed Sec13p accumulation in the Golgi area upon co-expression of ΔC436-GFP with Sar1p(H79G). A similar result was obtained when ΔC237-GFP, in which most of the coiled coil domains including the rab1 interacting region were removed (*68*), was expressed, but not with GFP (Table 2, Fig. 8, B and C), suggesting that this effect specifically resulted from the removal of p115 from the Golgi apparatus. Mannosidase II was still localized in the Golgi area together with GRASP65 in cells in which only Sar1p(H79G) was injected (unpublished observation). Therefore, much of the Golgi resident proteins were not absorbed into the ER in this time point. These results also argue against the relocalization of GM130 to ER exit sites.

We also tested whether the accumulated Sec13p could be dissociated by the expression of ΔC436-GFP. For this purpose, Sar1p(H79G) was expressed by adenovirus infection, and ΔC436-GFP was expressed by plasmid microinjection. In cells expressing ΔC436-GFP, the accumulated Sec13p was dispersed from the Golgi area (Fig. 7C, arrowhead). The dispersal was more evident in cells showing higher levels of ΔC436-GFP expression (Fig. 7 arrows, Table 3, Fig. 8D). Concomitantly, p115 was effi-

Table 2. **Number of the cells showing the accumulation of Sec13p positive structures to the Golgi apparatus.**

construct	GFP[a]	Sar1p[b]	both[c]
ΔC436-GFP	0[d]/30[e](0%[d])	43/61 (70%)	3/13 (23%)
	0/16 (0%)	34/50 (68%)	6/24 (25%)
	0/20 (0%)	43/63 (79%)	4/16 (14%)
ΔC237-GFP	1/47 (2%)	34/48 (71%)	7/21 (33%)
	1/40 (3%)	33/39 (84%)	4/18 (25%)
	1/59 (2%)	37/43 (86%)	6/18 (33%)
GFP	0/15 (0%)	45/52 (87%)	9/12 (75%)
	0/30 (0%)	49/63 (78%)	7/ 9 (77%)
	0/43 (0%)	55/70 (79%)	23/30 (77%)

Experiments were done as described in the legend to Fig. 6. The data in each line were from independent experiments. [a]Cells positive only for the GFP construct. [b]Cells positive only for the Sar1p(H79G). [c]Cells positive for the GFP construct and Sar1p(H79G). [d]Number of cells showing the accumulation of Sec13p to the Golgi apparatus. [e]Total cells counted. [d]% cells showing a Golgi pattern.

Table 3. **Number of cells showing the accumulation of Sec13p-positive structures to the Golgi apparatus under high and low expression ΔC436-GFP.**

Control (%)[a]	Low (%)	High (%)
91[b]/100[c] (91%[d])	8/12 (67%)	13/25 (52%)
88/100 (88%)	7/10 (70%)	6/20 (30%)
89/100 (89%)	14/20 (70%)	26/40 (65%)

Experiments were done as described in the legend of Fig. 6E. The data in each line were from independent experiments. [a]Cells not expressing ΔC436-GFP. [b]Number of cells showing the accumulation of Sec13p in the Golgi apparatus. [c]Total cells counted. [d]% cells showing a Golgi pattern.

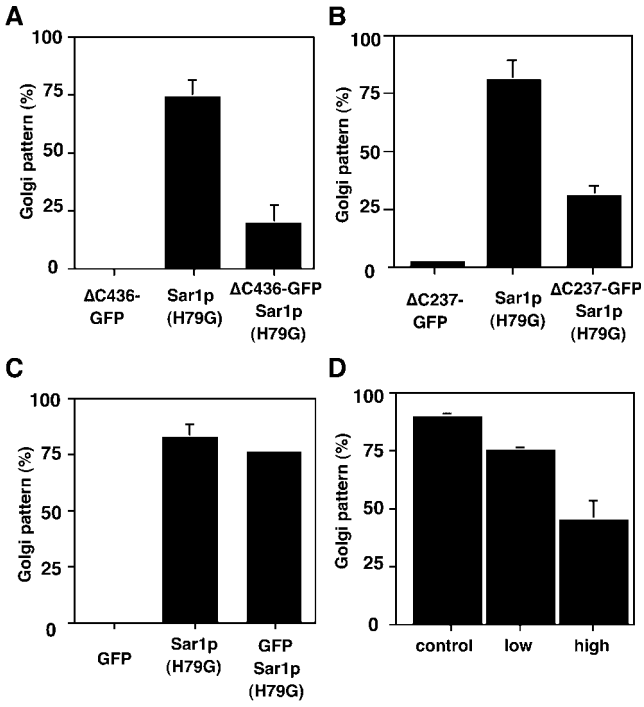

Fig. 8. **Quantitation of Sec13p accumulation to the Golgi apparatus.** (A) ΔC436-GFP, ΔC237-GFP or GFP were expressed and Sar1p(H79G) was injected into NRK-52E cells. The cells were then processed as described in the legend to Fig. 7. The cells were categorized into three groups [GFP-positive, Sar1p(H79G) positive and both] and the number of cells showing Sec13p clusters accumulated in the Golgi area was counted for each category of cells (see Table 2). (B) The number of cells showing Sec13p clusters accumulated in the Golgi area was counted for cells not expressing (control) or expressing low level and high level of ΔC436-GFP in Fig. 7D (see Table 3). The relative number (%) for all the examined cell number was calculated and presented. The results are the average of three experiments. The error bars indicate standard deviation.

ciently removed from Golgi remnants even in the presence of Sar1p(H79G) (unpublished observation). These results suggest that the binding of p115 to the Golgi apparatus is important for the accumulation of Sec13p clusters in the vicinity of the Golgi apparatus.

DISCUSSION

Inhibition of ER to Golgi Transport by Sar1p Mutants—The introduction of Sar1p(H79G) into cells prevents the uncoating of COPII coated vesicles, and VTCs decorated by COPII-like coats thus accumulate (*51*, *53*, *69*). These results and our own data (Fig. 7) suggest that preventing uncoating of the COPII coat with Sar1p(H79G) causes COPII components to accumulate on VTCs. Therefore, it is reasonable to assume that consumption of free COPII components is the reason why ER to Golgi transport is inhibited. Ward *et al.* reported Sar1p(H79G) on vesicular tubular membranes, often in direct continuity with the ER, by immuno-EM (*53*), and suggested that the ER exit sites move and accumulate around the centrosome. They found a similar relocalization of GM130 and interpreted this as the relocalization of GM130 to the ER exit site. We do not think this is the case for the following reasons. (i) When we analyzed the localization of Sec13p by both conventional EM and immuno-EM, continuity of the coated vesicular profiles with the ER was not obvious (Fig. 6 and unpublished observations). (ii) We found by immuno-fluorescence analysis that the smaller Sec13p clusters first became larger clusters and that these gradually accumulated around the Golgi apparatus (Fig. 6). The accumulation was complete within 1 h, while mannosidase II was still retained in the Golgi apparatus. Therefore, it is less likely that Golgi matrix proteins relocate quickly to the ER leaving the bulk of Golgi resident proteins behind. (iii) The accumulation of Sec13p clusters in the Golgi remnants was inhibited when p115 was removed from these Golgi remnants (Fig. 7). Under these conditions, *cis*-Golgi matrix proteins remain associated with the Golgi remnants. Therefore, it is unlikely that *cis*-Golgi matrix proteins associate with ER exit sites.

Our interpretation is as follows: COPII vesicles form and detach from the ER but do not fully uncoat and thus accumulate as COPII-like vesicular clusters. These COPII-like vesicular clusters then move and dock to the Golgi apparatus in a p115-mediated manner. As a result, most COPII components accumulate in COPII-like vesicular clusters and are depleted at ER exit sites (Fig. 5, A and B). Concomitantly, the number of VTCs or ERGIC/IC seem to be reduced judging from the accumulation of p58/ERGIC53 in the ER (Fig. 5D). After longer incubation, the Golgi resident proteins start to relocate to the ER. The removal of the Golgi resident proteins and part of the cisternal membranes reduces the size of the Golgi cisternae and, eventually, the Golgi cisternae become fragmented to produce dispersed Golgi remnants (Figs. 2 and 6). As COPII-like vesicular clusters are closely associated with GM130-positive Golgi derived vesicles throughout this process (Figs. 5 and 6), it is less likely that cis-Golgi matrix proteins dissociate from the Golgi cisternae and re-associate with the COPII-like vesicular clusters. COPII like vesicles probably trap membrane components such as COPII coat receptors and SNAREs that function in vesicle fusion to the Golgi membranes (Fig. 6). Apparently, these COPII like vesicles cannot fuse with Golgi remnant membranes or the ER membrane even after the disruption of the tether between the COPII like vesicles and Golgi remnants (Figs. 7 and 8). Probably, the vesicle fusion machinery, such as SNAREs, is covered by the locked COPII coat and can not function in the presence of Sar1p(H79G). Importantly, the Golgi remnants can not fuse with the ER membrane even after the disruption of the tether with the COPII like vesicles (Figs. 6–8). These results suggest that components necessary for fusion to the ER membrane are depleted from the Golgi remnants without the supply of anterograde vesicles.

In contrast, the structure of the Golgi apparatus was less affected even when larger amounts of Sar1p(T39N) protein were introduced into the NRK cells (Fig. 1 and unpublished observation). This is reasonable because Sar1p(T39N) is a GDP-bound inactive form and thought to be recessive under the co-existence of wild type Sar1p. Importantly, GM130 was retained in cytoplasmic punctate structures in many cells where most of the mannosidase II had relocated to the ER (Fig. 1C). A very small number of cells showed dispersal of GM130 into the cytoplasm (Fig. 1C). Therefore, the introduction of larger amounts of Sar1p(T39N) is thought to be necessary to

disassemble GM130 containing structures. How can the dispersal of GM130 in the cytoplasm be explained? Larger amounts of Sar1p(T39N) are thought to compete with the endogenous Sar1p and inhibit the accumulation of the COPII coat at the ER exit sites, leading to the inhibition of anterograde vesicular transport. Similar to the effect of Sar1p(H79G), this causes the relocalization of much of the Golgi proteins back to the ER *via* retrograde transport (Fig. 1). Under the higher level expression of Sar1p(T39N), the binding of the COPII coat is thought be completely prevented and SNAREs that function in the fusion with the Golgi membranes are not segregated by the COPII coat and remain on the ER membrane. The SNAREs on the ER membrane are then thought to be eventually activated, leading to the fusion with the Golgi remnant membranes. In the end, Golgi matrix proteins relocate to the ER membrane. Alternatively, Golgi matrix proteins may dissociate from the membrane because the special organization of the Golgi membrane where matrix proteins associate would be lost. We are now trying to confirm where Golgi matrix proteins relocate under the expression of Sar1p(T39N).

Tethering of COPII-Like Vesicular Clusters with Golgi Apparatus—The binding of p115 to the Golgi apparatus appeares to be necessary for the accumulation of COPII-like vesicular clusters around the Golgi apparatus (Fig. 7). How does p115 mediate this accumulation? It has been reported that p115 mainly localizes to intermediate compartment/VTC and *cis*-Golgi cisternae (*28*, *29*, *67*). It has also been reported that p115 is directly recruited to budding COPII vesicles (*31*). Considering the proposed function of p115 in vesicle tethering at the Golgi cisternae (*27*), it is probable that p115 also functions in the tethering of the ER to Golgi carrier vesicles/VTC to the *cis*-Golgi cisternae. Therefore, it is reasonable to think that the COPII-like vesicular clusters generated by Sar1p(H79G) retain the character of the ER to Golgi carrier vesicles, and that p115 is recruited on these vesicles or the Golgi cisternae to function in the tethering of COPII-like vesicular clusters to the *cis*-Golgi cisternae. COPII-like vesicular clusters are thought to be tethered to the Golgi apparatus mainly by p115-GM130 complex formation. However, giantin may also be involved in the tethering because N-terminal fragments of GM130 (Δ237 or Δ436) compete with p115 binding not only with GM130 but also, perhaps, with giantin (*71*). We are now analyzing the contribution of these proteins in COPII vesicle tethering.

Dynamics of Cis-Golgi Matrix Proteins—The resistance of *cis*-Golgi matrix proteins to retrograde flow strongly suggests the importance of the *cis*-Golgi matrix composed of the GM130-GRASP65 complex in the organization of the Golgi apparatus. Actually, Vasile *et al.* recently reported that GM130 has an important role in maintaining the structure and function of the Golgi apparatus (*72*). They have shown that GM130 is undetectable in a temperature–sensitive, conditional mutant, ldlG cells. In ldlG cells, the organization of the *cis*-Golgi elements is perturbed even at the permissive temperature (34°C), and furthermore, that the whole Golgi apparatus is completely disassembled at the restrictive temperature (39.5°C). Probably, GM130 functions to tether transport vesicles to the Golgi apparatus and supports the efficient import of vesicles to the Golgi apparatus. The lack of tethering machinery at the *cis*-Golgi reduces the efficiency of vesicle fusion to the Golgi apparatus. This may somehow be compensated for at the permissive temperature only to cause perturbation of the *cis*-Golgi elements. At the restrictive temperature, the tethering machinery at the *cis*-Golgi may become essential and the complete disassembly of the Golgi apparatus occurs.

GM130 and GRASP65 have been found to form a tight complex and are thought to co-migrate during membrane fractionation (*18*). However, there are some small differences in the behaviors of GM130 and GRASP65. Firstly, there is a subpopulation of GRASP65 that does not co-localize with GM130 in control cells (Fig. 3). In agreement with this, we have recently shown that GRASP65 can bind to the Golgi membrane independent of GM130 (*37*). Secondly, after the expression of Sar1p(H79G), a significant amount of GRASP65 is recovered in the cytoplasmic fraction, whereas very little GM130 is recovered in a cytoplasmic fraction in the presence or absence of Sar1p(H79G) (Fig. 4). This may be caused either by the accumulation of newly synthesized GRASP65 in the cytosol or by the release of pre-existing GRASP65 from the Golgi membrane following the gross relocation of Golgi resident proteins to the ER. In either case, the association of GRASP65 with the Golgi membrane may require GM130 and the properly organized stacked cisternal structure. In normal interphase cells, newly synthesized GRASP65 may be recruited from the cytosol to regions of the Golgi membrane where Golgi resident proteins and Golgi matrix proteins are concentrated and organized. While we cannot exclude the possibility that GRASP65 dissociated during the fractionation procedure due to the conditions used, we can at least say that the membrane binding of GRASP65 is clearly reduced in the presence of Sar1p(H79G). Ward and co-workers reported that there is loosely membrane bound GRASP65 and this is in dynamic equilibrium with a cytoplasmic pool of GRASP65 (*53*). The increase in this fraction of GRASP65 in the presence of Sar1p(H79G) suggests that GRASP65 membrane association is coupled in some way to ER to Golgi vesicle transport.

Dynamics of Medial-Golgi Matrix Proteins—It has previously been shown that golgin-45 and GRASP55 are key components in the maintenance of the Golgi structure, and that they do not relocate back to the ER following one hour of BFA treatment (*19*, *36*). Therefore, it is somewhat surprising that golgin-45 and GRASP55 partially relocate to the ER after the expression of Sar1p(H79G). These results may indicate that some fraction of golgin-45 and GRASP55 continuously recycles between the ER and Golgi apparatus together with other Golgi resident proteins such as mannosidase II. However, most golgin-45 and GRASP55 remain associated with the Golgi remnant vesicles suggesting that either (i) there are subpopulations of golgin-45 and GRASP55 that co-localize with *cis*-Golgi matrix proteins and are excluded from retrieval to the ER or (ii) the retrieval of golgin-45 and GRASP55 is much slower than that of other Golgi resident proteins. Immunofluorescence analysis revealed that a fraction of Golgin-45 and GRASP55 that did not relocate to the ER, was closely localized with GM130 and GRASP65, suggesting their association with Golgi remnant vesicles.

The precise localization of these proteins is under investigation.

Giantin also partially relocated to the ER, but significant amounts remained in the Golgi remnants after the expression of Sar1p(H79G) (Figs. 3F and 4). Although it has been reported that giantin is efficiently incorporated into COPI vesicles and tethered to the Golgi cisternae (*33*), we think it is unlikely that giantin was present in COPI vesicles that accumulated around the Golgi remnants because β-COP was removed from the Golgi remnants. Preliminary immuno-EM analysis that showed giantin on larger Golgi remnant vesicles supports this view. The partial relocalization of giantin to the ER suggests that it might slowly recycle between the ER and Golgi apparatus in a manner similar to golgin-45 and GRASP55.

Dynamics of Trans-Golgi Network Proteins—The TGN was also affected by Sar1p(H79G) treatment. After the expression of Sar1p(H79G), golgin 97 relocated to cytoplasmic punctate structures distinct not only from GM130 positive Golgi remnants, but also from endocytic compartments (Fig. 5D and unpublished observation). Much of this golgin-97 remained associated with membranes (Fig. 4). Golgin-97 binds to the TGN membrane *via* a GRIP domain at its C-terminus. It was recently reported that the removal of GRIP domain-containing proteins from the TGN by over-expression of GRIP domain-containing fragments perturbs the localization of integral TGN proteins including TGN46 and Furin (*73*). Therefore, we believe that golgin-97 is a good candidate for a TGN matrix protein that regulates the structure and function of TGN.

A part of TGN38 also relocated to the ER (Fig. 2G), although detectable amounts of TGN38 remained in golgin-97-positive structures 4 h after the microinjection of Sar1p(H79G). TGN38 disappeared from golgin-97-positive structures after longer incubations (Fig. 2F). A similar result was reported by Miles *et al.* (*52*). These results suggest that TGN38 relocates to the ER at a much lower rate than mannosidase II. However, the possibility that a fraction of TGN38 was trapped in golgin-97-positive structures and then slowly delivered to lysosomes for degradation can not be excluded. In fact, biochemical analysis could not detect the relocalization of the mature form of TGN38 to the ER (Fig. 3A). Unfortunately, the higher turnover rate and accumulation of newly synthesized protein hamper a more detailed analysis.

Importantly, the degree of co-localization of TGN38 and golgin-97 4 h after the microinjection of Sar1p(H79G) clearly indicates that golgin-97 is associated with residual *trans*-Golgi network membranes or vesicles at this time point. The final TGN38-free golgin-97-positive structures are thought to be some form of vesicles because most golgin-97 can be floated with the membrane (Fig. 4). Further analysis of these membranes is in progress.

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