Coordination of Golgin Tethering and SNARE Assembly

GM130 BINDS SYNTAXIN 5 IN A p115-REGULATED MANNER*

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During membrane traffic, transport carriers are first tethered to the target membrane prior to undergoing fusion. Mechanisms exist to connect tethering with fusion, but in most cases, the details remain poorly understood. GM130 is a member of the golgin family of coiled-coil proteins that is involved in membrane tethering at the endoplasmic reticulum (ER) to Golgi intermediate compartment and cis-Golgi. Here, we demonstrate that GM130 interacts with syntaxin 5, a t-SNARE also localized to the early secretory pathway. Binding to syntaxin 5 is specific, direct, and mediated by the membrane-proximal region of GM130. Interestingly, interaction with syntaxin 5 is inhibited by the binding of the vesicle docking protein p115 to a distal binding site in GM130. The interaction between GM130 and the small GTPase Rab1 is also inhibited by p115 binding. Our findings suggest a mechanism for coupling membrane tethering and fusion at the ER to Golgi intermediate compartment and cis-Golgi, with GM130 playing a central role in linking these processes. Consistent with this hypothesis, we find that depletion of GM130 by RNA interference slows the rate of ER to Golgi trafficking in vivo. The interactions of GM130 with syntaxin 5 and Rab1 are also regulated by mitotic phosphorylation, which is likely to contribute to the inhibition of ER to Golgi trafficking that occurs when mammalian cells enter mitosis.

Membrane traffic within the endomembrane system is mediated by transport carriers that dock with the correct target compartment prior to delivering their contents by membrane fusion. Specificity in docking and fusion is ensured through the combined actions of many proteins that act in a temporally and spatially controlled manner. The initial attachment of a carrier to the target membrane is known as tethering and is mediated by small GTPases of the Rab and Arl families together with so-called tethering factors, which are typically long-coiled-coil proteins or multi-subunit complexes (1–4). Tethering is usually followed by the bridging of SNARE proteins across the apposing carrier and target compartment membranes, which serves not only to ensure specific recognition but also directly contributes to the fusion process itself (5, 6).

A number of tethering factors have been identified at the Golgi apparatus, including members of the golgin family of cytoplasmically orientated coiled-coil proteins (1–3). One of the most extensively studied golgins is GM130, which is present on the cis-face of the Golgi apparatus and in higher eukaryotes is also found in the ER2 to Golgi intermediate compartment (ERGIC) (7, 8). GM130 is anchored to the membrane via its extreme C terminus through binding to the myristoylated protein GRASP65, whereas the basic N terminus binds to the acidic C-terminal tail of the vesicle docking protein p115 (9, 10). p115 can also interact with another golgin called giantin, and it has been proposed that p115 bridges vesicles containing giantin with target membranes containing GM130 (11, 12). An alternative view is that GM130 and giantin bind p115 independently in a mutually exclusive fashion (13). Both GM130 and p115 interact with active Rab1, which in the latter case is important for membrane recruitment (14–18).

Most evidence supports a role for GM130 in trafficking between the ER and Golgi apparatus, where it appears to act both in the homotypic tethering of ERGIC membranes as well as in the tethering of ERGIC membranes to the cis-Golgi (8, 19–21). An alternative view is that GM130 is not required at all for trafficking but instead participates in the lateral tethering of Golgi cisternae required for Golgi ribbon formation in mammalian cells (22–24). Studies in a mutant cell line lacking GM130 suggest that it may be important for ensuring optimum trafficking efficiency and Golgi integrity under certain conditions (25), which also appears to be the case for the closest yeast homologue Bug1p (26).

In addition to GM130, giantin, and Rab1, p115 can also bind directly to ERGIC and Golgi SNAREs (14, 27). This is believed to link SNAREs on opposing membranes and promote their assembly into SNAREpin complexes, thereby directly connecting tethering with downstream SNARE-mediated fusion events (27). The interaction with unassembled SNAREs also helps recruit p115 to the membrane, which may ensure that tethering only occurs at sites where active unassembled SNAREs are present (17, 18). Although it is clear p115 binds to GM130, the functional role of this interaction in the context of tethering and fusion is debated. In one view, binding of GM130 to p115 directly tethers the vesicle to the target membrane (9, 28). In another view, the GM130-p115 interaction is not required for

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2 The abbreviations used are: ER, endoplasmic reticulum; ERGIC, ER to Golgi intermediate compartment; RLG, rat liver Golgi; IP, immunoprecipitation; GTP·(thio)triphosphate; siRNA, small interfering RNA; EndoH, endoglycosidase H; RNAi, RNA interference; VSV-G, vesicular stomatitis virus glycoprotein.
tethering (22–24). Instead, it may simply help recruit p115 to the membrane, allowing it to engage Rab1 and/or unassembled SNAREs. In line with this hypothesis, it has recently been shown that binding to GM130 induces a conformational change in p115, converting it from an autoinhibited state to one that is able to bind active Rab1 (29). The subsequent interaction of p115 with Rab1, together with binding to unassembled SNAREs, is probably important for stable association of p115 with the membrane (17). In this scenario, GM130 may mediate tethering independently of p115, either via homotypic GM130 interactions or through binding to other tethering components (21, 24).

When mammalian cells enter mitosis, secretory trafficking is arrested, and the Golgi apparatus is extensively fragmented, which allows equal partitioning of the organelle into the two daughter cells (30). These changes require phosphorylation of Golgi proteins by mitotic kinases. GM130 is phosphorylated in early mitosis on serine 25 by CDK1-cyclin B (9, 31, 32). Phosphorylation of serine 25 abolishes binding to p115, resulting in the redistribution of p115 from Golgi membranes to the cytosol (31–33), which probably contributes to the trafficking inhibition and Golgi fragmentation seen in mitotic cells.

The interaction between p115 and SNAREs is important for ER to Golgi transport and Golgi structure (23, 27). Most studies place GM130 upstream of p115 and SNARE assembly, yet the mechanisms by which these various components are linked remain poorly understood. To gain further insights into this process, we examined whether GM130, like p115, can physically associate with the SNARE machinery. We found a direct and specific interaction between GM130 and the major Golgi t-SNARE syntaxin 5. Interestingly, binding of GM130 to syntaxin 5 as well as to Rab1 was inhibited when p115 bound to GM130, suggesting an additional mechanism for coupling vesicle tethering and fusion at the cis-Golgi. Depletion of GM130 by RNA interference significantly inhibited ER to Golgi transport, consistent with an important role for these interactions in vivo. We also found that mitotic phosphorylation of GM130 increased syntaxin 5 binding but reduced Rab1 interaction, indicating that GM130 regulation in mitosis is more complex than previously thought.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies**—All materials were from Sigma or Merck unless otherwise stated. Protease inhibitors (mixture set III, used at 1:250) were from Calbiochem. MLO7 (anti-N73pep) rabbit polyclonal anti-GM130 and NN7 rabbit anti-p115 (9), 4A3 monoclonal anti-GM130 (34), rabbit anti-phospho-GM130 (PS25) (31), and sheep anti-golgin 84 (84) antibodies were obtained previously. Monoclonal anti-GM130 C terminus and anti-golgin 84 antibodies were from Transduction Laboratories. JSEE1 was raised in rabbits against the GST-tagged cytoplasmic domain of rat syntaxin 5 and purified against the immunogen coupled to glutathione-Sepharose beads (Amer- sham Biosciences). Rabbit anti-CASP antibodies were raised against His-tagged CASP cytoplasmic domain and affinity-purified against GST-tagged CASP cytoplasmic domain coupled to glutathione-Sepharose beads. Monoclonal anti-Myc tag (9E10) was from Cancer Research UK. Monoclonal anti-VSV-G (P5D4) and anti-His tag were purchased from Sigma. Monoclonal antibody UH-4 to GalNAcT2 was a kind gift from Dr. Henrik Clausen (University of Copenhagen, Denmark). Monoclonal anti-VSV-G ectodomain antibody was a kind gift from Dr. Rainer Pepperkok (EMBL, Heidelberg, Germany). Monoclonal rabbit anti-giantin antibodies were a gift from Prof. Manfred Renz (Institute of Immunology and Molecular Genetics, Karlsruhe, Germany). Fluorophore and horseradish peroxi-
dase-conjugated secondary antibodies were purchased from Molecular Probes and Tago Immunologicals, respectively.

**Molecular Biology and Yeast Two-hybrid Analysis**—Standard molecular biology techniques were used for all constructs; primer sequences are available upon request. DNA encoding cytoplasmic domains of human syntaxin 5 (residues 1–274), GS27 (residues 1–184), or syntaxin 1 (residues 1–264) or full-length constitutively inactive or active mutant RabS was inserted into the BamHI and EcoRI sites of pGEX 4T-2 vector for expression of GST-tagged protein in *Escherichia coli* (Amersham Biosciences). DNA encoding wild-type or truncated versions (ΔN74) of rat GM130 cDNA or GOS28 (1–226) cytoplasmic domain were inserted into BamHI and EcoRI sites of pTrcHis (Invitrogen) for *E. coli* expression of His-tagged protein. Plasmids encoding His-tagged full-length p115, TA (951–961), and T- (951–933) domains were described previously (29). Full-length rat GM130 cDNA was cloned into pBAC-2cp (Novagen) for expression in insect cells. Myc-GM130 constructs in pcDNA3.1 were kindly provided by Dr. Francis Barr (University of Liverpool, UK). DNA encoding cytoplasmic domains of human syntaxin 5 (residues 1–274), syntaxin 1 (residues 1–264), GS15 (residues 1–81), GS27 (residues 1–184), GOS28 (residues 1–226), Bet1 (residues 1–86), Ykt6 (residues 1–87), or Sec22B (residues 1–185) was inserted into the yeast two-hybrid activation domain vector pGADT7. DNA encoding wild-type rat GM130 cDNA or human golgin-84 cytoplasmic domain (residues 1–698) was inserted into the yeast two-hybrid DNA-binding domain vector pGBK7. The plasmids were co-transformed into the yeast reporter strain AH109 on synthetic medium lacking leucine and tryptophan (low selection) and then restreaked onto synthetic medium lacking leucine, tryptophan, histidine, and adenine with 2% glucose as the carbon source (high selection), according to the Clontech protocol handbook.

**Protein Preparation**—Rat liver p115 was purified according to Levine et al. (33). Recombinant His/protein S-tagged GM130 and OCR11 were made in High Five cells (Invitrogen) using the BacVector system (Novagen) according to the manufacturer’s instructions and purified using Ni^{2+}-nitrilotriacetic acid beads (Novagen). Plasmids encoding GST-tagged SNAREs or RabS or His-tagged GM130 or p115 were transformed into *E. coli* BL21 CodonPlus cells (Stratagene). Cells were induced with 0.1 mM isopropyl β-D-thiogalactoside for 3–4 h at 27 °C, and recombinant proteins were purified on glutathione-Sepharose beads (Amer-

**In Vitro Phosphorylation of Golgi Membranes**—Rat liver Golgi (RLG) membranes were incubated with desalted interphase and mitotic HeLa cytosol (9 mg/ml in buffer A; 20 mM β-glycerophosphate, 15 mM EGTA, 50 mM KOAc, 10 mM MgOAc, 2 mM ATP, 1 mM dithiothreitol, 0.2 mM succrose) in the presence of 10 mM creatine phosphate and 20 μg/ml creatine.
kinase for 30 min at 30 °C (35). Where indicated, staurosporine was used at a 10 μM final concentration. Golgi membranes were reisolated by centrifugation through a layer of 0.4 M sucrose (in buffer A) for 10 min at 55,000 rpm in a TLA55 rotor.

Salt Washing of Golgi Membranes—RLG membranes were salt-washed by incubation at 0.2 mg/ml in 1M NaCl buffer (20 mM Hepes-KOH, pH 7.4, 0.1 M NaCl, 5 mM MgCl₂, 0.2 M dithiothreitol, 0.5% Triton X-100) containing protease inhibitors for 15 min at 4 °C and clarified by centrifugation at 13,200 rpm for 10 min. Two micrograms of purified antibodies to GM130 (4A3), syntaxin 5 (JSEE1), or golgin-84 or 2 μl of anti-giantin serum were added and incubated at 4 °C for 2 h followed by 10 μl of protein A or protein G-Sepharose (Zymed Laboratories Inc.) for a further 1 h at 4 °C. After washing three times with IP buffer, the bound proteins were eluted by boiling in SDS sample buffer and analyzed by Western blotting with appropriate antibodies.

Cross-linking—Salt-washed RLG membranes were resuspended in PBS (at 1 mg/ml) containing either a 250 μM concentration of the reducible sulfhydryl-reactive cross-linker dithiobismaleimidoethane (Pierce) or Me₂SO carrier and incubated for 20 min at room temperature. Cross-linking was stopped by the addition of cysteine to 5 mM, and membranes were recovered by centrifugation at 13,200 rpm for 15 min at 4 °C prior to extraction in IP buffer lacking dithiothreitol and immunoprecipitation.

Limited Proteolysis—Reisolated interphase or mitotic Golgi membranes were resuspended in trypsin buffer (20 mM Hepes, pH 7.4, 0.1 M KCl, 5 mM MgCl₂, 0.2 M dithiothreitol, 0.5% Triton X-100) containing protease inhibitors for 20 min at room temperature. Limited proteolysis was stopped by the addition of SDS sample buffer containing protease inhibitors and boiling and then analyzed by Western blotting with appropriate antibodies.

Pull-down Experiments—GST-tagged cytoplasmic domains of syntaxin 5, GS27, syntaxin 1 (2 μg, for 175 nM in the binding
reaction) were coupled to glutathione-Sepharose beads (Amer sham Biosciences) and incubated for 2 h at 4 °C with either salt-washed untreated, interphase, or mitotic RLG membrane extracts, purified recombinant proteins, or HeLa cell extracts. HeLa cell extracts were prepared by incubating a confluent 10-cm dish of cells with 1 ml IP of buffer containing protease inhibitors for 15 min on ice and clarified by centrifugation at 13,200 rpm. After washing three times with IP buffer containing 0.25% Triton X-100, bound proteins were eluted by boiling in SDS sample buffer and analyzed by immunoblotting with appropriate antibodies. Rab binding was performed according to Ref. 35 with some modifications. GST-Rabs (50 μg; 5 μM) were coupled to glutathione-Sepharose beads and loaded with GDP or GTPγS before incubation with purified recombinant proteins or Golgi extracts in IP buffer containing 0.25% Triton X-100 and either 100 μM GDP or GTPγS for 2 h at 4 °C. Beads were washed three times with IP buffer supplemented with 10 μM GDP or GTPγS. Bound proteins were eluted with SDS-PAGE sample buffer and analyzed by immunoblotting with appropriate antibodies.

Cell Culture and RNA Interference—Adherent HeLa cells were cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin G, and 100 μg/ml streptomycin sulfate. Suspension HeLa cells were grown at 37 °C and 5% CO2 in RPMI 1640 medium supplemented as Dulbecco’s modified Eagle’s medium. Mitotic HeLa cells were prepared by incubating in 100 ng/ml nocodazole for 24 h. RNA interference was performed on adherent HeLa cells using Oligofectamine (Invitrogen) and siRNA oligonucleotides (Dharmacon Research) as described previously (35). Luciferase (GL2; Eurogentec) was used as a negative control. GM130 was targeted with the oligonucleotide GM130 siRNA 1 (AAGUUAGAGAUGACGGAACUC) (24). Syntaxin 5 was targeted with the oligonucleotide AAGCUGGAGAAGCUGACAAUC. Cells were analyzed 72 h after transfection.

Immunofluorescence Microscopy—Cells were grown on coverslips and fixed in 100% methanol at −20 °C for 4 min. Coverslips were incubated with primary antibodies diluted into PBS containing 0.5 mg/ml bovine serum albumin for 20 min at room temperature, washed, and incubated with PBS/bovine serum albumin containing fluorophore-conjugated secondary antibodies for an additional 20 min at room temperature. Coverslips were mounted in Mowiol and analyzed by conventional epifluorescence microscopy using an Olympus BX60 upright microscope with a CoolSNAP EZ CCD camera (Roper Scientific) driven by Metaview software (Universal Imaging Corp.).

VSV-G Trafficking—Cells were grown in 3-cm dishes and transfected with siRNA as appropriate. Forty-eight hours after transfection, cells were washed and incubated in 400 μl of Dulbecco’s modified Eagle’s medium supplemented with 5% FBS containing 0.5 μl high titer adenovirus encoding tsO45-VSV-G-EGFP (36) for 1 h at 37 °C in a humidified incubator. Prewarmed Dulbecco’s modified Eagle’s medium supplemented with 10% FBS (2 ml) was added, and the cells were transferred to 40 °C overnight. Cells were rapidly transferred to 32 °C by exchanging the medium for medium containing 0.1 mg/ml cycloheximide precooled to 32 °C and incubating in an incubator at this temperature for the appropriate time. Transport to the cell surface was monitored using antibody to the ectodomain of VSV-G as described previously (35). For the biotinylation and endoglycosidase H (EndoH) assays, adenovirus encoding untagged tsO45-VSV-G was made according to the

FIGURE 2. p115 regulates the GM130-syntaxin 5 interaction. A, S-GM130 (3.5 nM) was incubated with beads containing GST-Syntaxin 5 (175 nm) in the presence of increasing concentrations of His-Rab1Q70L (0–200 nm) or purified rat liver p115 (0–35 nm). Bound proteins were analyzed by Western blotting with antibodies to GM130 (MLO7) or p115.B, left, His-p115FL or His-p115ΔA (3.5 nm) was incubated with beads containing GST-Syntaxin 5 or GST-GS27 (175 nm), and bound proteins were analyzed by Western blotting with anti-His antibodies. Middle panel, His-p115FL or His-p115ΔA (3.5 nm) was incubated with beads containing S-GM130 (35 nm), and bound proteins were detected by Western blotting with antibodies to p115. Right, S-GM130 (3.5 nm) was incubated with beads containing GST-syntaxin 5 (175 nm) in the presence of increasing concentrations of His-p115FL or His-p115ΔA (0–35 nm). Bound proteins were detected by Western blotting with antibodies to GM130 (ML07) or p115. C, His-GM130FL (3.5 μM) was incubated with beads containing GST-syntaxin 5 (175 nm) in the presence of increasing concentrations of His-p115TA or p115ST (0–17.5 nm), and bound proteins were detected by Western blotting with antibodies to p115. D, His-GM130FL or His-GM130ΔN74 (3.5 nm) was incubated with beads containing GST-syntaxin 5 (175 nm) in the presence of increasing concentrations of His-p115FL or His-p115ΔA (0–17.5 nm), and bound proteins were detected by Western blotting with anti-His antibodies. E, His-GM130FL alone (1), His-GM130FL mixed with His-p115TA (2), or His-p115ST (3), respectively, at a molar ratio of 1:2 was digested with 0, 0.02, 0.1, or 0.5 μg of trypsin for 20 min at 25 °C. Reactions were stopped by the addition of SDS sample buffer and boiling, and samples were analyzed by Western blotting with the 4A3 monoclonal anti-GM130 antibody.
**Results**

The Golgin GM130 Binds to the SNARE Syntaxin 5—To determine whether the golgins GM130 or golgin-84 can interact with SNAREs, we exploited the yeast two-hybrid system. Full-length GM130 or the cytoplasmic domain of golgin-84 was screened against the cytoplasmic domains of several SNAREs, including the major SNAREs found at the ERGIC and/or Golgi apparatus. As shown in Fig. 1A, GM130 bound to the t-SNARE syntaxin 5A (short form, herein referred to as syntaxin 5) but not to other ERGIC or Golgi SNARES tested. GM130 also failed to interact with the neuronal plasma membrane t-SNARE syntaxin 1A (herein referred to as syntaxin 1). Golgin-84 failed to interact with any of the SNARES tested. To verify the interaction between GM130 and syntaxin five pull-down experiments were performed. GM130 present in Golgi membrane extracts bound to immobilized syntaxin 5, but not GS27 or syntaxin 1 (Fig. 1B, left). Importantly, membranes were salt-washed prior to extraction to remove p115, indicating that binding was not via this protein, which can bind independently to both GM130 and syntaxin 5 (9, 27). As seen in the yeast two-hybrid assay, golgin-84 failed to bind any of the SNAREs tested. We also failed to detect the golgins TMF1 or giantin bound to the syntaxin 5 beads (data not shown). To assess for direct interaction, purified recombinant versions of GM130 and syntaxin 5 were incubated together. As shown in Fig. 1B (right), immobilized syntaxin 5 but not GS27 bound to purified recombinant GM130, indicating a direct interaction between the proteins.

To determine the region of GM130 responsible for syntaxin 5 binding, truncated versions of GM130 were co-expressed with syntaxin 5 in the yeast two-hybrid system, and binding was monitored by growth on high selection medium. Constructs containing the C-terminal region of GM130 including coiled-coil regions 4–6 (M5, ΔN679) bound to syntaxin 5 but not the negative control syntaxin 1 (Fig. 1C). Constructs lacking this region failed to bind either syntaxin. To confirm these results, full-length and truncated Myc-tagged versions of GM130 were expressed in HeLa cells, and binding to syntaxin 5 beads was analyzed. As shown in Fig. 1D, full-length GM130 bound to syntaxin 5, as did the C-terminal construct containing coiled-coils 4–6 (ΔN679). In contrast, neither coiled-coil 3 of GM130 nor full-length golgin-84 bound to syntaxin 5. We therefore conclude that the syntaxin 5 binding site resides in the C-terminal region of GM130, which contains coiled-coils 4–6. Further mapping analysis in the yeast two-hybrid system indicated
GM130 Binding to Syntaxin 5

A

Luciferase RNAi | Syntaxin 5 RNAi | GM130 RNAi

GM130

Syntaxin 5

CASp

32 °C shift (min)

0 30 45 60

B

Luciferase RNAi | GM130 RNAi | Syntaxin 5 RNAi

GM130

Syntaxin 5

VSVG

EndoH

32 °C shift (min)

0 30 45 60

C

% VSV-G in Gag

| Condition       | 30 min | 45 min | 60 min |
|-----------------|--------|--------|--------|
| Luciferase RNAi | 28.35  | 44.6   | 49.4   |
| GM130 RNAi      | 3.85   | 27.5   | 43.25  |
| Syntaxin 5 RNAi | 0      | 0      | 1.1    |

D

Surface VSV-G | Total VSV-G

Luciferase RNAi

GM130 RNAi

Syntaxin 5 RNAi

E

Surface/Total VSV-G (normalized)

| Condition       | 30 min | 60 min | 90 min |
|-----------------|--------|--------|--------|
| Luciferase RNAi | 32.85  | 71.2   | 100    |
| GM130 RNAi      | 15.8   | 40.25  | 93.4   |
| Syntaxin 5 RNAi | 6.2    | 16.1   | 21.25  |

F

Biotinylated VSV-G | Total VSV-G (5%)

Luciferase RNAi

GM130 RNAi

Syntaxin 5 RNAi

G

% VSV-G on surface (normalized)

| Time (min) | Luciferase RNAi | GM130 RNAi | Syntaxin 5 RNAi |
|------------|-----------------|------------|-----------------|
| 0          | 9.6             | 10.45      | 3.6             |
| 20         | 10             | 10.45      | 4.95            |
| 40         | 12.85           | 13.6       | 5.05            |
| 60         | 18.65           | 14.65      | 5.15            |
| 80         | 26.65           | 16.66      | 6.6             |
| 100        | 35.5            | 20.4       | 9.5             |
that coiled-coil 6 was sufficient to bind GM130 (data not shown). However, this region was insufficient to bind GM130 in biochemical binding experiments, suggesting weak or transient interaction, either because this construct was improperly folded or because additional regions outside coiled-coil 6, most likely coiled coils 4 and 5, contribute to GM130 binding.

We next studied whether endogenous GM130 and syntaxin 5 interact with each other. Golgi membranes were extracted under native conditions and immunoprecipitated with various antibodies. Antibodies to GM130 co-precipitated syntaxin 5 and vice versa (Fig. 1E). Neither antibody precipitated golgin-84, and golgin-84 antibodies failed to precipitate significant amounts of either syntaxin 5 or GM130. To confirm that GM130 and syntaxin 5 interact in the context of the membrane, cross-linking was performed prior to extraction and immunoprecipitation. As shown in Fig. 1F, the addition of DTME cross-linker noticeably increased the amount of co-precipitating GM130 and syntaxin 5, indicative of an interaction between the proteins in intact membranes. The interaction also appeared specific, since golgin-84 was absent from immunoprecipitates of cross-linked membranes. Together, the above results indicate a direct and specific interaction between the C terminus of GM130 and syntaxin 5 that occurs in the membrane.

p115 Regulates the GM130-Syntaxin 5 Interaction—Since p115 binds to both syntaxin 5 and GM130 (9, 27), we decided to test whether it could influence the interaction between these proteins. To investigate this possibility, binding of GM130 to immobilized syntaxin 5 was performed in the presence of increasing concentrations of p115. We also tested a constitutively active form of Rab1, which can bind to GM130 as well as to p115 (14–16). Strikingly, the addition of purified p115 inhibited the interaction between GM130 and syntaxin 5 in a dose-dependent manner (Fig. 2A). In contrast, Rab1 had no effect on the GM130-syntaxin 5 interaction. To characterize the mechanism underlying the p115-mediated inhibition of GM130-syntaxin 5 interaction, a mutant version of p115 lacking the acidic tail (ΔAA) comprising the GM130 binding site was used (37). This construct is still able to bind syntaxin 5 but is deficient in GM130 binding (Fig. 2B, left and middle), as was expected (27, 37). In contrast to full-length p115, p115ΔAA had little effect on the binding of GM130 to syntaxin 5 (Fig. 2B, right). Similar results were obtained using a truncated p115 construct containing only the coiled-coil and acidic tail domains (p115TA; Fig. 2C, left). Again, removal of the GM130 binding site in this construct (p115T) abolished the inhibition of GM130-syntaxin 5 binding (Fig. 2C, right). These results suggest that p115 binding to GM130 reduces the affinity of GM130 for syntaxin 5. To further test this hypothesis, binding experiments were carried out using a mutant GM130 lacking the N-terminal p115 interaction domain. This construct bound to syntaxin 5 as efficiently as full-length GM130 (Fig. 1B, right), but binding was noticeably less sensitive to the presence of added p115 (Fig. 2D). We therefore conclude that p115 binding to the N terminus of GM130 causes a reduction in the binding affinity of the C-terminal region for syntaxin 5.

A possible explanation for the above results is that p115 binding induces a conformational change in GM130, resulting in a reduced ability to bind syntaxin 5. We used limited trypic digestion to probe the conformation of GM130, either in the absence of p115 or in the presence of p115 TA or p115T. As shown in Fig. 2E, the digestion of GM130 was altered in the presence of p115TA compared with in its absence or in the presence of p115T. This could either reflect masking of trypic sites in GM130 by bound p115TA or a change in GM130 conformation. We favor the latter explanation, since the addition of an antibody that binds to the same region of GM130 as p115 (9) had no effect on GM130 digestion (data not shown). Importantly, this antibody was quantitatively bound to GM130, as indicated by the inability of p115TA to bind GM130 in its presence (data not shown). Thus, our data suggest p115 can induce a conformational change in GM130.

p115 Regulates the GM130-Rab1 Interaction—In addition to p115 and syntaxin 5, GM130 can also interact with the small GTPase Rab1 (15, 16). Rab1 binds to GM130 via a central coiled-coil region (coiled-coil 3) that lies downstream from the N-terminal p115 binding site (16). Since p115 binding to GM130 could influence the distal binding of syntaxin 5, most likely through an induced conformational change, we decided to test whether it could also influence GM130 binding to Rab1. We also tested the effect of GM130 on p115 binding to Rab1, since a previous study has shown that the affinity of p115 for Rab1 is increased following its association with GM130, most likely by relieving an autoinhibitory conformation of p115 (29). As shown in Fig. 3A, recombinant full-length GM130 and p115 bound to GST-Rab1 but not Rab6 in a GTP-dependent manner, as expected (Fig. 3A). p115 binding to Rab1 was relatively poor compared with that of GM130. However, the addition of GM130 greatly increased its binding affinity for Rab1 (Fig. 3B). In contrast, mutant GM130 lacking the p115 binding site (ΔN74), which can still bind Rab1, albeit in a less GTP-dependent manner compared with the wild-type protein (Fig. 3A), did not stimulate the p115–Rab1 interaction (Fig. 3B). These results

FIGURE 5. GM130 and syntaxin 5 are required for efficient ER to Golgi transport. A. HeLa cells were subjected to RNAi with oligonucleotides targeting luciferase (control), GM130, or syntaxin 5 for 72 h and analyzed by Western blotting with antibodies to GM130, CASP, or syntaxin 5. Note that syntaxin 5 runs as two bands, corresponding to isoforms of 42 and 35 kDa. B. HeLa cells were transfected with siRNA targeting luciferase, GM130, or syntaxin 5, incubated for 48 h, infected with adenovirus encoding ts-O45 VSV-G, and incubated at 40 °C overnight. Cells were shifted to 32 °C for various times as indicated before lysis and treatment without or with EndoH prior to Western blotting with PD4 anti-VSV-G antibody. C, quantitation of VSV-G transport, assessed by the percentage of total VSV-G that was EndoH-resistant. Results are expressed as the average ± S.D. (n = 2). D, RNAi-treated HeLa cells were infected with adenovirus encoding ts-O45 VSV-G-EGFP and incubated at 40 °C overnight before shifting to 32 °C for various times and labeling with anti-VSV-G ectodomain antibody. The example shown is at 60-min release. Bar, 10 μm. E, quantitation of VSV-G-EGFP transport, assessed by the ratio of surface to total VSV-G fluorescence at the indicated times of release. Results are normalized to the control luciferase RNAi at 90 min and expressed as the average ± S.D. (n = 2). F, RNAi-treated HeLa cells were infected with adenovirus encoding ts-O45 VSV-G and incubated at 40 °C overnight before shifting to 32 °C for the indicated times. Transport to the surface was assessed by biotinylation and surface (biotinylated), and total VSV-G was detected by Western blotting with PD4 antibody. G, quantitation of VSV-G transport, assessed by surface biotinylation. Results are expressed as the percentage of total VSV-G transported to cell surface (biotinylated) at the indicated time points, normalized to the control luciferase RNAi at 100 min. The results are expressed as the average ± S.D. (n = 2). H, RNAi-treated cells were analyzed 72 h post-transfection by immunofluorescence microscopy with antibodies to GM130 (red) and GaINAcT2 (green). Bar, 10 μm.
confirm the findings of Beard et al. (29) and are consistent with GM130 binding to p115 stimulating the latter’s interaction with Rab1.

To assess whether there is a reciprocal effect of p115 on the GM130-Rab1 interaction, additional binding experiments were performed. As shown in Fig. 3C, binding of GM130 to Rab1 was reduced by the addition of p115. In contrast, binding of GM130AN74 to Rab1 was unaffected by the addition of p115, indicating that this effect is due to p115 binding to GM130 rather than Rab1 (Fig. 3C). Binding of p115 to GM130 therefore reduces the affinity of GM130 for both Rab1 and syntaxin 5.

*GM130 Inhibits Syntaxin 5 Interaction with SNAREs*—It has previously been shown that p115 can directly bind specific ERGIC/Golgi SNAREs and promote their assembly into SNARE complexes (27). Since GM130 also binds syntaxin 5, we were interested in whether it too could influence SNARE interactions. Binding of GOS28 to immobilized syntaxin 5 was therefore monitored in the presence of increasing amounts of GM130 or p115, which served as a positive control. As expected, p115 increased the binding of GOS28 to syntaxin 5 (Fig. 4). The addition of GM130 had the opposite effect, with a reduction in SNARE association, whereas various control proteins had no effect (Fig. 4) (data not shown). GM130 binding to syntaxin 5 therefore reduces its ability to form SNARE complexes.

*Both GM130 and Syntaxin 5 Are Required for Efficient ER to Golgi Transport*—The extent to which GM130 participates in ER to Golgi trafficking has been a matter of some debate (8, 19–22, 24). Furthermore, a recent report claimed that depletion of syntaxin 5 did not affect the trafficking of cargo from ER to Golgi, which was somewhat surprising given the well defined function of this protein in membrane fusion (38). In light of these issues and our finding that GM130 and syntaxin 5 directly interact, we decided to reinvestigate whether GM130 and syntaxin 5 are required for ER to Golgi trafficking. Each protein was depleted by RNA interference (Fig. 5A), and ER to Golgi trafficking of the well characterized cargo protein tsO45-VSV-G was monitored by acquisition of EndoH resistance, which occurs when delivery to the medial Golgi has occurred (Fig. 5B). Depletion of syntaxin 5, which exists as two isoforms in vivo (39), gave a complete block in transport of VSV-G from the ER to the Golgi apparatus (Fig. 5, B and C). Depletion of GM130 did not block but did significantly delay Golgi delivery of VSV-G.

To further corroborate these findings and exclude the possibility that altered EndoH sensitivity was due to effects upon glycosyltransferase activity rather than cargo transport, we studied delivery of VSV-G to the cell surface using both immunofluorescence microscopy and surface biotinylation. Surface delivery of VSV-G as monitored by immunofluorescence microscopy was slower in GM130-depleted cells, whereas in syntaxin 5-depleted cells it was almost completely blocked (Fig. 5, D and E). Surface biotinylation gave a similar result. Delivery of VSV-G to the cell surface was delayed in cells depleted of GM130 and blocked in cells depleted of syntaxin 5 (Fig. 5, F and G). Analysis of Golgi morphology in the depleted cells indicated that the ribbon was disrupted by GM130 depletion, as previously reported (24), whereas a more extensive fragmentation was observed upon depletion of syntaxin 5, again as reported previously (38) (Fig. 5H). Together, these results indicate that GM130 is required for efficient ER to Golgi transport in vivo and that syntaxin 5 is critical for this process.

*Mitotic Regulation of GM130 Interactions with Syntaxin 5 and Rab1*—In order to gain further insight into the physiological relevance of the GM130-syntaxin 5 interaction, we studied whether this interaction is subject to mitotic regulation. Several Golgi proteins are phosphorylated in mitosis, including GM130, which is phosphorylated by CDK1-cyclin B on serine 25 (31, 32). Phosphorylation prevents p115 binding, leading to the dissociation of p115 from mitotic Golgi membranes (31–33). To determine whether GM130 phosphorylation also influences syntaxin 5 interaction, binding experiments were performed with interphase or mitotic Golgi extracts. In contrast to p115, whose interaction with GM130 is inhibited in mitosis (31–33), binding of GM130 to syntaxin 5 was dramatically increased under mitotic conditions (Fig. 6A). This was probably due to phosphorylation of GM130, which could be observed by blotting with an anti-phosphoserine 25 antibody (PS25) rather than syntaxin 5, since binding was performed under conditions where mitotic kinases are inactive. Moreover, we did not observe any phosphorylation of syntaxin 5 after incubation with the Golgi extracts (data not shown). These effects were also independent of p115, since Golgi membranes were salt-washed to remove this protein prior to extraction.

Binding of GM130 to Rab1 was also affected by mitotic phosphorylation (Fig. 6B). In this case, binding was reduced. This was not due to Rab1 phosphorylation, since mitotic kinases were inactivated prior to performing the binding. GM130 extracted from salt-washed mitotic membranes also bound less to Rab1, although the effect was less pronounced than with unwashed membranes. This may be due to some of the binding to Rab1 being mediated through p115, which is lost from mitotic membranes after salt-washing or because the conformation of mitotic GM130 is altered by salt washing such that its binding to p115 is restored. Another Rab1 interaction partner is giantin (29, 40). We could show that this protein is efficiently phosphorylated under mitotic conditions, both in vitro and in vivo (Fig. 6C). However, in contrast to GM130, giantin phosphorylation failed to affect its binding to Rab1 (Fig. 6B). Although Rab1 can be efficiently phosphorylated in mitosis, we failed to observe any effects upon its interaction either with GM130 or giantin (not shown). Tryptic digests of interphase and mitotic Golgi membranes indicated that mitotic GM130 altered in its sensitivity to trypsin compared with the interphase protein (Fig. 6D). This could reflect an altered conformation of the protein due to mitotic phosphorylation, which may then explain its altered binding to both syntaxin 5 and Rab1 under these conditions.

**DISCUSSION**

We report here a novel interaction between the membrane-tethering protein GM130 and syntaxin 5, a major t-SNARE localized to the ERGIC and Golgi apparatus. The syntaxin 5 binding site was mapped to a C-terminal region encompassing coiled-coils 4–6, which is distinct from the interaction sites of other proteins, which bind either to the extreme N terminus (p115), coiled-coil 1 (YSK1), coiled-coil 3 (Rab1), or the
An obvious concern is that the binding between GM130 and syntaxin 5 is nonspecific, simply reflecting the stabilization of the α-helical SNARE motif in syntaxin 5 by the coiled-coil regions of GM130. We believe this is not the case for several reasons. First, GM130 failed to bind any of the other seven SNAREs tested, whereas syntaxin 5 failed to interact with the extensively coiled-coil proteins golgin-84, TMF1, or giantin. Second, syntaxin 5 binds only to the C-terminal region of GM130 and not to other coiled-coil regions 1–3. Third, binding can be detected in the context of the membrane using chemical cross-linking. Fourth, the interaction of GM130 with syntaxin 5 is regulated, either through the interaction of GM130 with p115 or by mitotic phosphorylation. Finally, GM130 and syntaxin 5 co-localize extensively in cells, both under steady state conditions and after disruption of Golgi organization by brefeldin A treatment, consistent with the proteins interacting in vivo (7).

Binding of p115 to GM130 inhibited its binding to syntaxin 5 and Rab1. Since p115 binds to a site on GM130 (the extreme N terminus) distal to that used by syntaxin 5 (coiled-coils 4–6) and Rab1 (coiled-coil 3), we believe the most likely explanation for these effects is that p115 induces a conformational change in GM130 that alters its binding properties. Consistent with this idea, we observed an altered tryptic digestion pattern of GM130 when p115 was bound to its N terminus. We failed to observe this effect when antibodies were bound to the same site, suggesting that masking of tryptic sites by bound p115 is not the reason for altered digestion pattern. However, to confirm that GM130 does change conformation upon p115 binding, additional structural studies will be required.

One might predict that a conformational change is necessary for tethering to couple to fusion. GM130 is an elongated rod of ~100 nm,3 anchored at one end to the membrane and interacting with p115 at the opposite end. For p115 to engage SNARE and Rab

3 A. Diao and M. Lowe, unpublished observations.
GM130 Binding to Syntaxin 5

Our data lead us to propose the following model for linking membrane tethering and fusion at the ERGIC and cis-Golgi (see Fig. 7). GM130 binds syntaxin 5 and Rab1 at the target membrane, which keeps these proteins in close proximity to the tether. Binding of p115 to the N terminus of GM130 triggers a conformational change in GM130, causing the dissociation of both Rab1 and syntaxin 5. Simultaneously, p115 will change conformation, converting to a form that can now bind Rab1 with high affinity (29). The activation of p115 by GM130 may promote association of p115 with vesicle-bound Rab1, allowing tethering to occur. An alternative possibility is that p115 is already bound to the vesicle, with tethering occurring when p115 first engages GM130. The conformational change in GM130 triggers dissociation of both Rab1 and syntaxin 5, allowing membrane fusion to occur. An alternative scenario (not depicted) is that the GM130-p115 interaction does not directly tether the vesicle to the target membrane; this may be carried out by homotypic GM130 interactions or via other proteins (22–24). In this scheme, the role of the GM130-p115 interaction would be to help recruit p115 to the membrane, activating it to allow Rab1 binding and bringing it closer to the SNARE proteins.

Using an in vitro assay that reconstitutes homotypic COPII vesicle tethering and fusion, it was recently found that SNAREs play a role not only in membrane fusion but also in the upstream tethering reaction (18). Unassembled SNAREs appear important for the membrane recruitment of both p115 and GM130 (17, 18), which may help ensure that tethers are formed only at sites where active unassembled SNAREs reside. Although the majority of GM130 is tightly bound to membranes through binding to GRASP65, fluorescence recovery after photobleaching experiments suggest that these proteins undergo constant exchange with the cytoplasm (42). Our data suggest that GM130 may be recruited from the cytoplasm through a direct physical interaction with free syntaxin 5. Alternatively, GM130 already on the membrane may interact with syntaxin 5; here we can think of tethers recruiting SNAREs to sites on the membrane where incoming vesicles will first bind (as indicated in Fig. 7).

Depletion of GM130 slowed the rate of trafficking of VSV-G from the ER to the medial Golgi apparatus, as monitored by acquisition of EndoH resistance. It was recently reported that VSV-G trafficking is unaffected when GM30 is depleted by RNA interference (24). It was also suggested that depletion of GM130 may lead to altered glycosylation of cargo through loss of Golgi ribbon integrity (24), which could account for the altered EndoH sensitivity of VSV-G observed in our experiments. We therefore studied VSV-G transport using two additional methods, both independent of the glycosylation status of VSV-G. In both cases, we observed a significant delay in VSV-G transport to the cell surface when GM130 was depleted, consistent with a role for the protein in anterograde trafficking. The reasons for the discrepancy between our findings and those of Puthenveedu et al. (24) are unclear but may reflect differences in knockdown efficiency, with GM130 depleted to a greater extent in our hands. This would fit with the idea that GM130 is required for optimum trafficking efficiency, with defects only becoming apparent when the protein is depleted beyond a certain threshold. A role for GM130 in ER to Golgi trafficking is consistent with the observed interactions with Rab1 (14, 16), p115 (9), and syntaxin 5 (this study) and several other in vitro and in vivo studies (8, 19–21). Depletion of syntaxin 5 caused a block in ER to Golgi transport of VSV-G. This is contrary to the recent study of Suga et al. (38), who reported that VSV-G was still delivered from the ER to the cell surface upon syntaxin 5 depletion. It should be noted, however, that the amount of VSV-G transport was not quantitated by Suga et al. (38), and it therefore remains possible that trafficking may indeed have been affected in these experiments. The discrepancy between our findings and those of Suga et al. (38) may also be due to differences in the efficiency of syntaxin 5 depletion. Our results fit with the established function of syntaxin 5 as a core component of the membrane fusion machinery and are consistent with studies demonstrating a key role for the protein in ER to Golgi trafficking (5, 6).

Under mitotic conditions, NSF disassembles Golgi SNAREs, but these appear unable to assemble into SNARE complexes, suggesting that mechanisms exist to prevent SNARE assembly in mitosis (43). The increased binding of GM130 to syntaxin 5 observed under mitotic conditions could at least partially...
explain this phenomenon, since GM130 prevents syntaxin 5 from binding to its cognate SNAREs. Phosphorylation of GM130 is likely to contribute to this process in other ways. Most obviously, phosphorylation of GM130 releases p115 from the membrane (31, 33), which would be expected to reduce SNARE assembly given the importance of p115 in catalyzing this process (27). The significance of Rab1 dissociation from GM130 in mitosis is currently unclear but is also likely to contribute to the inhibition of trafficking or Golgi fragmentation observed in mitotic cells. Since Rab1 remains membrane-bound in mitosis (44), it may retain some functions at the ERGIC and Golgi apparatus in mitotic cells. These could include recruitment of mitotic kinases, such as Plk1 (45), or participation in tethering reactions mediated by proteins other than GM130 (e.g. those mediated by giantin, which retains Rab1 binding in mitosis). Further studies will be required to determine the precise functions of Rab1 in tethering and fusion both in interphase and mitosis.

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