

## The Amino-terminal Domain of the Golgi Protein Giantin Interacts Directly with the Vesicle-tethering Protein p115\*

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**Giantin is thought to form a complex with p115 and Golgi matrix protein 130, which is involved in the reassembly of Golgi cisternae and stacks at the end of mitosis. The complex is involved in the tethering of coat protomer I vesicles to Golgi membranes and the initial stacking of reforming cisternae. Here we show that the NH<sub>2</sub>-terminal 15% of Giantin suffices to bind p115 *in vitro* and *in vivo* and to block cell-free Golgi reassembly. Because Giantin is a long, rod-like protein anchored to the membrane by its extreme COOH terminus, these results support the idea of a long, flexible tether linking vesicles and cisternae.**

In eukaryotic cells proteins and lipids are conveyed to different intracellular compartments via vesicular carriers. Coated vesicles carrying selected cargo bud from the donor compartment and fuse with the appropriate acceptor compartment delivering their content (1). COPII<sup>1</sup> vesicles transport newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus (2–4). COPI vesicles bud from the Golgi and transport back to the endoplasmic reticulum molecules that need to be salvaged or recycled (5–7). In addition, COPI vesicles have been shown to be involved in anterograde transport (8–10) and, more recently, in endocytosis (11–13).

Correct intracellular transport demands that both COPI and COPII vesicles are targeted to and fuse with specific membranes. Membrane fusion requires NSF, a set of soluble proteins, the SNAPs, and pairing between two sets of cognate proteins, the SNAREs, one set being on the vesicle (v-SNARE), the other on the target membrane (t-SNARE) (14–17). This association is thought to be positively regulated by the Ypt/Rab family of GTPases (18–21) and negatively regulated by the

munc-18/sec1 group of proteins (22, 23). *In vitro* studies employing yeast vacuoles suggest that after SNARE pairing a phosphatase-dependent (24) and a Ca<sup>2+</sup>/calmodulin-dependent step (25) are required for fusion.

It has become increasingly clear that before SNARE pairing COP vesicles become tethered to target membranes. Tethering is thought to be mediated by rod-like proteins with extensive coiled-coil (23, 26–28). One such protein is p115, which was identified originally because it is required for transport within the Golgi apparatus (29). Subsequently, it was implicated in fusion of transcytotic vesicles with the plasma membrane (30) and in docking COPI vesicles to Golgi membranes (31). The p115 yeast homolog, Uso1p, is involved in endoplasmic reticulum to Golgi transport (32) and is essential for docking of endoplasmic reticulum-derived vesicles (33). Both proteins form dimers with two globular heads, a long coiled-coil domain and a short acidic tail (34, 35).

Further understanding of p115 function came from studying the Golgi apparatus during the cell cycle. During interphase p115 binds Golgi membranes via the Golgi matrix protein GM130 (36). At the onset of mitosis cdc2/cyclin B phosphorylates GM130, and this inhibits the binding of p115 to the Golgi apparatus *in vitro* (36–38) and *in vivo* (39). This may explain the extensive vesiculation of the Golgi complex observed during mitosis (40–42). The loss of p115 would prevent the tethering and hence fusion of COPI vesicles. Continued budding in the absence of fusion would help to vesiculate the Golgi apparatus (42–44).

Another receptor for p115 is Giantin (31, 36). In addition to residing on Golgi membranes, Giantin is also incorporated into budding COPI vesicles (31), whereas GM130 is largely excluded. *In vitro* binding experiments showed that p115 binds Giantin on COPI vesicles and GM130 on Golgi membranes. It has therefore been suggested that p115 tethers COPI vesicles by connecting GM130 on the Golgi membrane to Giantin on the COPI vesicle (31). Experiments employing a Golgi reassembly assay (45, 46) lend support to this idea. They showed that p115, GM130, and Giantin play a crucial role in Golgi cisternal re-growth (47), and more recently they have been implicated in stacking Golgi cisternae (48).

Because tethers operate before SNARE pairing (33), one could imagine that they act at a greater distance. They would perhaps allow the vesicle to sample the membrane before bringing it closer to permit v- and t-SNARE interaction. Such a sampling function would be critically dependent on the length of the tethering complex. Both GM130 and Giantin appear to be long, rod-like proteins. GM130 is about 50 nm in length by negative staining,<sup>2</sup> whereas Giantin has a predicted length,

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<sup>1</sup> The abbreviations used are: COPII, coat protomer II; COPI, coat protein complex I; NSF, N-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; GM130, Golgi matrix protein 130; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); PCR, polymerase chain reaction; MALDI, matrix-assisted laser desorption and ionization.

<sup>2</sup> M. Lowe, R. Newman, and G. Warren, unpublished observation.

based on sedimentation analysis, of 250 nm (49, 50). Furthermore, GM130 binds to Golgi membranes at its COOH-terminal end and to p115 at its NH<sub>2</sub>-terminal end. Giantin is anchored by its COOH terminus to the membrane, but the binding site for p115 has not been mapped. Given the importance of this site in conceptualizing the tethering complex we have mapped the p115 binding site on Giantin and analyzed its properties *in vitro* and *in vivo*.

#### EXPERIMENTAL PROCEDURES

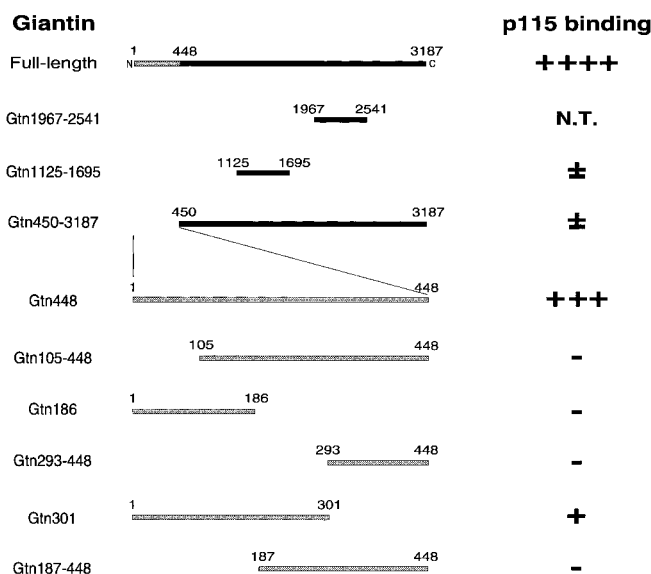
**Antibodies**—The following antibodies were used in this study: mouse monoclonal 4H1 against p115 (29), mouse monoclonal against GM130 (Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-myc tag (Santa Cruz Biotechnology, Santa Cruz, CA), Texas Red-X goat anti-mouse (Molecular Probes, Eugene, OR), and Alexa Fluor 488 goat anti-rabbit (Molecular Probes).

**SDS-PAGE and Western Blotting**—Proteins were solubilized in SDS-PAGE sample buffer, boiled for 4 min, and analyzed on 6, 10, or 12% SDS-polyacrylamide gels (51, 52). For Western blotting, proteins were transferred onto Hybond C blots (Amersham Pharmacia Biotech, Uppsala, Sweden) using a semidry blotter. Blocking and antibody incubations were performed in phosphate-buffered saline containing 5% (w/v) low fat skim milk powder and 2% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20). Secondary antibodies were horseradish peroxidase conjugates (Tago, Buckingham, U. K.) and were detected with an ECL kit (Amersham Pharmacia Biotech).

**Constructs for *in Vitro* Transcription/Translation**—The construct used for *in vitro* transcription/translation of full-length Giantin was pGCP364/pSG5, kindly provided by Dr. Y. Ikehara (Fukuoka University, Japan). pGCP364/pSG5 contains the full-length cDNA of rat Giantin cloned into the *Eco*RI restriction site of the eukaryotic expression vector pSG5 (Stratagene, La Jolla, CA). Gtn450–3187 was obtained by *in vitro* transcription/translation of pGL88, which contains an ~8.5-kb *Xba*I fragment from pGCP364/pSG5 in the cloning vector pSTBlue-1 (Novagen, Madison, WI) oriented with its 5'-end toward the T7 promoter. Plasmids encoding for Gtn448, Gtn105–448, Gtn186, Gtn293–448, Gtn301, and Gtn187–448 were obtained by PCR using primers with *Bam*HI and *Eco*RI restriction sites, the high fidelity DNA polymerase *Pfu* turbo (Stratagene), and pGCP364/pSG5 as a template. Each PCR product was subcloned in pBluescript II (Stratagene) between the restriction sites *Bam*HI and *Eco*RI and sequenced by the I.C.R.F. sequencing facility using an Applied Biosystems Prism 377 DNA sequencer (Perkin Elmer, Norwalk, CT). Gtn448 was translated *in vitro* from pGL108, which contains the NH<sub>2</sub>-terminal 1.3 kb of the rat Giantin cDNA. This fragment was obtained using the primers GL2, 5'-CTCAGCTCCTCCAGCGAATTCGAACCTAAGGAGCAAGG-3', and GL48, 5'-CCATGGAGCGCTCCTGGATCCATGCTGAGCCG-3'. To translate Gtn105–448 *in vitro* we made pGL100 using the primers GL2 and GL61, 5'-CTTCATGCAAAGGCCGGATCCATGCTGCTTGAACAAACA-3'. To obtain Gtn186 we made pGL109 (primers GL48 and GL58, 5'-CTGCTCATCAGCTAGAAATTCCTCCATCTCCGC-3'). We translated Gtn293–448 *in vitro* from pGL102 (primers GL2 and GL63, 5'-CTGATGAAAAGGTAGATCCGAAATGGCAGAGAGG-3'), Gtn301 from pGL101 (primers GL48 and GL62, 5'-CTCCAAGTGTCCCTAGAATTCATACAGCTCTTC-3'), and Gtn187–448 from pGL110 (primers GL2 and GL59, 5'-GCGGAGATGGAGGGATCCATCTGATGAGGCAG-3').

**Constructs for Recombinant Proteins**—Gtn448, Gtn1967–2541, and Gtn1125–1695 were obtained as His<sub>6</sub>-tagged recombinant proteins in bacteria. To express Gtn448, a PCR fragment was obtained using *Pfu* turbo DNA polymerase and the primers GL1, 5'-CCATGGAGCGCTCCTGGTACCATGCTGAGCCG-3', and GL2 and subcloned in the prokaryotic expression vector pTrcHis (Invitrogen, Carlsbad, CA) between the restriction sites *Kpn*I and *Eco*RI, generating pGL70. A ~1.7-kb *Kpn*I-*Hind*III fragment encoding amino acids 1967–2541 and a ~1.7-kb *Hind*III fragment encoding amino acids 1125–1695 were obtained by restriction digestion of pGCP364/pSG5 and subcloned in pTrcHis to generate pTrc#1 and pTrc#2, respectively. The joins between the histidine tag and Giantin fragments and the following 300–500 base pairs at the 3'-end were sequenced.

**Constructs for Immunofluorescence**—The construct used to express Gtn448 in normal rat kidney cells was pGL140, which was made in the following way. We excised a ~1.35-kb *Kpn*I-*Eco*RI fragment from pGL70 encoding Gtn448, and we subcloned it in the eukaryotic expression vector pcDNA3.1(+) (Invitrogen). We then inserted the PCR-generated 9E10 myc tag sequence (53) between the restriction sites *Nhe*I and *Kpn*I. To express Gtn1125–1695 we constructed pGL142. We used

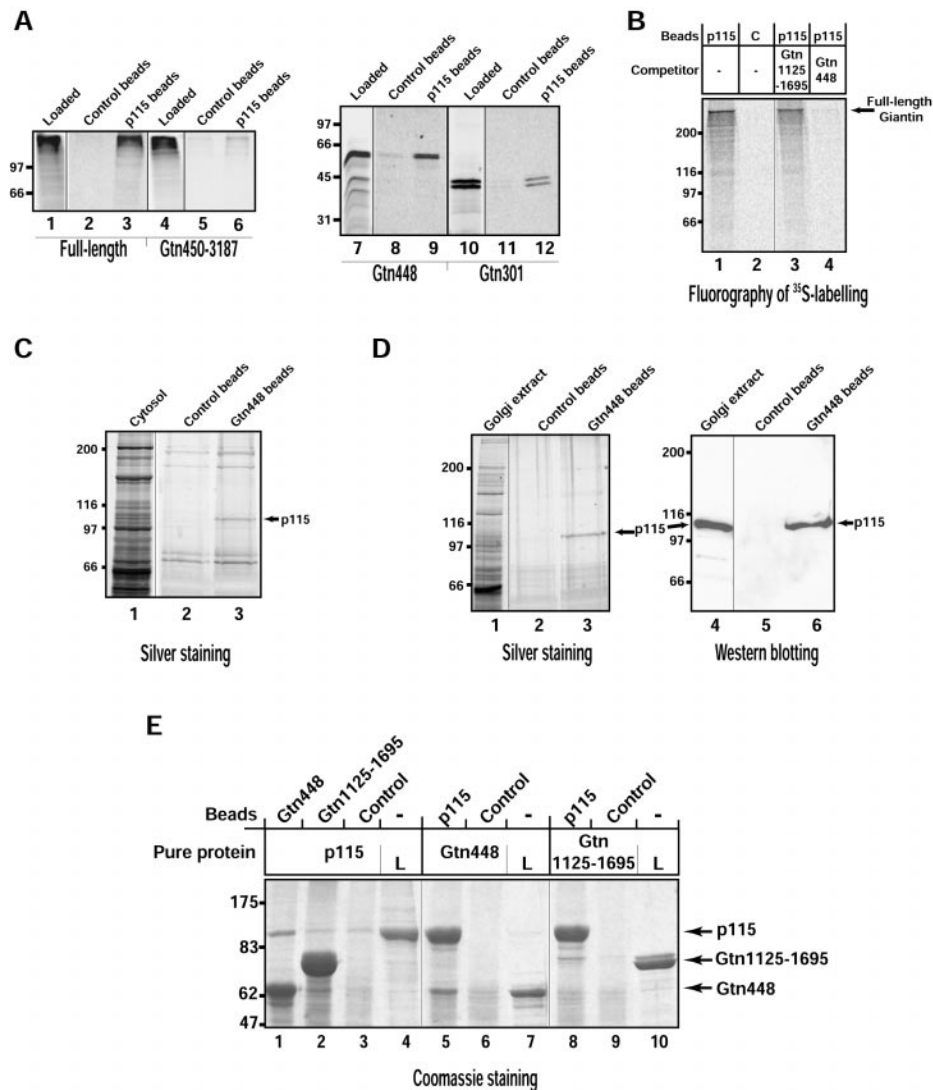


**FIG. 1. Schematic representation of the Giantin fragments used to map the binding site for p115.** The fragments were generated by *in vitro* transcription/translation using [<sup>35</sup>S]methionine as the label with the exception of Gtn1125–1695, which was prepared as a recombinant protein from *E. coli*. Each fragment was analyzed for binding to biotinylated p115 immobilized on neutravidin beads and compared with binding of full-length Giantin. The results from a number of experiments (some of which are presented in Fig. 2) are summarized on the right. +++++ indicates binding of full-length Giantin to immobilized p115; +++, strong binding; +, moderate binding; ±, poor binding; -, no detectable binding; N.T., not tested.

*Pfu* turbo DNA polymerase to PCR amplify a ~1.7-kb fragment from pGCP364/pSG5 using primers GL98, 5'-GACTCAGGATCCAATGAAGCTTCAAGAAGCCTTAATTTCC-3', which contains a *Bam*HI restriction site and a starting codon, and GL99, 5'-CCTGAGCTTCTACTGAGAATTCAGATTACGAGTCTCTTC-3', which contains an *Eco*RI restriction site. We cloned this fragment in pcDNA3.1(+), *Bam*HI-*Eco*RI. We then inserted the myc tag sequence between *Kpn*I and *Bam*HI to obtain pGL142. To express Gtn1967–2541 we made pGL141. We used *Pfu* turbo DNA polymerase to amplify a ~1.7-kb fragment from pGCP364/pSG5 using primers GL37, 5'-CCGAGCTCGAGATCGATCCATGTACCTCGGCAGGATCAGTGCC-3', which contains a *Bam*HI restriction site and a starting codon, and GL97, 5'-CAACTGCTCCTTGAATTCCTGATCTCTTTAGACAAACC-3', which contains an *Eco*RI restriction site. We cloned this fragment in pcDNA3.1(+), *Bam*HI-*Eco*RI. This mutation was verified by DNA sequencing. We then inserted the myc tag sequence between *Kpn*I and *Bam*HI to obtain pGL141. To express Gtn450–3187 we made pGL147. We used the Quickchange kit (Stratagene) to insert a stop codon in pGL88, which eliminated the last 24 amino acids of Giantin, including its transmembrane domain. We then subcloned the *Bam*HI-*Not*I ~8.1-kb fragment from this construct in pcDNA3.1(+) with a myc tag between *Kpn*I and *Bam*HI, to generate pGL147.

***In Vitro* Transcription/Translation**—Constructs for *in vitro* transcription/translation were in pSG5 (Stratagene) for full-length Giantin, in pSTBlue-1 (Novagen) for Gtn450–3187, and in pBluescript II for all other Giantin fragments. 50- $\mu$ l reactions were performed using a T7 RNA polymerase kit following the manufacturer's instructions (Promega, Madison, WI) using 1  $\mu$ g of plasmid DNA, the amino acid mix without methionine, and 4  $\mu$ l of L-[<sup>35</sup>S]methionine, 10 mCi/ml (ICN Biomedicals, Basingstoke, U. K.). Reactions were incubated for 2–4 h at 30 °C, and 5  $\mu$ l of each reaction was used for binding experiments.

**Binding Assays with Immobilized Proteins**—Native p115 was purified from rat liver cytosol (29, 38). Gtn448 and Gtn1125–1695 were prepared from bacterial cultures (*Escherichia coli*) following the pTrcHis instruction manual (Invitrogen). All three proteins were biotinylated using sulfo-*N*-hydroxysulfosuccinimide-long chain biotin (Pierce) for 2 h on ice (20 mol of biotin/mol of protein) and incubated with Ultralink immobilized neutravidin or streptavidin beads (Pierce) (0.3–1  $\mu$ g of biotinylated protein/ $\mu$ l of beads) in HKTID buffer (20 mM Hepes, pH 7.4, 100 mM KCl, 0.25% Triton X-100, 1 mM dithiothreitol) for 2 h at 4 °C. Beads were then washed with HKTID buffer and incubated, in the presence of protease inhibitors, with rat liver Golgi membranes



**FIG. 2. Association of full-length Giantin and Giantin fragments with p115.** Panel A, coupled *in vitro* transcription/translation using [<sup>35</sup>S]methionine as the label was performed of plasmids encoding full-length Giantin (lanes 1–3), full-length Giantin lacking the NH<sub>2</sub>-terminal 449 residues of a 25-fold molar excess of Gtn448 (lane 4), full-length Giantin lacking the NH<sub>2</sub>-terminal 449 residues (Gtn450–3187, lanes 4–6), the first 448 amino acids of Giantin (Gtn448, lanes 7–9), or the first 301 amino acids of Giantin (Gtn301, lanes 10–12). A fraction of each product was run alone (lanes 1, 4, 7, and 10), added to control beads (lanes 2, 5, 8, and 11), or added to p115 beads (lanes 3, 6, 9, and 12). Bound proteins were analyzed by SDS-PAGE and fluorography. Lanes 1 and 4, 25% of total; lanes 7 and 10, 10% of total; all other lanes, 100% of total. Panel B, coupled *in vitro* transcription/translation using [<sup>35</sup>S]methionine as the label was performed of a plasmid encoding full-length Giantin, and the product was added to p115 beads (lanes 1, 3, and 4) or control beads (lane 2) in the absence (lanes 1 and 2) or presence of a 25-fold molar excess of Gtn448 (lane 4) or a control Giantin fragment Gtn1125–1695 (lane 3). Bound proteins were eluted and analyzed by SDS-PAGE and fluorography. Panel C, rat liver cytosol was cleared by centrifugation and then incubated with biotinylated Gtn448 immobilized on neutravidin beads or with control beads. The clarified cytosol (lane 1, 20% of total) and proteins bound to control beads (lane 2, 50% of total) or Gtn448 beads (lane 3, 50% of total) were fractionated by SDS-PAGE and silver stained. p115 in lane 3 was identified using MALDI with electrospray mass spectrometry. Panel D, rat liver Golgi membranes were solubilized, cleared by centrifugation, and then incubated with biotinylated fragment Gtn448 immobilized on neutravidin beads or with control beads. The clarified Golgi extract (lanes 1 and 4, 20% of total) and proteins bound to control beads (lanes 2 and 5, 50% of total) or Gtn448 beads (lanes 3 and 6, 50% of total) were fractionated by SDS-PAGE and either silver stained (lanes 1–3) or blotted with an anti-p115 antibody (lanes 4–6). Molecular mass markers are in kDa. Panel E, Gtn448 (lane 1), Gtn1125–1695 (lane 2), or p115 (lanes 5 and 8) was biotinylated and immobilized on neutravidin beads. These and control beads were incubated with purified p115 (lanes 1–3), Gtn448 (lanes 5 and 6), or Gtn1125–1695 (lanes 8 and 9). Beads were washed and eluted with sample buffer. Bound proteins were detected by SDS-PAGE and Coomassie staining. L, protein loaded (2 μg each).

(100 μg of protein) prepared as described in Ref. 54 and solubilized in buffer containing 0.5% Triton X-100, rat liver cytosol (100 μg) at a protein concentration of 0.5 mg/ml and cleared by centrifugation, p115 (2 μg), Gtn448 (2 μg), Gtn1125–1695 (2 μg), or with 5 μl of *in vitro* translated protein along with bovine serum albumin as a carrier protein for 1–2 h at 4 °C. Beads were then washed three times with HKTD buffer and either boiled in sample buffer or incubated with elution buffer (HKTD buffer with KCl added to 1 M). Eluted proteins were then precipitated with 10% trichloroacetic acid. Proteins were analyzed by SDS-polyacrylamide gel followed by Coomassie Blue or silver staining. For experiments involving *in vitro* translated proteins we used a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) for analysis. Protein identification was done starting from zinc-imidazole-SDS reversed stained gels (55). Protein bands were excised, *in gel* digested with

trypsin, and the generated peptides were analyzed by MALDI–time-of-flight mass spectrometry using a Bruker Reflex III instrument (Bruker Instruments Inc. Bremen, Germany) operating under delayed extraction conditions. Proteins were first identified by peptide mass fingerprinting using an aliquot of the complete digestion mixture (56). Protein assignments were confirmed further by selecting a well resolved peptide ion from which post-source decay fragments were recorded using the reflectron mode (57). The information contained in these post-source decay spectra was subject to a search in a nonredundant protein data base or an expressed sequence tags data base using the SEQUEST algorithm (58). This analysis confirmed our previous assignment.

**Transient Expression and Immunofluorescence**—Normal rat kidney cells were microinjected using the Eppendorf Transjector 55246 and the Eppendorf Micromanipulator 5171 (Eppendorf, Hamburg, Germany) in

conjunction with a Zeiss Axiovert-10 microscope (Carl Zeiss, Oberkochen, Germany). Plasmid DNA (pGL140, pGL141, pGL142, or pGL147) was injected into nuclei at a concentration of 0.1 mg/ml. After injection the cells were incubated at 37 °C for 3 h, then fixed and permeabilized with cold methanol and incubated with the following antibodies: monoclonal 4H1 (against p115), monoclonal antibody against GM130, or polyclonal anti-myc tag, followed by the secondary antibodies Texas Red-X goat anti-mouse and Alexa Fluor 488 goat anti-rabbit. Coverslips were then mounted in Moviol 4–88 (Harco, Harlow, U. K.). Fluorescence analysis was performed using a Zeiss Axiovert-135TV microscope, and images were captured on a cooled CCD camera (Princeton Instruments, Trenton, NJ). Images were analyzed using IPLab Spectrum V3.1 software (Signals Analytics Corp., Vienna, VA).

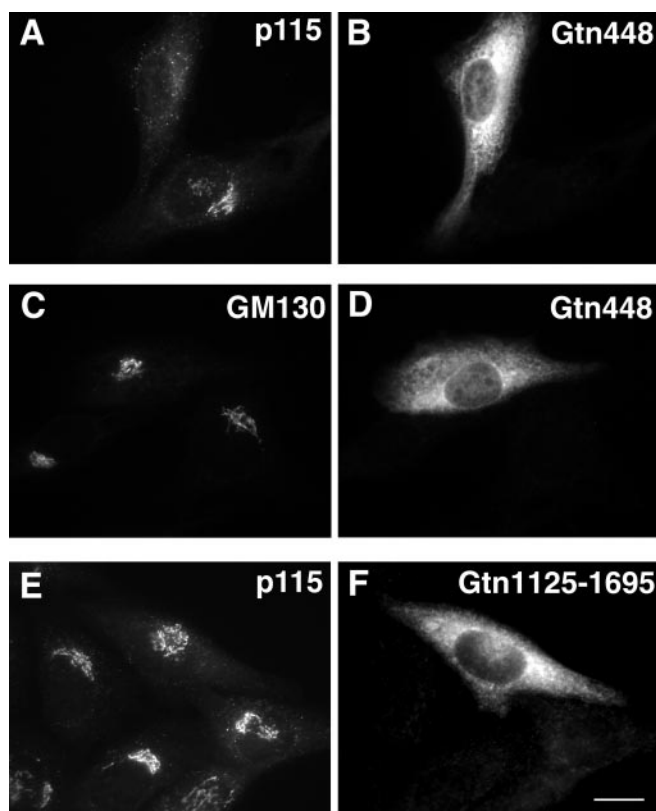
**Golgi Reassembly Assay**—The Golgi reassembly assay was performed essentially as in Ref. 48. Rat liver Golgi membranes and HeLa mitotic cytosol were prepared as described previously (54, 59). Mitotic Golgi fragments were generated by incubating rat liver Golgi membranes with mitotic cytosol for 20 min at 37 °C. The mitotic Golgi fragments were recovered by centrifugation through a 0.5 M sucrose cushion (48) and subsequently preincubated on ice for 15 min with either Giantin fragment buffer (20 mM Hepes/KOH, pH 7.4, 100 mM KCl, 1 mM magnesium acetate, and 0.1 mM dithiothreitol), Gtn448, or Gtn1967–2541 to achieve a final concentration of Giantin fragment of 3.25 μM in the reassembly reaction. In some experiments increasing concentrations of Gtn448 were used to pretreat the mitotic Golgi fragments. The pretreated mitotic Golgi fragments were then resuspended at 0.75 μg/μl with NSF (100 ng/μl; final concentration), α-SNAP (25 ng/μl), γ-SNAP (25 ng/μl), p115 (30 ng/μl), and an ATP regeneration system in a final reaction volume of 20 μl and incubated for 60 min at 37 °C. Reactions were then fixed and processed for electron microscopy (46) and the amount of cisternal regrowth determined (36).

## RESULTS

**The NH<sub>2</sub>-terminal Domain of Giantin Binds Directly to p115**—The p115 binding site on Giantin was mapped by generating NH<sub>2</sub>-terminal and COOH-terminal deletions of the protein as well as internal fragments (see Fig. 1). These were generated by *in vitro* transcription/translation using [<sup>35</sup>S]methionine as the label and then tested for binding to p115 immobilized by biotinylation and binding to streptavidin or neutravidin beads. As shown in Fig. 2A, full-length Giantin bound to p115 beads but not to control beads. Removal of the first 448 amino acids was sufficient to abolish most of this binding. In contrast, the first 448 amino acids alone were sufficient to bind p115 almost as well as the full-length protein. Quantitation from two experiments gave a binding efficiency of 80% compared with the full-length protein. Further mapping within this region generated mostly inactive fragments (not shown; for a summary, see Fig. 1), although a fragment of the NH<sub>2</sub>-terminal 301 amino acids did bind weakly to p115 (about 25% compared with full-length Giantin; *n* = 2).

Because these results suggested that most of the p115 binding activity resided in the NH<sub>2</sub>-terminal 448 amino acids of Giantin, a recombinant Gtn448 protein was generated and characterized as well as two control peptides from internal regions of the rest of the protein (Gtn1125–1695 and Gtn1967–2541, see Fig. 1). Gtn448 prevented the binding of *in vitro* transcribed/translated Giantin to p115 beads, whereas the control peptide Gtn1125–1695 did not (Fig. 2B). This shows that Gtn448 has an active p115 binding site. This was confirmed by immobilizing Gtn448 on beads and incubating them either with cytosol or extracts of Golgi membranes. A band of 115 kDa was the only additional protein to be isolated from cytosol compared with control beads (Fig. 2C). This protein band was identified unambiguously by peptide mass fingerprinting, confirmed by MALDI postsourc e decay analysis and data base searching as p115. A similar result was obtained using extracted Golgi membranes, and in this instance the identity of the protein was confirmed by Western blotting (Fig. 2D).

Purified Giantin fragments and p115 were used to show a direct interaction (Fig. 2E). Immobilized Gtn448 bound p115,

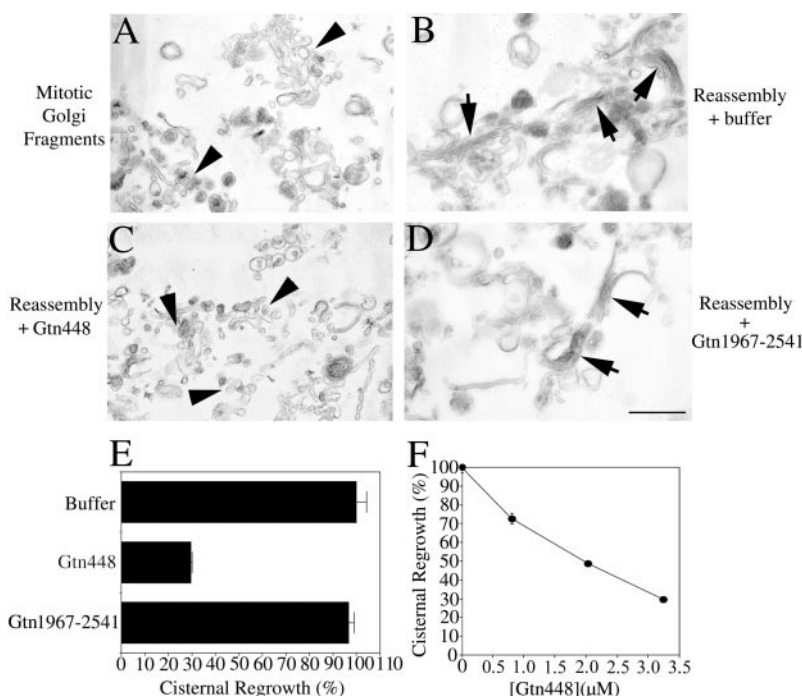


**FIG. 3. Overexpression of rat Giantin fragment Gtn448 inhibits p115 binding to the Golgi apparatus.** Myc-tagged Giantin fragment Gtn448 (panels A–D) or myc-tagged Giantin fragment Gtn1125–1695 (panels E and F) were transiently expressed in normal rat kidney cells by microinjection of the respective plasmids into the nuclei. After 3 h, the cells were fixed and permeabilized with cold methanol. Cells were then double labeled with polyclonal antibodies against the myc tag (panels B, D, and F) and with a monoclonal antibody against p115 (panels A and E) or GM130 (panel C) followed by fluorescently tagged secondary antibodies Alexa Fluor 488 (panels B, D, and E) or Texas Red-X (panels A, C, and E). Bar, 20 μm.

whereas beads and an immobilized control peptide did so only weakly. In the converse experiment, immobilized p115 bound Gtn448 but not the control peptide. Beads alone bound neither. These results clearly show that p115 and Gtn448 interact directly; however, the interaction was not stoichiometric, suggesting that other parts of the Giantin molecule or additional proteins may be needed.

**Overexpression of Gtn448 Removes p115 from the Golgi Apparatus *in Vivo***—p115 is a cytosolic protein (30) that localizes to the Golgi apparatus (29, 36, 39) and peripheral vesicular tubular clusters (60). To determine whether Gtn448 binds p115 *in vivo*, myc-tagged Gtn448 was transiently expressed in normal rat kidney cells by nuclear microinjection of the respective plasmids and detected with polyclonal antibodies against the myc tag (53). In noninjected cells the p115 antibody stained a juxtannuclear ribbon corresponding to the Golgi complex (Fig. 3) and peripheral, punctate structures. Overexpression of Gtn448 resulted in removal of p115 from the Golgi apparatus (Fig. 3A) but not from the peripheral structures. Overexpression did not result in disruption of the Golgi as detected by an anti-GM130 antibody (Fig. 3C). The effect on p115 was specific because overexpression of the Giantin fragments Gtn1125–1695 (Fig. 3, E and F) or Gtn1967–2541 (not shown) did not remove p115 from the Golgi. In addition, overexpression of a construct lacking the NH<sub>2</sub>-terminal 449 amino acids and the transmembrane domain (to make it cytoplasmic) did not remove p115 from the Golgi (not shown).

**FIG. 4. Rat Giantin fragment Gtn448 inhibits the NSF-catalyzed fusion of mitotic Golgi fragments.** Highly purified rat liver Golgi membranes were incubated with mitotic cytosol for 20 min at 37 °C and the mitotic Golgi fragments (MGF, panel A) isolated by centrifugation. These were then preincubated for 15 min on ice with buffer (panel B), 3.25  $\mu$ M Gtn448 (panel C), or 3.25  $\mu$ M Gtn1967–2541 (panel D) and then incubated for 60 min at 37 °C with NSF,  $\alpha$ -SNAP,  $\gamma$ -SNAP, and p115. Samples were fixed and processed for electron microscopy and the amount of cisternal regrowth determined (panel E). Arrows denote reassembled Golgi stacks, and arrowheads denote tubulovesicular membranes. Bar, 0.5  $\mu$ m. Panel F, increasing amounts of Gtn448 were titrated into the reassembly reaction and the amount of cisternal regrowth determined.



**Gtn448 Inhibits the NSF-catalyzed Fusion of Mitotic Golgi Fragments**—The disassembly of the Golgi apparatus during mitosis can be mimicked in a cell-free system by incubating highly purified Golgi membranes with mitotic cytosol. The Golgi apparatus disassembles, forming a population of vesicles and short tubular structures (45). By removing the cytosol and adding the purified components NSF,  $\alpha$ -SNAP,  $\gamma$ -SNAP, and p115 in the presence of ATP and GTP, these Golgi fragments grow to become bigger cisternae, which are then restacked to form the typical Golgi structure (46–48, 61, 62). Giantin is involved in p115-mediated Golgi reassembly because antibodies against Giantin inhibit cisternal growth and stacking (48). We therefore tested whether Gtn448 had an effect on this process. Mitotic Golgi fragments (Fig. 4A) were preincubated with reassembly buffer or with reassembly buffer plus recombinant His<sub>6</sub>-tagged Gtn448 at a concentration approximately 50-fold that of endogenous Giantin. Samples were fixed, and cisternal regrowth was quantitated by electron microscopy (48). When preincubated with reassembly buffer alone, the Golgi fragments formed flattened, stacked cisternae (Fig. 4B). However, in the presence of Gtn448 cisternal regrowth was inhibited drastically (Fig. 4C), and this inhibition was dose-dependent (Fig. 4F). In contrast, in the presence of the control Giantin peptide Gtn1967–2541, Golgi reassembly was not affected (Fig. 4, D and E). These results show that Gtn448 interferes with cisternal regrowth and strongly suggest that this Giantin domain participates in Golgi reassembly.

#### DISCUSSION

In this study we have shown that Giantin interacts directly with the vesicle-tethering protein p115. We have mapped the binding site to the NH<sub>2</sub>-terminal domain of Giantin and found that overexpressing it in living cells removes p115 from the Golgi apparatus. Finally, we have shown that this fragment has a crucial role in reassembling the Golgi apparatus using a well established cell-free assay.

The NH<sub>2</sub>-terminal 448 amino acids was the minimum fragment of Giantin (Gtn448) which bound p115 almost as well as full-length Giantin. Deletions of this NH<sub>2</sub> terminus resulted in inactive fragments. Deletions within this NH<sub>2</sub> terminus also abolished binding. The only exception was a fragment compris-

ing the first 301 amino acids, which bound p115 but did so very weakly (see Fig. 1). We therefore conclude that the first 448 NH<sub>2</sub>-terminal amino acids are needed for maximal binding to p115. This region is predicted to be mostly coiled-coil, and it does not seem to have any particular features apart from a stretch of basic amino acids around position 100 which is similar but not identical to that found in the p115 binding site of GM130 (36). However, because Gtn186, which contains this basic stretch, did not associate with p115, it is either not required or not sufficient for p115 binding.

Although Gtn448 bound p115 almost as well as full-length Giantin, the binding was not stoichiometric. There are several possible explanations for this. First, Gtn448 might not be a dimer because it lacks the COOH-terminal region that is thought to be necessary for Giantin to oligomerize. This could result in less efficient binding to p115. Second, additional proteins could be needed for optimal binding to p115. Examples would include Sec35p (63), Sec34p (64), and polypeptides of the transport protein particle (65). Third, other regions of Giantin might have some affinity for p115. The evidence is against this, however, because there was only one peptide (Gtn1967–2541) that associated weakly with p115 *in vitro*, and this peptide did not interfere with Golgi reassembly or remove p115 from the Golgi *in vivo*. Fourth, the interaction might be sensitive to post-translational modification of the proteins. There is evidence to suggest that p115 binds less efficiently to Giantin on membranes compared with vesicles (31). There is also evidence that phosphorylation/dephosphorylation of p115 might be important for its functioning (66). Finally, it is of course possible that the low affinity between p115 and Giantin could be part of the tethering mechanism. COPI vesicles appear to have more than one Giantin per vesicle (31) so that strong tethering could result from multiple weak interactions. This would also make it easier to break the interactions once tethering is completed.

The NH<sub>2</sub>-terminal 448 amino acids of Giantin appear to be the major p115 binding site *in vivo*. Overexpressed Gtn448 removed p115 from the Golgi, but other Giantin domains or Giantin missing the NH<sub>2</sub>-terminal 449 amino acids did not. The NH<sub>2</sub>-terminal domain also appears to be the major one responsible for Giantin function in Golgi reassembly because

Gtn448 inhibited cisternal regrowth to an extent similar to that of Giantin polyclonal antibodies (48). Together these data strongly argue that the Giantin NH<sub>2</sub> terminus is functionally significant and active *in vivo*.

The fact that the p115 binding site is at the NH<sub>2</sub> terminus is consistent with the idea of Giantin being a long tether linking the Golgi membrane at this end to the vesicle at the other. Several lines of evidence support this idea. First, Giantin is anchored to membranes via its 25 COOH-terminal amino acids (50). Second, many fibrous proteins with predicted coiled-coil structure similar to that of Giantin are elongated structures with NH<sub>2</sub> and COOH termini at opposite ends. These include the nuclear mitotic apparatus protein (NuMA) (67), the synaptonemal complex protein 1 (SCP1) (68), myosin (69), and intermediate filament proteins such as keratins, lamins, vimentin, plectin, and desmin (70, 71). Third, in agreement with its predicted coiled-coil structure, calculations based on glycerol velocity gradients estimate Giantin to be a long rod measuring 3.5 × 250 nm (50). This would be long enough to pass through the COPI coat (10–15 nm) and protrude out into the cytoplasm (8). Finally, previous experiments suggested that Giantin is indeed present in most, if not all, COPI-coated vesicles and is likely present in multiple copies (31).

Based on these data it is possible to propose a model in which Giantin plays a crucial role in tethering COPI vesicles to Golgi membranes. COPI vesicles would bud from the Golgi carrying multiple copies of Giantin. The NH<sub>2</sub> terminus, the most distant part from the vesicle coat, would tether the vesicle to Golgi membranes by binding to p115 (which in turn would bind GM130). The binding site at the NH<sub>2</sub>-terminal end of Giantin would permit interaction at some distance from the membrane, perhaps permitting the vesicle the freedom to sample the membrane for cognate SNAREs. Based on its sequence, Giantin should have a good degree of flexibility, which would then allow the vesicle to come closer to Golgi membranes so that v- and t-SNAREs could pair and eventually catalyze fusion. This model fits all available data and suggests that vesicle tethering might be a highly orchestrated process. Much more study, however, will be needed to work out the details.

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