THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 27, Issue of July 8, pp. 25840–25848, 2005

Printed in U.S.A.

A Cryptic Rab1-binding Site in the p115 Tethering Protein*[S] ♦

Received for publication, April 11, 2005, and in revised form, May 5, 2005
Published, JBC Papers in Press, May 6, 2005, DOI 10.1074/jbc.M503925200

Matthew Beard‡, Ayano Satoh§¶, James Shorter‖, and Graham Warren§**

From the ‡Department of Physiology and Cellular Biophysics, Columbia University, New York, New York 10032, the §Department of Cell Biology, Ludwig Institute for Cancer Research, Yale University School of Medicine, New Haven, Connecticut 06520, and the ‖Lindquist Lab, Whitehead Institute, Cambridge, Massachusetts 02142

Small GTPases and coiled-coil proteins of the golgin family help to tether COPI vesicles to Golgi membranes. At the *cis*-side of the Golgi, the Rab1 GTPase binds directly to each of three coiled-coil proteins: p115, GM130, and as now shown, Giantin. Rab1 binds to a coiled-coil region within the tail domain of p115 and this binding is inhibited by the C-terminal, acidic domain of p115. Furthermore, GM130 and Giantin bind to the acidic domain of p115 and stimulate p115 binding to Rab1, suggesting that p115 binding to Rab1 is regulated. Regulation of this interaction by proteins such as GM130 and Giantin may control the membrane recruitment of p115 by Rab1.

Targeting of transport vesicles to the correct membrane compartment is a multilayered process consisting of tethering, docking, and fusion. SNARE[1] proteins are the best characterized components of the docking and fusion machinery. Cognate SNARE pairs are thought to provide the core specificity for membrane fusion (1–3). Tethering components act before SNAREs and are thought to provide an initial interaction between a vesicle and target membrane (4–6).

Tethering is essential for transport and is mediated by a diverse array of proteins including: GTPases of the Ypt/Rab and Arl families, coiled-coil proteins that can link membranes together, and large multiprotein assemblies recently termed quatrefoil tethering complexes (7–10). At the entry face of the Golgi apparatus, multiprotein complexes include TRAPP and COG (11, 12), the Rab family GTPases are Rabs 1 and 2 (13), and the coiled-coil proteins are p115, Giantin, and GM130. The latter are members of the golgin protein family, initially identified as antigens in certain autoimmune diseases (14). The precise roles of tethering proteins, their mechanisms of action, and how they interact, however, remain unclear.

The best characterized Golgi tethering proteins are mammalian p115 and its yeast homologue Uso1p. These proteins are myosin-shaped, homodimeric molecules, each polypeptide of which comprises an N-terminal globular head, a coiled-coil tail, and a short C-terminal acidic domain (15–18). Uso1p is essential for exocytic transport (19) and tethers COPII vesicles to Golgi membranes in yeast (20–22). p115 is essential for both exocytic transport and maintenance of the stacked structure of mammalian Golgi membranes (23). It acts during ER to Golgi and intra-Golgi transport, as well as post-mitotic Golgi reassembly (15, 24). p115 tethers COPI vesicles to Golgi membranes (25). These data suggest that p115/Uso1p functions to tether vesicles, although the molecular mechanism is still unclear.

Our working model for the mechanism by which p115 tethers COPI vesicles to Golgi membranes has been that it forms a "bridge," simultaneously binding and linking Giantin in COPI vesicle membranes to GM130 on the Golgi (25). This model for a *cis*-golgin tethering complex arose from two important ideas: 1) GM130 acts as the Golgi membrane anchor for p115; 2) p115 (anchored by GM130) tethers by simultaneously binding to Giantin in vesicle membranes.

The first idea originated from studies into the mitotic disassembly of mammalian Golgi stacks. GM130 is a mitotically regulated, p115-binding protein present in highly purified Golgi membranes (26–29). The N-terminal domain of GM130 binds to the acidic C-terminal domain of p115 (27, 30–32). GM130 is regulated by the mitotic kinase, CDK1/cyclin B (28). CDK1/cyclin B-mediated phosphorylation of GM130 inhibits GM130 binding to p115 (27), and this correlates with the inhibition of p115 binding to Golgi membranes *in vivo* (33) and *in vitro* (34). Furthermore, p115 localization to the Golgi region of cells is disrupted either by microinjection of a GM130 N-terminal peptide (N73), which inhibits p115 binding to GM130, or by overexpression of a truncated GM130 that lacks the p115-binding domain (35).

The second idea, that p115 links GM130 to Giantin, arose from the observation that GM130 and Giantin are asymmetrically distributed between Golgi membranes and vesicles. Giantin (but not GM130) is incorporated into COPI vesicles, during *in vitro* budding reactions. p115 stimulates binding of these vesicles to Golgi membranes (25). Giantin, like GM130, is a major p115-binding protein in Golgi extracts (25). However, whereas anti-GM130 antibodies (or the peptide N73) prevent p115 binding to Golgi membranes, antibodies against Giantin do not (25). In contrast, anti-Giantin antibodies do prevent p115 binding to COPI vesicles, but anti-GM130 antibodies do not. Inhibition of p115 binding to either GM130 on Golgi membranes or Giantin in vesicles is sufficient to abolish the p115 effect on tethering (25, 36). p115 is also necessary to link Giantin and GM130 during co-immunoprecipitation experiments from Golgi extracts (32). The functional importance of these interactions is apparent because agents that inhibit p115 binding to Giantin or to GM130 block tethering and fusion during an *in vitro* assay for Golgi reassembly after mitosis (36).

* This work was supported in part by National Institutes of Health and the Ludwig Institute for Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

♦ This article was selected as a Paper of the Week.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Table S1.

¶ Supported by the American Heart Association.

** To whom correspondence should be addressed: Dept. of Cell Biology, Ludwig Inst. for Cancer Research, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520-8002. Tel.: 203-785-5058; Fax: 203-785-4301; E-mail: graham.warren@yale.edu.

[1] The abbreviations used are: SNARE, soluble NSF attachment protein receptors (where NSF is *N*-ethylmaleimide-sensitive factor); ER, endoplasmic reticulum; MBP, maltose-binding protein; GST, glutathione *S*-transferase; DTT, dithiothreitol; PI, protease inhibitor; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

Furthermore, GM130 and Giantin function *in vivo*, because microinjected antibodies against these proteins inhibit exocytic transport (24). The most parsimonious explanation for these results is that p115 tethers by linking GM130 on one membrane to Giantin in the other.

However, several lines of evidence are inconsistent with these interpretations. First, antibodies against p115, GM130, and Giantin cause different phenotypes when microinjected. Although antibodies to GM130 or Giantin inhibit transport, they do so at a later stage than antibodies against p115 (24). In other experiments, microinjection of anti-Giantin antibodies led to degradation, but this did not inhibit progression through mitosis or Golgi reassembly in the daughter cells, at least as assessed by immunofluorescence microscopy. Both of these processes are believed to depend on tethering and fusion. Similarly, microinjected anti-GM130 antibodies did not affect mitosis or Golgi reassembly. In contrast, microinjected anti-p115 antibodies led to p115 degradation and collapse of the Golgi structure (37). These results suggest a function for p115 that is independent of its interactions with GM130 and Giantin and were presented as arguing against the working model for tethering by p115.

Second, a prediction of the tethering model is that p115 constructs without the GM130 and Giantin-binding sites should neither localize to the Golgi apparatus nor function to tether membranes. Indeed, inhibition of GM130 binding to p115 (by microinjection of the N73 peptide or by truncation of GM130) does block p115 localization to the Golgi apparatus (35). Confusingly, however, an initial study showed that p115 truncations lacking the GM130 and Giantin-binding domain do localize to the Golgi region of transfected cells (38). This finding has recently been confirmed and extended by a gene replacement approach showing that truncated p115, without the binding domain for GM130 or Giantin, is sufficient to rescue Golgi morphology and transport in cells where endogenous p115 has been knocked down by RNA interference (23). It seems, therefore, that experiments in which p115 can no longer bind to GM130 (and Giantin) contradict those in which GM130 can no longer bind to p115. Furthermore, the acidic C-terminal domain of p115 is absent in *Drosophila* p115 and is much shorter in *C. elegans* (39). Furthermore, a conditional lethal CHO cell line that contains no detectable GM130 immunoreactivity has been described that has no apparent defect in Golgi structure or transport when grown at the permissive temperature (40). Together these data are difficult to reconcile with the model that p115 tethers by directly linking GM130 to Giantin.

A resolution for these discrepancies might be related to the function of Rab1, which also acts during ER to Golgi and intra-Golgi transport and binds directly to p115 and golgin tethering proteins (41–43). Rab family GTPases function throughout the exocytic pathway and several family members are implicated in tethering (4, 6, 7, 44). Rab proteins undergo a cycle of GTP binding and hydrolysis that switches between their active and inactive states, respectively. In the active state they bind effectors that are either membrane proteins (43, 45, 46) or that become recruited to membranes by the Rab itself (41, 47). After GTP hydrolysis, Rab proteins are removed from the membrane by Rab-GDP dissociation inhibitor. This allows recycling of GDP bound Rab for further rounds of transport (48).

Rab1 recruits p115 to COPII vesicles during *in vitro* assays for ER to Golgi transport (41). It also binds GM130 and acts on the Golgi membrane (42). The yeast homologue, Ypt1p, recruits Uso1p (p115) to membranes and acts in tethering during ER to Golgi and intra-Golgi transport (21, 22, 49, 50).

While mapping the Rab1-binding site on p115 we noticed a dramatic enhancement in the apparent affinity of interaction when the p115 C-terminal domain was removed. Further experiments showed that the p115 C-terminal domain bound to and competed for the Rab1-binding site on p115. The inhibition of Rab1 binding was relieved by either GM130 or Giantin, both of which bind directly to p115 and to Rab1. This raises the possibility that binding of these golgins serves a regulatory role instead of, or in addition to, a structural role in tethering.

EXPERIMENTAL PROCEDURES

Antibodies—For anti-Giantin, antibodies (against 1–448 and 1125–1695) were raised in rabbits, against hexahistidine His_6-tagged immunogens. Sera were concentrated (40% ammonium sulfate precipitation) and dialyzed against 25 mM Tris (pH 8.0), 150 mM KCl. Anti 1125–1695 was affinity-purified against immunogen covalently linked to cyanogen bromide-activated Sepharose (GE Healthcare). Other antibodies were as follows: GM130, monoclonal (Transduction Laboratories); p115, monoclonal (15); GRASP65 (7E10), monoclonal (Francis Barr, Max Planck Institute of Biochemistry, Martinsried, Germany); GRASP65, polyclonal (51).

Plasmids—Rab1a/pGEX4T3, Rab2/pGEX, Rab6/pGEX, and Rab11/pGEX were gifts from Tommy Nilsson (Göteborg University, Göteborg, Sweden), Francis Barr (Max Planck Institute of Biochemistry), and David Sheff (University of Iowa), respectively.

Rab1a(S25N), Rab1a(Q70L), Giantin 1–500/pET23a, Giantin 121–500-maltose-binding protein (MBP)/pET23a, his-CC1,2,3 (p115 652–812)/pQE9, his-CC1,2 (p115 652–776)/pQE9, his-CC1 (p115 652–701)/pQE9, and his-CC2,3,4 (p115 704–933)/pQE9 were made by QuikChange® mutagenesis (Stratagene). Giantin 1–1197/pET23a, Giantin 1–500-MBP/pET23a, GM130/pET23a, p115/pGEX6P1, and p115-A/pGEX6P1 were made by PCR and subcloning (see supplemental Table S1 for primer sequences). Constructs were verified by sequencing.

Other plasmids used in this study were as follows: pGCP364/pSG5, pGL88, pGL108, pGL141, pGL147, pGL101 (30), pBSGM130 (26), GRASP65-his/pET30a (51), his-H (p115 1–650)/pQE9, his-TA (p115 651–961)/pQE9, and his-T (p115 651–933)/pQE9 (36).

Recombinant Proteins—Recombinant proteins were purified on glutathione-Sepharose 4B (Amersham Biosciences) or nickel-nitrilotriacetic acid-agarose (Qiagen). GM130-his was further purified by ion-exchange chromatography (Hi-Trap SP column (pH 7.4)) (Amersham Biosciences) and gel filtration (Hi-Prep 16/60 Sephacryl S-300 column) (Amersham Biosciences). The GST moiety was removed from GST-p115 using PreScission protease (Amersham Biosciences).

Rabs were loaded as described previously (52). Briefly, Rab was washed with exchange buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM EDTA, 5 mM $MgCl_2$, 1 μM DTT, 1 μM guanosine nucleotide) and then incubated (three times, 30 min, room temperature) in exchange incubation buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM EDTA, 5 mM $MgCl_2$, 1 μM DTT, 1 mM guanosine nucleotide). After washing in stabilization buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 μM DTT, 1 μM guanosine nucleotide) the Rab was then incubated (30 min, room temperature) in stabilization incubation buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 μM DTT, 1 mM guanosine nucleotide).

Superose 6 Chromatography—Recombinant p115, his-TA, or his-T were filtered (0.45 μm) to remove any particulate matter and then gel-filtrated on a Superose 6 HR 10/30 column (Amersham Biosciences), equilibrated in column buffer (25 mM HEPES (pH 7.4), 200 mM KCl, 1 mM DTT, 10% glycerol), at 0.2 ml/min. Half-ml fractions were collected, and aliquots were analyzed by SDS-PAGE with Coomassie staining. Thyroglobulin (669 kDa, Stokes radius 85.0 Å), ferritin (440 kDa, Stokes radius 61.0 Å), catalase (232 kDa, Stokes radius 52.2 Å), and aldolase (158 kDa, Stokes radius 48.1 Å) were run as standards.

Velocity Sedementation—Recombinant p115, his-TA, or his-T were layered onto a linear glycerol gradient (10–30% (w/v)) in gradient buffer (25 mM HEPES (pH 7.4), 200 mM KCl, 1 mM DTT). Thyroglobulin (20.0 S), catalase (11.4 S), and bovine serum albumin (4.6 S) were run as standards. The gradients were centrifuged for 5 h in an SW55 rotor (Beckman Coulter), at 4 °C and then fractionated from the top into ~0.4-ml fractions. Aliquots were analyzed by SDS-PAGE with Coomassie staining.

Golgi Membrane Extracts—Rat liver Golgi was purified from rat liver as described (53) and then extracted at 0.5 mg/ml with extraction buffer (20 mM HEPES (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100, 1 mg/ml soybean trypsin inhibitor, 1 μM DTT, EDTA free protease inhibitor (PI) mixture (Roche Applied Science)) for 30 min on ice and clarified

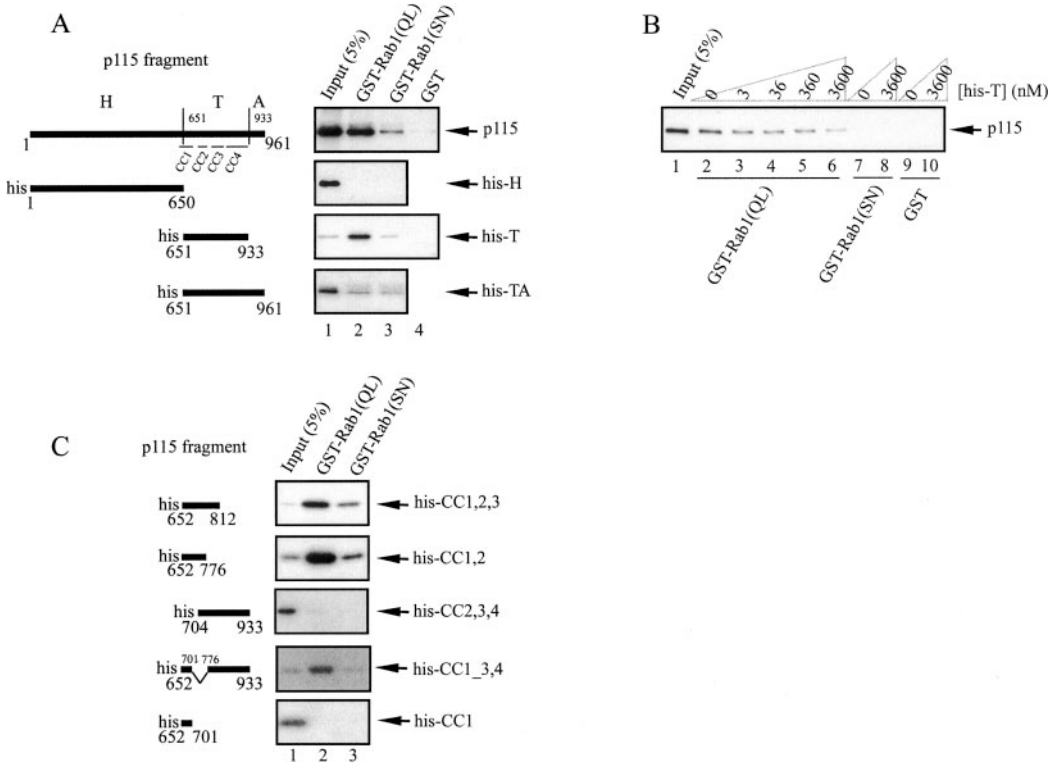

FIG. 1. **Mapping the Rab1-binding site on p115.** *A*, full-length p115 and a set of truncations were incubated with GST-Rab1(QL) (a GTP-locked, active mutant) and with GST-Rab1(SN) (a GDP-locked, inactive mutant). *B*, full-length p115 was incubated with GST-Rab1 in the presence of increasing concentrations of his-T. *C*, truncations corresponding to one or more coiled-coil regions within the p115 tail were incubated with GST-Rab1. In all cases bound proteins were analyzed by immunoblotting after SDS-PAGE. The schematics to the *left* of *A* and *C* are included to show the domain structure of p115 (*A*, *top*) and to indicate the regions covered by each truncation.

by centrifugation (20 min, 16,000 × *g*, 4 °C). For carbonate stripping, rat liver Golgi (0.2 mg/ml) was incubated in carbonate buffer (0.2 M sodium carbonate (pH 11) and PI) for 1 h on ice. Stripped rat liver Golgi was concentrated (30 min, 11,700 × *g*, 4 °C, on a swing out microcentrifuge) onto a 2 μl sucrose cushion (2 M), resuspended (0.2 mg/ml) in sucrose buffer (20 mM HEPES (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 200 mM sucrose, 1 μM DTT, PI), reconcentrated, and then extracted as above.

In Vitro Transcription/Translation—Twenty-five μl reactions were performed using the TNT T7 coupled reticulocyte lysate system (Promega) using 0.5 μg of plasmid DNA and 2 μl of [^{35}S]methionine (10 mCi/ml) per reaction. Two μl of each reaction was analyzed by SDS-PAGE. The remainder was frozen in liquid nitrogen and stored at −80 °C until use.

Binding Assays—Glutathione *S*-transferase (GST)-Rab1, immobilized on glutathione-Sepharose 4B, was incubated with putative binding proteins in binding buffer (20 mM HEPES (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 1 mg/ml soybean trypsin inhibitor, 1% Triton X-100, 1 μM DTT, PI supplemented with guanosine nucleotide (1 mM)) for 1 h, rotating at 4 °C. Beads were washed twice in the same buffer and then once in nucleotide-free binding buffer. Proteins were eluted (three incubations, 10 min, rotating, room temperature) in elution buffer (20 mM HEPES (pH 7.4), 100 mM KCl, 20 mM EDTA, 1% Triton X-100, 1 mM DTT, 5 mM GDP), pooled, concentrated by trichloroacetic acid precipitation, and then analyzed by SDS-PAGE.

GST-p115CT binding assays were performed as for Rab1 except: 10 μg of GST-p115CT (p115 886–961) was used. Beads were washed three times in the same buffer then resuspended in 2× sample buffer (100 mM Tris (pH 6.8), 3% SDS, 15% glycerol, 5% DTT, 0.01% bromphenol blue)

Quantification—Blots were quantified by scanning (Epson Expression 1680 scanner) or by directly measuring the ECL signal (Kodak Image Station 440CF). Signal intensity was compared with at least three standard samples of the same protein, loaded as a series of 2-fold dilutions, and detected on the same immunoblot. Signals were only quantified if their measured intensity lay within a range encompassed by the standards and over which there was a linear relationship between the amount of material loaded and signal intensity.

RESULTS

Rab1 Binds to the Coiled-coil Tail Region of p115—The Rab1-binding site on p115 had not previously been mapped. A set of truncations corresponding to structural and functional domains in p115 was therefore constructed and tested for binding to GST-Rab1 (Fig. 1*A*). p115 is thought to be a parallel homodimer of two polypeptide chains. Each is composed of an N-terminal globular head (*H*); a rod-like tail (*T*) containing four regions of predicted coiled-coil structure (*CC1*, *CC2*, *CC3*, *CC4*), and a short (28 amino acid), C-terminal acidic domain (*A*) (Fig. 1*A*) (17, 18).

Full-length p115 or truncations corresponding to the head (his-H), tail (his-T), or tail plus acidic (his-TA) domains (50 nM each) were incubated with immobilized GST or GST-Rab1 fusion proteins (1 nmol/200 μl reaction volume). Fusion proteins of either wild type Rab1a (preloaded with an appropriate guanosine nucleotide) or point mutations stabilized in the GTP bound (GST-Rab1(QL)) or the GDP bound (GST-Rab1(GDP)) conformations were tested. Specifically bound proteins were eluted by incubation with GDP/EDTA buffer. This technique has been used previously to measure effector binding to Rab1 (41–43, 45, 46) and other Rab family members (54–58).

In these assays, ~5% of the full-length p115 bound GST-Rab1(QL), whereas ≤0.5% bound to the inactive conformation GST-Rab1(SN) or to the GST controls (Fig. 1*A*). Similar results were obtained with wild type GST-Rab1 loaded with either GTPγS or GDP (data not shown). There was no detectable binding of his-H to GST-Rab1. In contrast, his-T bound strongly to GST-Rab1(QL), over 20% of the input bound (Fig. 1*A*). This shows that the T region in p115 contains a Rab1-binding site. The localization was confirmed by competition experiments to measure binding, between full-length p115 and GST-Rab1, in the presence of increasing concentrations of his-T

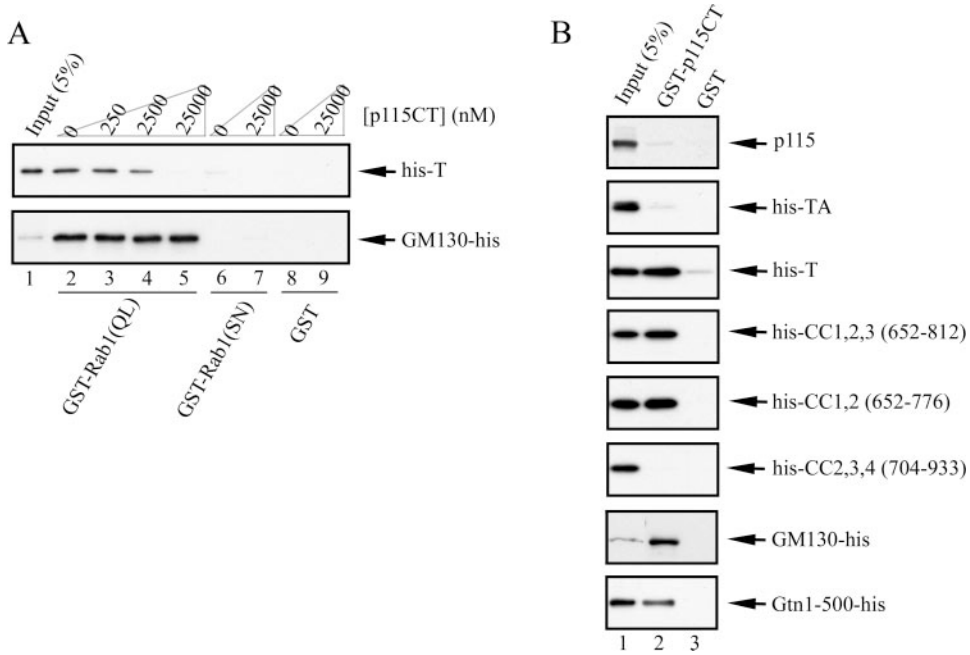

FIG. 2. **The C-terminal acidic region of p115 binds to the tail domain and inhibits Rab1 binding.** *A*, either his-T (*top*) or GM130-his (*bottom*) were incubated with GST-Rab1(QL) or GST-Rab1(SN) in the presence of increasing concentrations of a peptide corresponding to the extreme C-terminal region (residues 886–961) of p115 (p115CT). *B*, full-length p115 and a set of truncations were incubated with GST-p115CT or GST. Bound proteins were analyzed by immunoblotting.

(Fig. 1*B*). his-T competed efficiently with full-length p115 for binding to GST-Rab1(QL), supporting the localization of a Rab1-binding site in the T domain.

Further truncations and deletions within the T domain of p115 were then prepared, corresponding to one or more of the coiled-coil regions: his-CC1,2,3, his-CC1,2, his-CC2,3,4, his-CC1_3,4, and his-CC1 (Fig. 1*C*). Binding assays between GST-Rab1 and each of these proteins revealed robust binding of his-CC1,2,3, his-CC1,2, and his-CC1_3,4 to GST-Rab1(QL) (>20% input bound) (Fig. 1*C*). In contrast his-CC2,3,4 did not bind (Fig. 1*C*), suggesting that CC1 is the only region within the p115 tail that is necessary for binding. However, the truncation his-CC1 did not bind to GST-Rab1(QL). Together these results suggest that the CC1 domain is necessary but not sufficient for Rab1 binding (Fig. 1*C*); the Rab1-binding site on p115 includes CC1 of the p115 tail, but other structural features are also necessary for Rab1 binding.

The p115 C-terminal Domain Affects Rab1 Binding—While performing these mapping experiments we noticed that his-T, his-CC1,2,3, his-CC1,2, and his-CC1_3,4 all bound to GST-Rab1(QL) with a much higher apparent affinity than did full-length p115. Less than 5% of input p115 bound to GST-Rab1(QL) compared with over 20% of the truncations under the same conditions (Fig. 1, *A* and *C*). The high affinity binding truncations all lacked the C-terminal region of p115. In contrast to this, his-TA, that includes the C-terminal domain, bound GST-Rab1 so poorly that we were unable to detect a signal above background under any conditions that we tested (Fig. 1*A*). This led us to speculate that the C-terminal region of p115 might inhibit Rab1 binding and perhaps serves a regulatory function.

A trivial explanation for the observed differences in Rab1 binding between full-length p115 and the truncations might be that our preparations of recombinant full-length p115 and his-TA are predominantly misfolded but that our his-T preparations are not. To address this possibility we characterized our preparations of these three proteins by gel filtration and velocity sedimentation to compare our data with a previous characterization of purified p115 (15). Recombinant full-length p115 gel-filtered (Superose 6) with an apparent Stokes radius of 108 Å and sedimented with a coefficient of 8 S (10–30% glycerol gradients) (see supplemental Fig. S1*A*). These data and the calculated values for the coefficients correspond well with those previously reported for purified bovine p115 (83 Å Stokes radius, 6.8 S sedimentation coefficient) (15). The preparations of his-TA and his-T behaved almost indistinguishably from each other on gel filtration and velocity sedimentation (see supplemental Fig. S1, *B* and *C*). Stokes radius values of 60 and 59 Å and sedimentation coefficients of 5 and 4 S were calculated for his-TA and his-T, respectively. Furthermore, both p115 (data not shown) and his-TA are active in a functional assay that measures p115-dependent reassembly of stacked Golgi membranes from mitotic Golgi fragments (32).

The p115 C-terminal Domain Binds the p115 Tail—To test directly whether the p115 C-terminal domain could inhibit Rab1 binding we performed competition experiments. GST-Rab1 binding assays were performed at a fixed concentration of his-T (50 nM), in the presence of increasing concentrations of a peptide corresponding to the C-terminal 76 amino acids of p115 (p115CT). This peptide caused dose-dependent inhibition of binding between his-T and GST-Rab1(QL) (Fig. 2*A*, *top panel*). In similar experiments, p115CT did not inhibit GM130-his (50 nM) binding to GST-Rab1(QL) (Fig. 2*A*, *bottom panel*). Together these results suggest that the p115 C-terminal domain competes with Rab1 for binding to the p115 tail.

Binding assays were then performed between immobilized GST-p115CT and various other truncations in p115 (Fig. 2*B*). GM130-his and Gtn1–500-his were also tested as positive experiments, since these proteins are known to bind the p115 C-terminal domain (27). This set of experiments revealed that truncations in p115 that lacked the acidic domain, but that included the CC1 region, bound efficiently to GST-p115CT (Fig. 2*B*, his-T, his-CC1,2,3, his-CC1,2). In contrast, the truncation his-CC2,3,4 did not bind (Fig. 2*B*). This is the same pattern of binding as to GST-Rab1(QL) (Fig. 1*C*). Neither full-length p115 nor his-TA bound to GST-p115CT in these assays. Each of these molecules includes both the CC1 region but also the C-terminal domain. This may indicate an intramolecular inter-

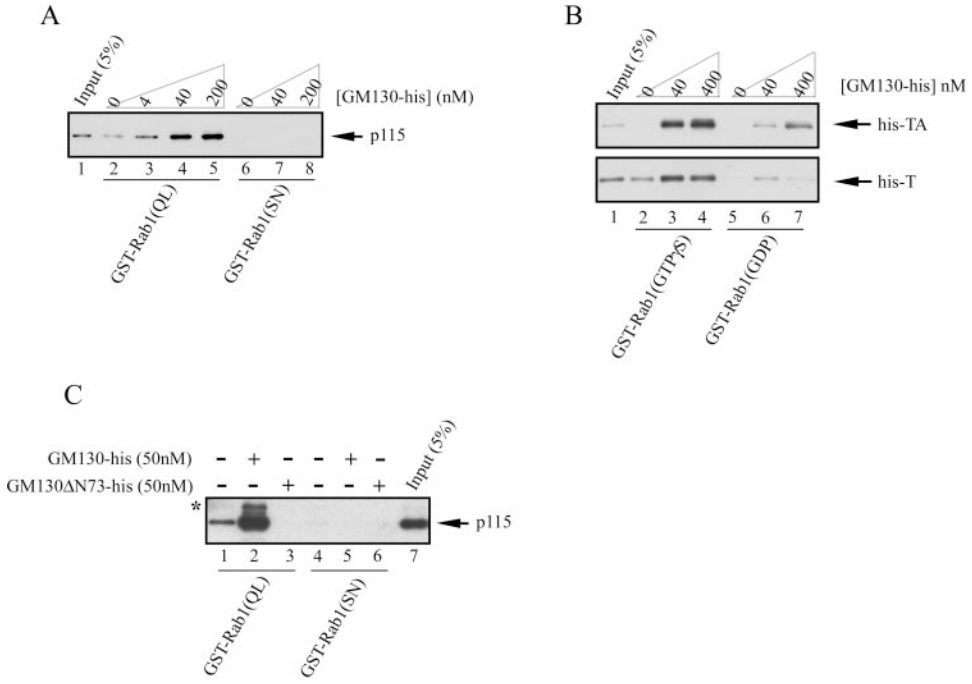

FIG. 3. **GM130 stimulates Rab1 binding to p115.** Full-length p115, his-TA, or his-T was incubated with active or inactive GST-Rab1 in the presence of various concentrations of GM130-his (*A* and *B*) or GM130ΔN73-his (*C*). Bound proteins were analyzed by immunoblotting. The asterisk in *C* indicates a minor, immunoreactive species, present in our recombinant p115 preparations. It likely arises from nonspecific cleavage by PreScission protease within the GST moiety of GST-p115 during purification.

action between the p115 C-terminal domain and CC1 region. Such an intramolecular interaction would be expected to efficiently compete with exogenous GST-p115CT, due to the very high local concentration of the binding partner.

GM130 Stimulates p115 Binding to Rab1—During preliminary experiments we had noted that GM130 (which binds to both Rab1 and p115) did not compete efficiently with p115 for binding to Rab1, at least not in the relative concentrations that we were able to test (data not shown). Rather, there seemed to be a stimulatory effect. In binding experiments between immobilized GST-Rab1 and a fixed concentration of p115 (50 nM), increasing concentrations of GM130-his caused a dose-dependent stimulation in p115 binding, up to a greater than 4-fold stimulation. Over 20% p115 binding was observed in the presence of 200 nM GM130-his, compared with 1–5% in its absence (Fig. 3*A*, *lanes 2–5*). GM130 also stimulated his-TA binding to GST-Rab1(QL), from undetectable levels in the absence to more than 10% binding in the presence of 400 nM GM130-his, whereas it had a much smaller effect on his-T (Fig. 3*B*). This stimulatory effect was related to the N-terminal (p115 binding) domain of GM130 because a truncation (GM130ΔN73-his) that lacked the N-terminal region did not stimulate the binding of p115 to Rab1 (Fig. 3*C*). This suggests that binding to the p115 C-terminal domain is necessary for stimulation.

Giantin Is a Rab1-binding Protein—Next we decided to examine the effects of Giantin on p115 and Rab1, because, like GM130, Giantin is a Golgi-localized p115-binding protein (30). Active conformation, immobilized GST-Rab1 specifically retains Giantin as well as p115, GM130, and GRASP65 from detergent extracts of rat liver Golgi membranes (see supplemental Fig. S2). Purified, recombinant GRASP65 did not bind to GST-Rab1 in either nucleotide bound conformation under any conditions that we tested (data not shown), suggesting that endogenous GRASP65 may bind GST-Rab1 indirectly, via its well characterized interaction with GM130 (59).

An interaction between Giantin and Rab1 had not been reported previously. To rapidly narrow down the region within Giantin that is necessary for Rab1 interaction, *in vitro* coupled transcription-translation was used to prepare full-length Giantin and a set of truncations, as ^{35}S-labeled proteins. Binding assays between the *in vitro* transcribed-translated proteins and immobilized GST-Rab1 showed that the N-terminal region (1–301) of Giantin is necessary and sufficient for efficient binding to the active conformation of GST-Rab1 in this system (Fig. 4*A*). Full-length Giantin, and the truncations 1–448, 1–500-his, and 1–301, all bound to GST-Rab1(QL) (>10% input bound), whereas those lacking the N-terminal region, 450–3187, myc-448–3163, myc-1967–2541, did not. No binding was detected in control assays with GST-Rab1(SN) or GST (Fig. 4*A*).

Next, to test for direct binding between Giantin and Rab1, we used a bacterial system to express and purify affinity-tagged proteins, corresponding to fragments of Giantin. We also used this system to further map the Rab1-binding region of Giantin in relation to the p115-binding site, which also lies within the N-terminal region (30). The Giantin N-terminal fragments, 1–500 and 121–500, both showed robust binding (>10% input bound) to GST-Rab1(QL) but not to GST-Rab1(SN). Similar results were obtained with wild type GST-Rab1 (data not shown). In contrast to this, neither the fragments 1–181 or 1125–1695 nor the internal deletion 1–500Δ181–301 showed detectable binding to GST-Rab1(QL) (Fig. 4*B*). Binding was not affected by the affinity tag because MBP- and His-tagged fragments showed similar levels of binding, whereas MBP alone did not bind (Fig. 4*B*). Together these results show that Giantin is a direct binding partner of activated Rab1 and map the binding site to the region 181–301 (Fig. 4, *A* and *B*).

The Rab1 and p115-binding Sites Map to Distinct Regions of Giantin—The p115-binding site on Giantin also lies within the N-terminal 500 amino acids of the protein. However, there is an apparent discrepancy in the literature about the precise identity of the site. One report showed that the N-terminal region, Giantin 47–117, was sufficient for p115 binding (31). However, another study found that the truncation Giantin 1–186 did not bind to p115, whereas a longer truncation Giantin 1–448 did bind under the same conditions (30). Therefore

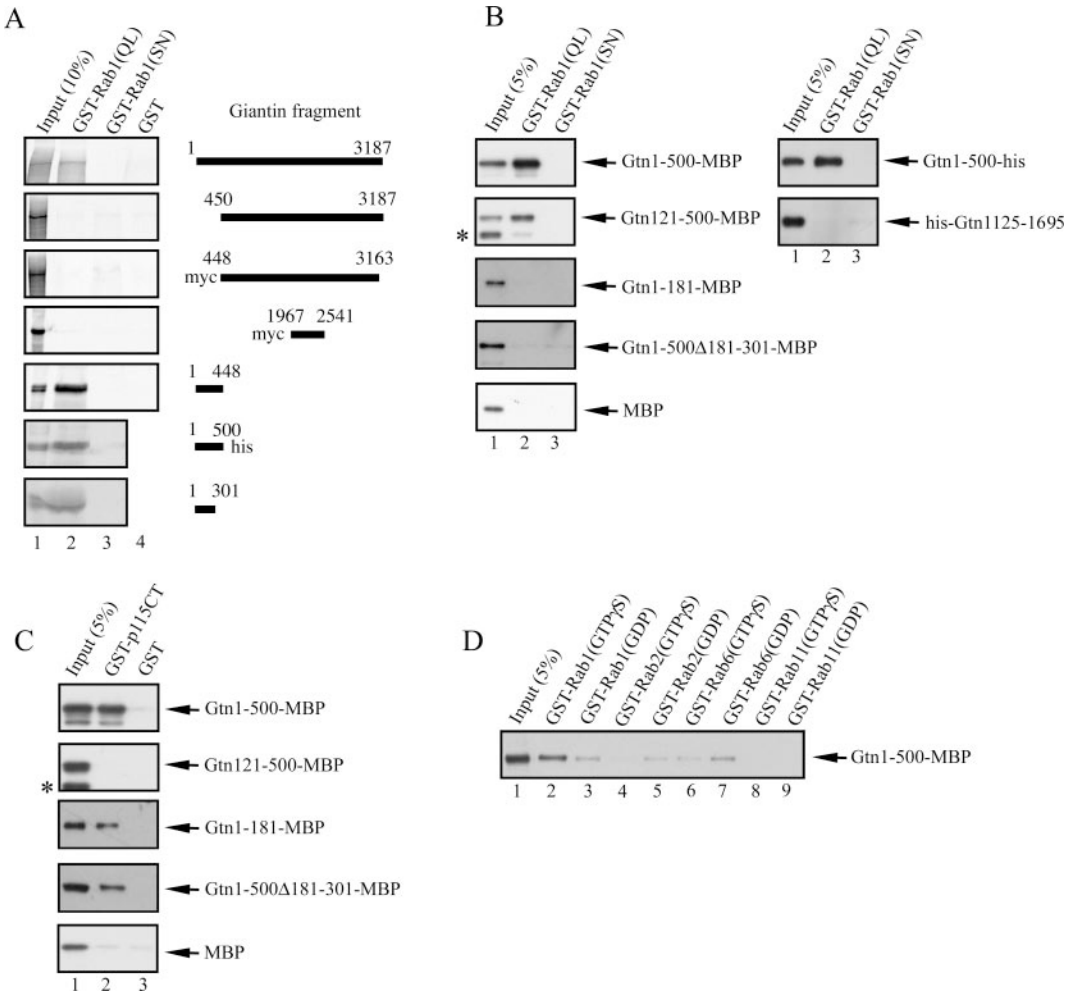

FIG. 4. **Giantin binds directly to active Rab1.** *A*, full-length Giantin and a set of truncations were prepared as ^{35}S-labeled proteins by *in vitro* transcription-translation, then incubated with GST-Rab1(QL), GST-Rab1(SN), or GST. The schematics to the *right* of *A* are included to show the position of each truncation within the primary structure of full-length Giantin. *B*, purified, tagged Giantin fragments were incubated with GST-Rab1(QL) or GST-Rab1(SN). The *asterisk* indicates a truncated, immunoreactive species present in preparations of Gtn121–500-MBP. *C*, truncated Giantin fragments, tagged with MBP, were incubated with immobilized GST or GST fused to the extreme C-terminal 76 residues of p115 (GST-p115CT). The *asterisk* indicates a truncated, immunoreactive species present in preparations of Gtn121–500. *D*, a recombinant Giantin fragment (Gtn1–500-MBP), containing the Rab1-binding site, was incubated with a range of immobilized GST-Rab fusion proteins, preloaded with either GTPγS or GDP. Bound material was analyzed by autoradiography (*A*) or immunoblotting (*B–D*).

we decided to test whether the p115-binding region of Giantin could be localized to a different fragment from the Rab1-binding region. To address this question, we tested each of our Giantin fragments for binding to GST-p115CT. This region of p115 includes the Giantin-binding, acidic C-terminal domain (32). In these binding experiments Giantin fragments 1–500, 1–181, and 1–500Δ181–301 all bound to GST-p115CT, whereas the fragment 121–500 did not (Fig. 4*C*). These results support the localization of the p115-binding site as closer to the N terminus of Giantin. Together these results map the p115-binding site on Giantin to a different region of the Giantin N-terminal region (residues 1–181) from the Rab1-binding site (residues 181–301).

Giantin Shows Selectivity for Rab1—To test whether Giantin shows selectivity for Rab1, compared with other Rab proteins, we tested Gtn1–500-his for binding to a panel of other Golgi and endosomal Rab proteins. This showed that interaction between Rab1 and Giantin is specific, since GST-Rab2, -6, and -11 fusion proteins all bound poorly to Gtn1–500-his (similar or lower binding than GST-Rab1(GDP)), irrespective of their nucleotide state (Fig. 4*D*).

Giantin Also Stimulates p115 Binding to Rab1—As with GM130, Giantin also causes a dose-dependent stimulation of p115 binding to GST-Rab1. Gtn1–500-his stimulated p115 binding to GST-Rab1(QL) ~5-fold, from 2–5% to over 20%, in the presence of 50 nM Gtn1–500-his (Fig. 5*A*, *lanes 2–4*). Similar results were also obtained using solubilized, carbonate-stripped, rat liver Golgi membranes as a source of enriched full-length Giantin (data not shown).

The stimulatory effects of GM130 and Giantin could also be mimicked by an anti-p115 monoclonal antibody (αp115Ab) (Fig. 5*B*) that binds within the CC4 region of p115 (812–886), assessed by immunoblotting (data not shown). More than a 4-fold stimulation was achieved, from <5% binding in the absence, up to ~20% in the presence, of 0.5 μg αp115 antibody. Similar results were also obtained in assays between his-TA and GST-Rab1. Binding was increased from undetectable levels in the absence of antibody to over 10% input bound in the presence of 0.5 μg of αp115 antibody (Fig. 5*C*, *top panel*). One might argue that the antibody could cross-link p115 or TA thereby stimulating binding to Rab1. However, the binding of T to Rab1 was not stimulated even though it too has the antibody binding and so should also be susceptible to cross-linking (Fig. 5*C*, *bottom panel*).

We also tested truncations in Giantin and GM130 that lacked Rab1-binding sites (Fig. 5, *D* and *E*). These also failed to stimulate p115- or his-TA-binding Rab1. Together these results

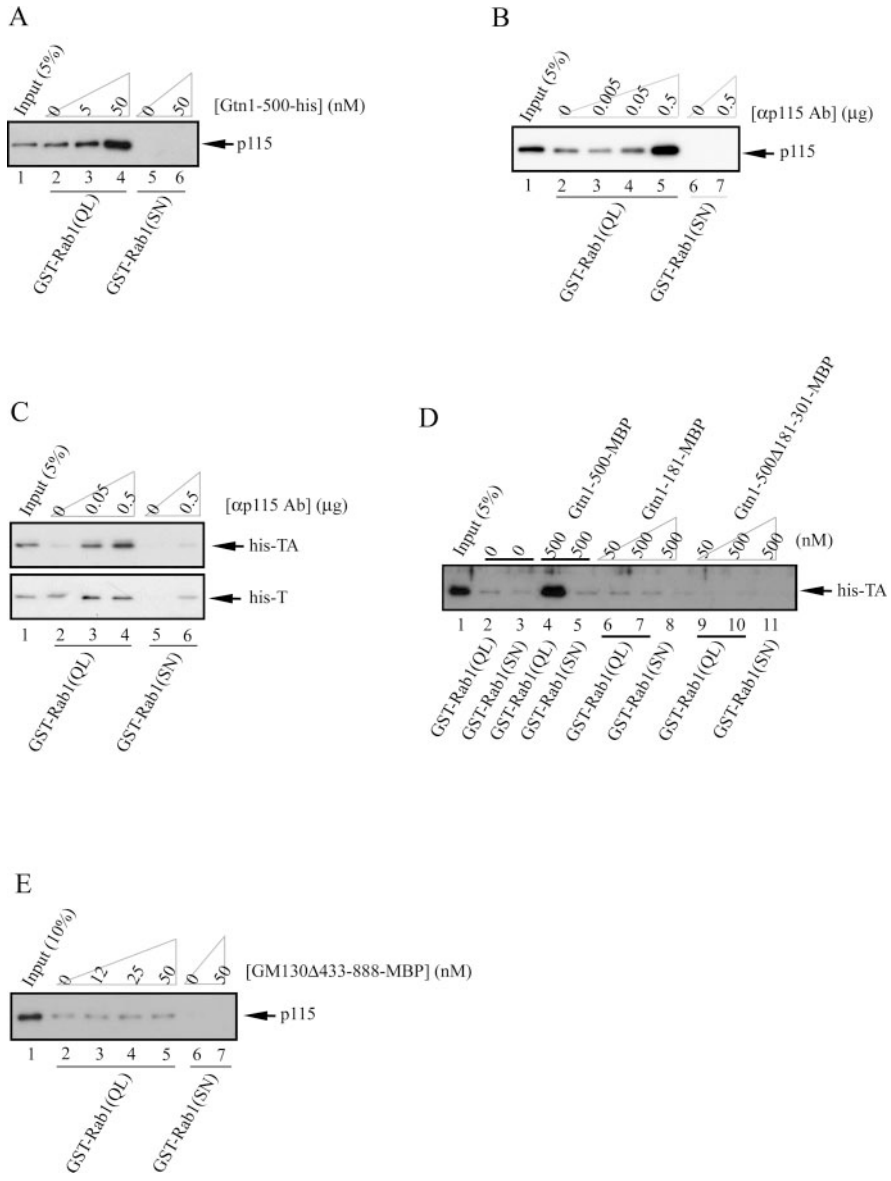

FIG. 5. **Giantin and anti-p115 antibodies stimulate Rab1 binding to p115.** Full-length p115, his-TA, or his-T was incubated with GST-Rab1(QL) or GST-Rab1(SN) in the presence of various concentrations of Gtn-1–500-his (*A*), αp115Ab (*B* and *C*), Giantin truncations or deletions lacking the Rab1-binding site (*D*), and a GM130 deletion lacking the Rab1-binding site (*E*). Bound proteins were analyzed by immunoblotting.

suggest that Giantin and GM130 must bind to both p115 and Rab1 to stimulate the interaction.

DISCUSSION

We have mapped the binding site for Rab1 on p115 and also shown that Rab1 interacts directly with Giantin. This means that all of the golgin components of the *cis*-golgin tether interact with each other and with Rab1. Importantly, the golgin interaction sites are, in each case, distinct from the Rab1-binding sites. A schematic of these interactions is illustrated in Fig. 6*A*, summarizing work by us and others (27, 30–32, 42, 43, 59).

The Rab1-binding site on p115 maps to the region CC1 in the p115 tail. Although CC1 in isolation (his-CC1 residues 652–701) is not sufficient for Rab1 binding, it is the only region that we found to be necessary. The shortest tested fragment with which we detected Rab1 binding included both CC1 and CC2 domains (residues 652–776). However, CC2 was not necessary to confer Rab1 binding activity (to CC1 containing truncations in p115), since an internal deletion of the p115 tail that included the CC1, CC3 and CC4 regions, but with CC2 deleted (652–933Δ701–776), also bound to Rab1. Nor was CC2 sufficient for Rab1 binding, since a truncation containing CC2, CC3, and CC4 regions only (704–993) did not bind. One possibility is that the homodimeric structure of p115 may be important for Rab1 binding. Since the CC1 peptide is predominantly monomeric in solution (36), this might explain why it did not bind to Rab1 in our assays. p115, GM130, and Giantin are all predicted to be homodimeric molecules. Alternatively some other structural feature of the p115 molecule that is not encoded within CC1 might be necessary for Rab1 binding.

While investigating the protein-protein interaction between Rab1 and p115, the most striking observation was that truncations in p115 that lack the C-terminal region bound Rab1 with a much higher apparent affinity than did full-length p115. Rab1 bound tightly to the tail domain (his-T, 651–933) but not at all to the tail plus acidic domain (his-TA, 651–961). It did bind weakly to full-length p115, suggesting the head domain plays a role in this interaction. However, we have not yet managed to assess this role since we have been unable purify a truncation containing only the head and tail. It is possible that the p115 head domain might interact with the C-terminal domain and partially relieve its inhibition of Rab1 binding. Another explanation could be that a second Rab1-binding site exists in the p115 head domain but was not detected in our binding assays.

The C-terminal region of p115 competes with Rab1 for binding to the p115 tail. This raises the possibility that the p115 C-terminal domain may serve a regulatory function by affect-

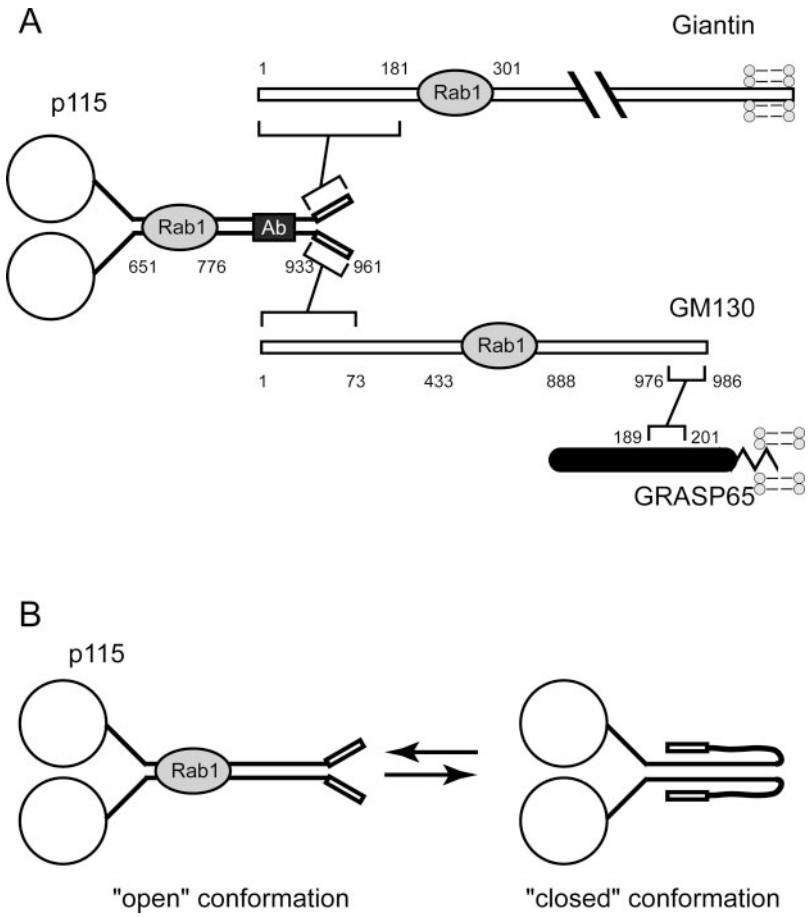

FIG. 6. **A schematic map of the interacting sites in the *cis*-golgin tethering complex.** *A*, mapped sites of interaction between golgins, GRASP65, and Rab1 constructed from data in this paper and published work (27, 30–32, 42, 43, 59). The binding site for the anti-p115 antibody (*Ab*) is also shown. *B*, schematic of a speculative model in which a closed conformation of p115 is converted to an open conformation, triggered by the binding of either GM130 and/or Giantin. The open conformation reveals a Rab1-binding site. p115 is depicted as a parallel homodimer with each chain composed of a head domain (*large circle*), a tail domain containing predicted coil-coil, and a C-terminal acidic domain (*open box*).

ing Rab1 binding. Binding of either GM130 or Giantin to the C-terminal domain of p115 stimulates p115 binding to Rab1. Together, these findings lead us to formulate a model in which p115 undergoes a conformational change from a "closed" to an "open" state. We hypothesize that Rab1 binding to the closed state p115 is inhibited by an intramolecular interaction between the p115 C-terminal domain and the Rab1-binding site. In the open state, however, the p115 C-terminal domain is displaced from the Rab1-binding site, leading to increased affinity for Rab1 (Fig. 6*B*).

The stimulatory effects of GM130 and Giantin on p115 binding to Rab1 are mimicked by an anti-p115 monoclonal antibody that binds near to the C-terminal domain (812–886). Since this antibody binding might be expected to disrupt the hypothesized intramolecular interaction, these data are consistent with the hypothesis that GM130, Giantin, and the anti-p115 antibody all stimulate p115 binding to Rab1, at least in part, by binding at or near to the p115 C terminus and causing a switch between the closed and open conformation.

This stimulation by GM130 and Giantin not only requires their p115-binding domains but also their Rab1-binding domains. Truncations in GM130 or Giantin that lack either the p115 or the Rab1-binding domains do not stimulate p115 binding to Rab1. This suggests that GM130 and Giantin must bind to both p115 and Rab1 to stimulate. What remains unclear is the number of Rab1 molecules that would be needed to form a productive complex. The binding sites of Giantin, p115, and GM130 on the Rab1 molecule have not yet been determined, other than to show that both the N- and C-terminal hypervariable regions are necessary for Rab1 binding to GM130 in yeast two-hybrid experiments (43). It is not, therefore, known whether more than one golgin molecule can simultaneously bind a single Rab1 molecule.

Although our proposed model for a regulated interaction between p115 and Rab1 is speculative, an attractive feature is that it does present a possible resolution for a major discrepancy in the literature. Currently it is perplexing that agents which act on GM130 or Giantin to disrupt their binding to p115 have much more severe effects on p115-mediated membrane tethering and fusion than do agents that act on p115 to disrupt its binding to GM130 and Giantin.

Rab1 recruits p115 to membranes, and this recruitment is essential for p115 function (22, 41, 42). The new model suggests that GM130 and Giantin regulate p115 recruitment (to Golgi and COPI vesicle membranes, respectively) rather than acting simply as membrane anchors (as suggested previously). At the Golgi membrane, for instance, GM130 bound to Rab1 (42) would be available to bind the C-terminal domain of p115 and trigger a switch from the closed to the open conformation. This would allow p115 recruitment by either the same or an adjacent Rab1. A similar recruitment to COPI vesicles might be triggered by the Rab1-Giantin complex.

This hypothesis fits well with the published data. It can account for the observation that full-length p115 does not localize to Golgi membranes when binding to GM130 is inhibited. Such conditions would prevent p115 switching from the closed to the open conformation at the Golgi. Microinjection of the GM130 N73 peptide, for instance, is sufficient to disrupt p115 localization and function at the Golgi (35). This peptide inhibits p115 binding to GM130 (27) but does not stimulate p115 binding to Rab1 (data not shown). In other words, this peptide can bind to the C-terminal domain of p115 (and so prevent binding to GM130) but is not sufficient to switch p115 into the open, high affinity binding conformation, hence, recruitment by Rab1 cannot occur.

Similarly, the model can explain why p115 truncations that

lack the acidic domain nevertheless rescue p115 function (23), despite being unable to bind GM130. In these truncations inhibition of Rab1 binding by the acidic domain has been removed. This should result in a constitutive open conformation. Rab1 would be expected to recruit such truncations even in the absence of their binding to GM130, bypassing this regulatory step.

What then is the role of this regulation? One possibility is that it increases the fidelity of vesicle targeting. There is an emerging model that suggests several independently targeted proteins must interact to mark sites for specific fusion (60). p115 lacking the acidic domain might be recruited to any Rab1 positive membrane even where no additional regulator, such as GM130 or Giantin, is present. Rab1 has a much wider distribution within the early exocytic pathway than GM130 or Giantin (24) so mistargeting of p115 might give rise to inappropriate fusion events. It is also noteworthy that Rab1 only binds to p115, GM130, and Giantin in its activated (GTP-bound) conformation. This implies that GTP hydrolysis by Rab1 may disrupt these interactions. Thus, Rab1 GTPase activity places an inherent time limit on these interactions and in so doing may increase the fidelity of COP I vesicle transfer.

The possibility of inappropriate fusion events derives from the known role of p115 in SNARE assembly. In addition to its role in binding Rab1, the CC1 region of p115 can also bind to certain SNARE proteins and promotes the formation of Syntaxin-5 containing SNARE-bundles (36). GM130 and Giantin would ensure that p115 is recruited to the Rab1 located at the *cis*-Golgi so that downstream fusion events would occur at the correct time and place. The binding of Rab1 to CC1 of p115 might regulate its action on SNARE bundling, perhaps by blocking the interaction with SNAREs until after the vesicle has docked with the membrane. Alternatively, or in addition, Rab1 may assist p115 in SNARE bundling.

The findings that p115, GM130, Giantin, and Rab1 all interact directly strengthens the hypothesis that they function together. Characterization of these interactions has given rise to model that provides a possible resolution for an apparent contradiction in the literature. Although the precise molecular mechanisms by which these proteins act to tether membranes remains unclear the findings presented in this paper should provide clues that may be important in elucidating their precise role.

Acknowledgments—We are indebted to the entire Warren/Mellman group, S. Ferro-Novick, and J. Rothman for discussion and support. We thank T. Nilsson, G. Lesa, M. Lowe, F. Barr, N. Nakamura, D. Sheff, G. Waters for generous provision of reagents.

REFERENCES

1. Rothman, J. E. (1994) *Nature* **372,** 55–63
2. Mellman, I., and Warren, G. (2000) *Cell* **100,** 99–112
3. Mayer, A. (2001) *Trends Biochem. Sci.* **26,** 717–723
4. Waters, M. G., and Hughson, F. M. (2000) *Traffic* **1,** 588–597
5. Lowe, M. (2000) *Curr. Biol.* **10,** R407–R409
6. Pfeffer, S. (2001) *Mol. Cell* **8,** 729–730
7. Zerial, M., and McBride, H. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 107–117
8. Barr, F. A., and Short, B. (2003) *Curr. Opin. Cell. Biol.* **15,** 405–413
9. Gillingham, A. K., and Munro, S. (2003) *Biochim. Biophys. Acta* **1641,** 71–85
10. Jackson, C. L. (2003) *Curr. Biol.* **13,** R174–R176
11. Sacher, M., Jiang, Y., Barrowman, J., Scarpa, A., Burston, J., Zhang, L., Schieltz, D., Yates, J. R., III, Abeliovich, H., and Ferro-Novick, S. (1998) *EMBO J.* **17,** 2494–2503
12. Whyte, J. R., and Munro, S. (2002) *J. Cell Sci.* **115,** 2627–2637
13. Tisdale, E. J., Bourne, J. R., Khosravi-Far, R., Der, C. J., and Balch, W. E. (1992) *J. Cell Biol.* **119,** 749–761
14. Seelig, H. P., Schranz, P., Schroter, H., Wiemann, C., and Renz, M. (1994) *J. Autoimmun.* **7,** 67–91
15. Waters, M. G., Clary, D. O., and Rothman, J. E. (1992) *J. Cell Biol.* **118,** 1015–1026
16. Sztul, E., Colombo, M., Stahl, P., and Samanta, R. (1993) *J. Biol. Chem.* **268,** 1876–1885
17. Sapperstein, S. K., Walter, D. M., Grosvenor, A. R., Heuser, J. E., and Waters, M. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 522–526
18. Yamakawa, H., Seog, D. H., Yoda, K., Yamasaki, M., and Wakabayashi, T. (1996) *J. Struct. Biol.* **116,** 356–365
19. Nakajima, H., Hirata, A., Ogawa, Y., Yonehara, T., Yoda, K., and Yamasaki, M. (1991) *J. Cell Biol.* **113,** 245–260
20. Sapperstein, S. K., Lupashin, V. V., Schmitt, H. D., and Waters, M. G. (1996) *J. Cell Biol.* **132,** 755–767
21. Barlowe, C. (1997) *J. Cell Biol.* **139,** 1097–1108
22. Cao, X., Ballew, N., and Barlowe, C. (1998) *EMBO J.* **17,** 2156–2165
23. Puthenveedu, M. A., and Linstedt, A. D. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1253–1256
24. Alvarez, C., Garcia-Mata, R., Hauri, H. P., and Sztul, E. (2001) *J. Biol. Chem.* **276,** 2693–2700
25. Sönnichsen, B., Lowe, M., Levine, T., Jamsa, E., Dirac-Svejstrup, B., and Warren, G. (1998) *J. Cell Biol.* **140,** 1013–1021
26. Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T. E., and Warren, G. (1995) *J. Cell Biol.* **131,** 1715–1726
27. Nakamura, N., Lowe, M., Levine, T. P., Rabouille, C., and Warren, G. (1997) *Cell* **89,** 445–455
28. Lowe, M., Rabouille, C., Nakamura, N., Watson, R., Jackman, M., Jamsa, E., Rahman, D., Pappin, D. J., and Warren, G. (1998) *Cell* **94,** 783–793
29. Lowe, M., Gonatas, N. K., and Warren, G. (2000) *J. Cell Biol.* **149,** 341–356
30. Lesa, G. M., Seemann, J., Shorter, J., Vandekerckhove, J., and Warren, G. (2000) *J. Biol. Chem.* **275,** 2831–2836
31. Linstedt, A. D., Jesch, S. A., Mehta, A., Lee, T. H., Garcia-Mata, R., Nelson, D. S., and Sztul, E. (2000) *J. Biol. Chem.* **275,** 10196–10201
32. Dirac-Svejstrup, A. B., Shorter, J., Waters, M. G., and Warren, G. (2000) *J. Cell Biol.* **150,** 475–488
33. Shima, D. T., Haldar, K., Pepperkok, R., Watson, R., and Warren, G. (1997) *J. Cell Biol.* **137,** 1211–1228
34. Levine, T. P., Rabouille, C., Kieckbusch, R. H., and Warren, G. (1996) *J. Biol. Chem.* **271,** 17304–17311
35. Seemann, J., Jokitalo, E. J., and Warren, G. (2000) *Mol. Biol. Cell* **11,** 635–645
36. Shorter, J., Beard, M. B., Seemann, J., Dirac-Svejstrup, A. B., and Warren, G. (2002) *J. Cell Biol.* **157,** 45–62
37. Puthenveedu, M. A., and Linstedt, A. D. (2001) *J. Cell Biol.* **155,** 227–238
38. Nelson, D. S., Alvarez, C., Gao, Y. S., Garcia-Mata, R., Fialkowski, E., and Sztul, E. (1998) *J. Cell Biol.* **143,** 319–331
39. Kondylis, V., and Rabouille, C. (2003) *J. Cell Biol.* **162,** 185–198
40. Vasile, E., Perez, T., Nakamura, N., and Krieger, M. (2003) *Traffic* **4,** 254–272
41. Allan, B. B., Moyer, B. D., and Balch, W. E. (2000) *Science* **289,** 444–448
42. Moyer, B. D., Allan, B. B., and Balch, W. E. (2001) *Traffic* **2,** 268–276
43. Weide, T., Bayer, M., Koster, M., Siebrasse, J. P., Peters, R., and Barnekow, A. (2001) *EMBO Rep.* **2,** 336–341
44. Barr, F. A. (1999) *Curr. Biol.* **9,** 381–384
45. Diao, A., Rahman, D., Pappin, D. J., Lucocq, J., and Lowe, M. (2003) *J. Cell Biol.* **160,** 201–212
46. Satoh, A., Wang, Y., Malsam, J., Beard, M. B., and Warren, G. (2003) *Traffic* **4,** 153–161
47. Short, B., Preisinger, C., Schaletzky, J., Kopajtich, R., and Barr, F. A. (2002) *Curr. Biol.* **12,** 1792–1795
48. Soldati, T., Riederer, M. A., and Pfeffer, S. R. (1993) *Mol. Biol. Cell* **4,** 425–434
49. Lupashin, V. V., Hamamoto, S., and Schekman, R. W. (1996) *J. Cell Biol.* **132,** 277–289
50. Cao, X., and Barlowe, C. (2000) *J. Cell Biol.* **149,** 55–66
51. Wang, Y., Seemann, J., Pypaert, M., Shorter, J., and Warren, G. (2003) *EMBO J.* **22,** 3279–3290
52. Christoforidis, S., and Zerial, M. (2000) *Methods* **20,** 403–410
53. Hui, N., Nakamura, N., Slusarewicz, P., and Warren, G. (1998) in *Cell Biology: A Laboratory Handbook* (Celis, J., ed) pp. 46–55, Academic Press, New York
54. Christoforidis, S., and Zerial, M. (2001) *Methods Enzymol.* **329,** 120–132
55. Short, B., Preisinger, C., Korner, R., Kopajtich, R., Byron, O., and Barr, F. A. (2001) *J. Cell Biol.* **155,** 877–883
56. Valsdottir, R., Hashimoto, H., Ashman, K., Koda, T., Storrie, B., and Nilsson, T. (2001) *FEBS Lett.* **508,** 201–209
57. Cullis, D. N., Philip, B., Baleja, J. D., and Feig, L. A. (2002) *J. Biol. Chem.* **277,** 49158–49166
58. Nagashima, K., Torii, S., Yi, Z., Igarashi, M., Okamoto, K., Takeuchi, T., and Izumi, T. (2002) *FEBS Lett.* **517,** 233–238
59. Barr, F. A., Nakamura, N., and Warren, G. (1998) *EMBO J.* **17,** 3258–3268
60. Söllner, T. H. (2002) *Dev. Cell* **2,** 377–378