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Evidence that Golgi structure depends on a p115 activity that is independent of the vesicle tether components giantin and GM130

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Inhibition of the putative coatomer protein I (COPI) vesicle tethering complex, giantin–p115–GM130, may contribute to mitotic Golgi breakdown. However, neither this, nor the role of the giantin–p115–GM130 complex in the maintenance of Golgi structure has been demonstrated in vivo. Therefore, we generated antibodies directed against the mapped binding sites in each protein of the complex and injected these into mammalian tissue culture cells. Surprisingly, the injected anti-p115 and antigiantin antibodies caused proteasome-mediated degradation of the corresponding antigens. Reduction of p115 levels below detection led to COPI-dependent Golgi fragmentation and apparent accumulation of Golgi-derived vesicles. In contrast, neither reduction of giantin below detectable levels, nor inhibition of p115 binding to GM130, had any detectable effect on Golgi structure or Golgi reassembly after cell division or brefeldin A washout. These observations indicate that inhibition of p115 can induce a mitotic-like Golgi disassembly, but its essential role in Golgi structure is independent of its Golgi-localized binding partners giantin and GM130.

Introduction

The Golgi apparatus mediates processing and sorting of newly synthesized proteins and lipids. As these reactions are essential for completing protein synthesis and targeting in the secretory pathway, mechanisms evolved to ensure accurate inheritance of the Golgi apparatus during cell division. At the onset of mitosis, the mammalian Golgi apparatus undergoes a dramatic breakdown of its interphase structure. Subcellular fractionation and microscopy experiments indicate that it is converted from a juxtanuclear ribbon of stacked membranes into a large number of small vesicles dispersed mostly throughout the mitotic cytoplasm (Lucocq et al., 1989; Misteli and Warren, 1995; Jesch and Linstedt, 1998; but see also Zaal et al., 1999). Uniform dispersal of Golgi-derived vesicles can explain accurate Golgi partitioning into daughter cells, but the mechanism of Golgi vesiculation is unknown.

Our current understanding concludes that kinases active at the G2/M transition, notably cyclin-dependent kinase I and mitogen-activated protein kinase kinase/extracellular signal–regulated protein kinase (Achárya et al., 1998; Lowe et al., 1998; Kano et al., 2000), carry out phosphorylation reactions that lead, either directly or indirectly, to the inhibition of Golgi vesicle docking and fusion, which are fundamental processes underlying transport in the Golgi stack. The transport of newly synthesized proteins, termed cargo, through the Golgi stack involves their progressive encounter with modifying enzymes enriched in either the cis-, medial-, or trans-cisternal subcompartments. This is in part due to both the vesicle-mediated transport of cargo between adjacent cisternae (Rothman, 1994) and the ongoing anterograde progression of cargo-containing cisternae through the stack (Bonfanti et al., 1998). In the latter case, cisternal progression occurs as retrieval vesicles carry the modifying enzymes from later subcompartments backward to earlier subcompartments (Glick et al., 1997; Love et al., 1998; Lanoix et al., 1999). Thus, at M-phase, the continued formation of vesicles containing cargo and/or enzymes without their docking and fusion would lead to vesiculation of the organelle (Warren et al., 1995). Because the identified mitotic Golgi vesicles contain Golgi enzymes and other Golgi residents, it follows that these vesicles are probably produced by the same sorting reaction that produces retrieval vesicles during normal interphase trafficking. Alternatively, these vesicles could be produced by a mitotic modification of the cargo–vesicle sorting reaction allowing the incorporation of Golgi residents (Warren, 1985). In either case, evidence indicates that the mitotic Golgi vesicle formation reaction is largely medi-

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ated by the coatomer protein I (COPI)* complex (Misteli and Warren, 1994, 1995), which suggests that COPI vesicle docking and fusion components may be inhibited at the G2/M transition.

Importantly, the proteins implicated in COPI vesicle docking undergo alterations in their phosphorylation state at mitosis. The mitotic phosphorylation of at least one of these, Golgi matrix protein 130 (GM130), reduces its ability to bind its putative docking partner p115 (Nakamura et al., 1997). Collectively, the proteins GM130, Golgi reassembly stacking protein 65 (GRASP65), p115, and giantin are referred to as tethering proteins because they are thought to form a long complex capable of linking COPI vesicles to the Golgi apparatus over considerable distances (Sönnichsen et al., 1998). Giantin is a COOH-terminally anchored Golgi integral membrane protein (Linstedt et al., 1995) at least partially present in COPI vesicles (Sönnichsen et al., 1998). Giantin has a long rod-shaped cytoplasmic domain containing a p115 binding site in its predicted outermost NH_2-terminal coiled-coil segment (Lesa et al., 2000; Linstedt et al., 2000). This site binds an acidic domain at the COOH terminus of the peripheral membrane protein p115 (Linstedt et al., 2000). This same acidic stretch in p115 also binds GM130 (Nelson et al., 1998; Linstedt et al., 2000). Giantin and GM130 compete to bind to p115 under certain in vitro conditions (Linstedt et al., 2000), however under different conditions, p115 is able to link giantin to GM130 (Dirac-Svejstrup et al., 2000). The simultaneous binding of p115 to Golgi cisterna–localized GM130 and COPI-localized giantin could link COPI vesicles to the Golgi membrane (Sönnichsen et al., 1998) because GM130 is stably bound to GRASP65, which is attached to the Golgi apparatus via a lipid anchor (Barr et al., 1997). Indeed, in vitro association of COPI vesicles to the Golgi membrane is blocked by anti-giantin antibody treatment of the COPI vesicles, anti-GM130 treatment of the Golgi membranes, or depletion of p115 (Sönnichsen et al., 1998). Therefore, inhibition of the p115–GM130 interaction by mitotic phosphorylation of GM130 by cyclin-dependent kinase I could account for mitotic Golgi vesiculation (Lowe et al., 1998).

Nevertheless, the precise role of the Golgi-localized tether proteins is complicated by their implication in a diverse set of reactions. The initial characterization of p115 was based on its essential role in an in vitro transport reaction designed to measure cis- to medial-Golgi transfer of vesicular stomatitis virus glycoprotein (Waters et al., 1992). Subsequently, it was also found to mediate fusion of transcytotic vesicles to acceptor membranes (Barroso et al., 1995). More recently, p115, giantin, GM130, and GRASP65 were revealed as a requirement for both cisternal regrowth and cisternal stacking (two subreactions during in vitro Golgi reassembly after mitotic fragmentation) (Shorter and Warren, 1999). GRASP65, as well as the related GRASP55, also play roles in Golgi stacking independent of GM130 (Barr et al., 1998; Shorter et al., 1999). p115 is also required for ER to Golgi transport in semi-intact cells, where it is recruited to COPII-coated vesicles by transient interaction with Rab1 followed by stable interaction with a vesicle SNARE protein complex containing syntaxin5, sly1, membrin, and rbet1 (Allan et al., 2000), and mediates translocation of ER–Golgi intermediate compartment (ERGIC) from peripheral sites to the Golgi stack (Alvarez et al., 1999). Interestingly, once transport intermediates reach the Golgi region, evidence suggests that GM130/GRASP65 functions first, perhaps in binding vesicle-localized p115, whereas subsequent steps involve giantin (Alvarez et al., 2000).

The diversity of these reactions suggests that p115 and its binding partners mediate a number of distinct steps involving membrane–membrane recognition. As the purpose of docking is to achieve a specific interaction between vesicle and target, it is important to determine whether specificity at each step is achieved by the use of distinct binding partners. Furthermore, the number of distinct in vitro reactions requiring tether proteins certainly raises the question of whether the corresponding in vivo reactions require them as well. For example, the belief that inhibition of p115–GM130 interactions causes mitotic Golgi breakdown is not supported by the finding that the microinjection of a peptide that prevents binding of p115 to GM130 leads to no apparent Golgi breakdown (Seemann et al., 2000). On the other hand, the microinjection of anti-p115 antibodies causes Golgi breakdown (Alvarez et al., 1999). This discrepancy leads to the hypothesis that p115 has an essential role in Golgi biogenesis independent of its interaction with GM130. As GM130 and giantin bind the same domain in p115, the injected GM130 NH_2-terminal peptide would presumably also inhibit p115–giantin complex formation. Therefore, p115's essential role could be independent of both giantin and GM130, the two proteins that it is thought to bridge during COPI vesicle docking.

We sought to test this hypothesis and further characterize the role of p115 in the regulation of Golgi structure in relation to its binding partners giantin and GM130 in vivo. Antibodies were made against the corresponding binding sites of each protein and injected into HeLa cells. Unlike giantin and GM130, the inhibition of p115 alone in interphase cells induced apparent COPI-dependent Golgi vesiculation. This suggests that p115 plays a role in maintenance of Golgi structure that may be relevant to mitotic Golgi breakdown, but this role is independent of its interactions with giantin and GM130.

Results

Anti-p115 antibody microinjection induces proteasome-dependent p115 degradation

To develop an inhibitory antibody against p115, polyclonal antibodies were generated against a glutathione *S*-transferase (GST)–p115 fusion protein containing the p115 COOH terminus. The acidic COOH terminus of p115 mediates its interaction with giantin and GM130 (Nelson et al., 1998; Linstedt et al., 2000). After affinity purification, the anti-p115 antibodies and a corresponding population of control

*Abbreviations used in this paper: BFA, brefeldin A; CBM, cyclohexanebis(methylamine); COP, coatomer protein; ERGIC, ER–Golgi intermediate compartment; GM130, Golgi matrix protein of 130 kD; GPP130, Golgi phosphoprotein of 130 kD; GRASP, Golgi reassembly stacking protein; GST, glutathione *S*-transferase; NRK, normal rat kidney.

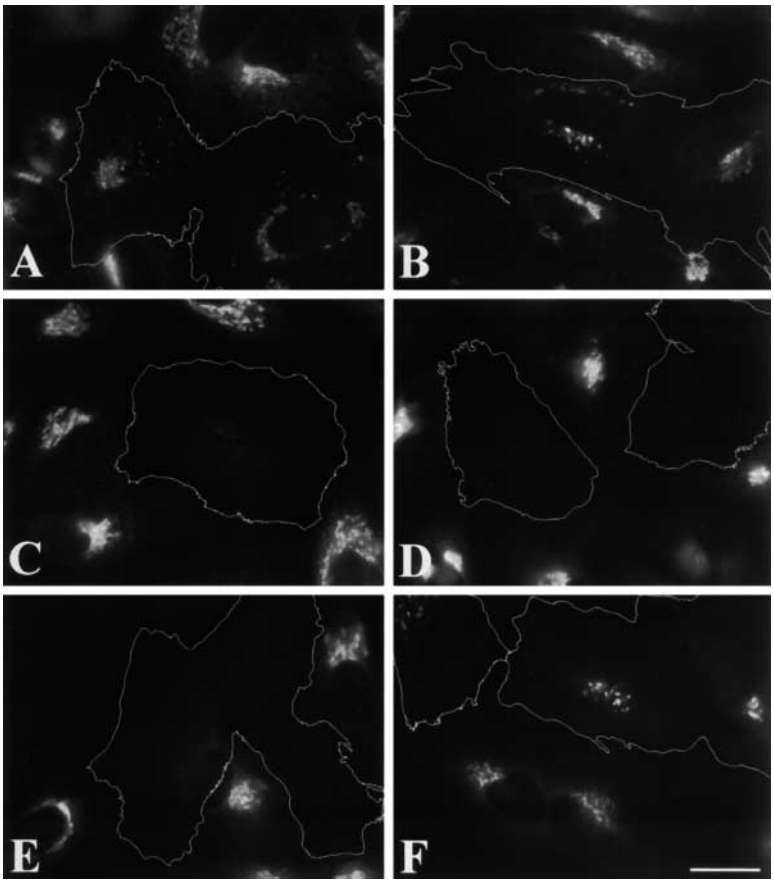


Figure 1. **Injection of anti-p115 antibodies induces proteasome-dependent degradation of p115.** HeLa cells were microinjected with affinity-purified polyclonal anti-p115 antibodies and stained for p115 using an anti-p115 mAb at 10 min (A), 30 min (B), 2 h (C), and 4 h (D) after injection. Injected cells were outlined as described in Materials and methods. Injected, but not uninjected, cells showed a loss of p115 staining over time. Also shown are cells that were incubated for 3 h after injection in the absence (E) or presence (F) of 50 μM MG132. Both incubations contained 0.005% DMSO, the solvent for the MG132 stock solution. Presence of MG132, a proteasome inhibitor, prevented anti-p115–induced loss of p115 staining. Bar, 10 μm.

anti-GST antibodies were microinjected into cultured HeLa cells. At various time points after injection, the cells were fixed and double stained to simultaneously detect the injected antibody as well as the cellular p115. As expected, uninjected cells (Fig. 1), as well as cells injected with the control anti-GST antibodies (unpublished data), exhibited the juxtanuclear and cytoplasmic staining pattern that is characteristic of p115, a peripheral membrane protein predominately localized to Golgi membranes. In contrast, injection of anti-p115 led to a time-dependent loss of the cellular p115. 10 min after injection, the cytoplasmic p115 staining became significantly more punctate, presumably due to cross-linking induced by antibody binding (Fig. 1 A). 30 min after injection, the juxtanuclear p115 pattern also became punctate and at this time point there was a marked loss of cytoplasmic staining (Fig. 1 B). 2 h after injection most, if not all, p115 staining was absent (Fig. 1 C), and p115 staining remained absent at 4 h (Fig. 1 D) and 6 h after injection (unpublished data). These observations suggest that the injected anti-p115 antibody cross-links cellular p115 and somehow induces its degradation.

This effect appeared specific to p115 as the abundance of other proteins in anti-p115–injected cells, including two of its known binding partners, was not detectably altered (see below). In addition, three observations argue against the possibility that binding of the injected polyclonal antibody blocked access of the monoclonal antibody used for detection of cellular p115. First, the monoclonal antibody binds to a region of p115 outside of the COOH-terminal portion used to generate the polyclonal antibody. Second, staining for the injected polyclonal antibody produced a juxtanuclear pattern at early time points, that became punctate at 30 min and disappeared at later time points (unpublished data). Thus, the apparent loss of p115 was also detected with the injected polyclonal antibody. Third, we obtained no evidence that the polyclonal antibody could block staining of cellular p115 by the monoclonal antibody in experiments where digitonin-permeabilized cells held on ice were first incubated with the polyclonal and then the monoclonal antibody. After fixation and detection with fluorescent secondary antibodies both the polyclonal and monoclonal antibody patterns colocalized on the Golgi membrane.

To test whether the loss of cellular p115 after anti-p115 microinjection was due to targeted degradation, we performed similar microinjection experiments in the presence and absence of a proteasome inhibitor. As expected, control cells examined 3 h after injection exhibited no detectable p115 staining (Fig. 1 E). In contrast, cells treated with the proteasome inhibitor MG132 during the 3 h after injection incubation exhibited readily detectable juxtanuclear p115 staining (Fig. 1 F). The staining pattern was markedly punctate suggesting that the injected antibodies had cross-linked p115 causing it to cluster on the Golgi membrane. In the absence of proteasome activity this failed to cause its degradation. These observations suggest that the binding of the injected antibody somehow targets p115 to the proteasome perhaps by inducing its recognition by a ubiquitin ligase. In any case, this provides an opportunity to study various cell functions in the apparent absence of p115.

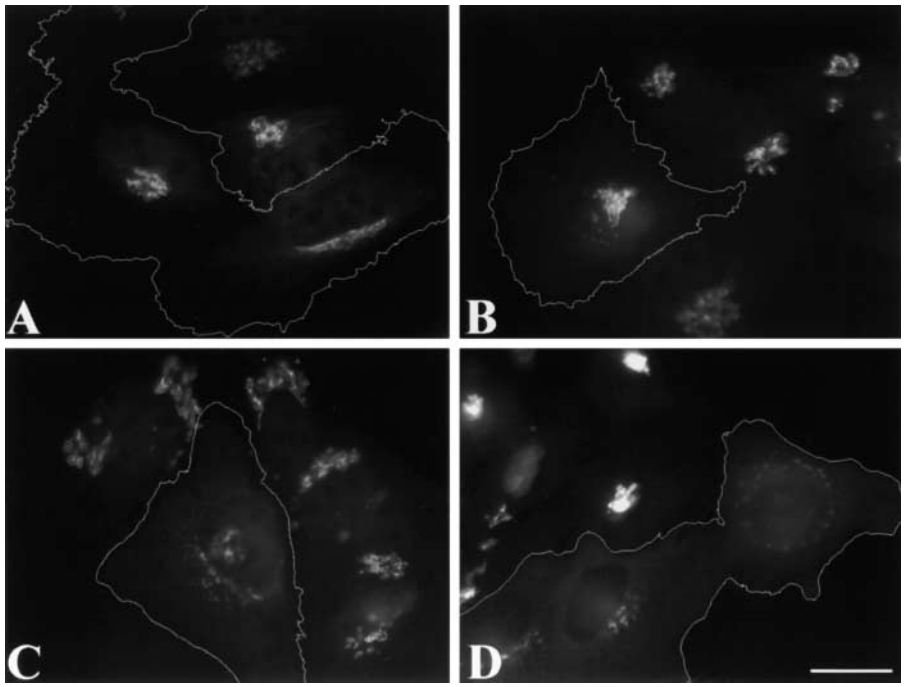


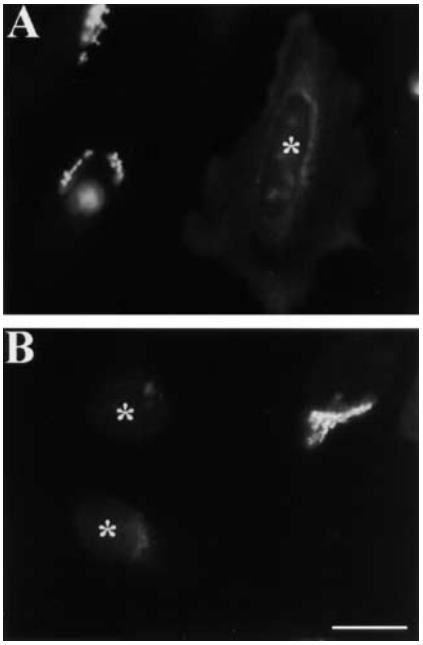
Figure 2. **Absence of detectable p115 induces Golgi breakdown.** HeLa cells were microinjected with anti-p115 and stained for the Golgi apparatus using anti-GPP130 mAb at 10 min (A), 30 min (B), 2 h (C), and 4 h (D) after injection. Injected cells are outlined. Note the prominent diffuse cytoplasmic Golgi staining at the last time point. Images are representative of three experiments with at least 25 cells examined in each experiment. Uninjected cells showed a normal Golgi apparatus at all time points. Bar, 10 μm.

Absence of p115 induces apparent COPI-dependent Golgi vesiculation

To test the effect of p115 loss on Golgi structure, we stained the Golgi apparatus in cells that had been incubated for various times after microinjection of anti-p115. The staining was performed using a monoclonal antibody that recognizes Golgi phosphoprotein 130 (GPP130), an integral membrane component of the cis-Golgi apparatus (Linstedt et al., 1997). The Golgi pattern remained normal 10 min after injection (Fig. 2 A), but 30 min after injection, the Golgi apparatus was noticeably fragmented (Fig. 2 B). 2 h after injection, the Golgi apparatus was present as dispersed fragments and there was a noticeable increase in diffuse staining (Fig. 2 C). 4 h after injection, the Golgi apparatus was almost entirely present as diffuse cytoplasmic staining with a few larger structures, or remnants, still visible (Fig. 2 D). A similar pattern of Golgi breakdown was observed when giantin or GM130 was used to mark the Golgi apparatus, and also when cells were allowed to pass through mitosis after injection, as described below (unpublished data). Injection of control anti-GST antibodies had no detectable effect on the Golgi apparatus at similar time points (unpublished data). Thus, loss of p115 led to a dramatic breakdown of at least the early Golgi structure.

Because Golgi breakdown in cells lacking detectable p115 resulted in diffuse Golgi staining, it was possible the anti-p115 microinjection induced Golgi vesiculation. However, it was also possible that the diffuse staining reflected redistribution of the Golgi apparatus into the ER membrane network, similar to that induced by brefeldin A (BFA) treatment. To distinguish between these possibilities, we performed a digitonin permeabilization of the injected cells. Because digitonin selectively permeabilizes the plasma membrane (Eilenberg et al., 1989), this treatment allows the release of small vesicles but not the large anchored ER network. The diffuse Golgi staining evident in cells 3 h after injection of anti-p115 (Fig. 3 A) was essentially lost if the cells were digitonin permeabilized before fixation (Fig. 3 B). Note that the Golgi apparatus present in adjacent, uninjected cells was unaffected by the permeabilization. In a quantitative analysis of these experiments, it was found that upon permeabilization the dispersed cytoplasmic fluorescence of the Golgi marker GPP130 in anti-p115–injected cells was reduced to the background level of cytoplasmic fluorescence in uninjected cells (Fig. 3 C). Two findings indicate that the ER was not released in these experiments. First, p63, an integral ER protein, was not released from uninjected or injected cells by digitonin treatment (Fig. 3 C). Second, after BFA treatment, ER-localized GPP130 was not released by digitonin treatment (Fig. 3 C). Thus, the Golgi breakdown pattern induced by the absence of detectable p115 did not reflect redistribution to the ER, but rather the accumulation of releasable structures likely to be small vesicles. This is consistent with the hypothesis that p115 mediates docking of Golgi-derived vesicles.

Because the mechanism most likely to mediate Golgi vesiculation is COPI vesicle formation, we next tested whether Golgi breakdown induced by anti-p115 antibody injection is inhibited by the presence of a COPI inhibitor. For these experiments we used the drug 1,3-cyclohexanebis(methylamine) (CBM), which causes displacement of COPI from the Golgi apparatus (Hu et al., 1999). The advantage of CBM is that, unlike other conditions that prevent COPI membrane association, CBM treatment does not cause Golgi redistribution into the ER. Nevertheless, CBM-treated cells remain competent to carry out a number of transport steps including bi-directional ER–Golgi transport (Hu et al., 1999). In our experiments, CBM treatment led to displacement of the COPI subunit, β-COP, from the Golgi apparatus, but did not cause Golgi collapse or prevent the BFA-induced redistribution of the Golgi system to the ER (unpublished data). As expected, control cells examined 3 h af-



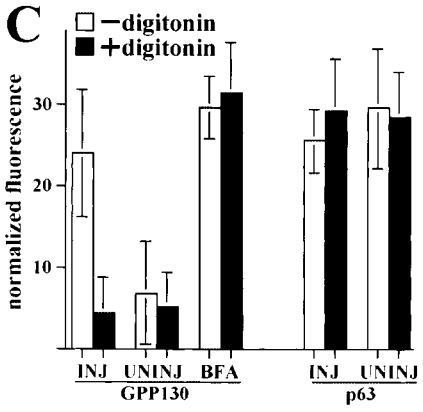


Figure 3. **Apparent release of Golgi vesicles upon digitonin permeabilization.** HeLa cells were injected with anti-p115 antibodies and were either fixed after 4 h (A) or were permeabilized with 0.04 mg/ml digitonin before fixation (B). Cells were single stained using anti-GPP130 to exclude any possibility of bleedthrough. Interphase cells that exhibited fragmented Golgi staining were only present in microinjected areas of the coverslips. Representative examples of these are marked with asterisks. (C) The level of cytoplasmic GPP130 fluorescence excluding the Golgi region was quantified as described in Materials and methods before (open bars) and after (filled bars) digitonin permeabilization for anti-p115–injected (INJ, $n = 65$), uninjected (UNINJ, $n = 23$), and BFA-treated cells (BFA, $n = 34$). The analysis was also performed for the ER marker p63 (INJ, $n = 28$; UNINJ, $n = 30$). Fluorescence of the dispersed Golgi complex, but not the ER, was reduced to background levels after digitonin permeabilization. Bar, 10 μm.

ter injection exhibited a significant Golgi breakdown and accumulation of the vesicle staining pattern (Fig. 4 A). In contrast, cells treated with CBM during the 3 h after injection retained an intact juxtanuclear Golgi apparatus that was morphologically similar to adjacent CBM-treated, but uninjected, cells (Fig. 4 B). The CBM block of Golgi vesiculation was most likely due to its effect on COPI rather than an indirect blockage of the antibody-induced p115 degradation, because the CBM-treated, microinjected cells exhibited the expected loss of p115 (unpublished data). Thus, the absence of p115 appears to induce COPI-mediated Golgi vesiculation.

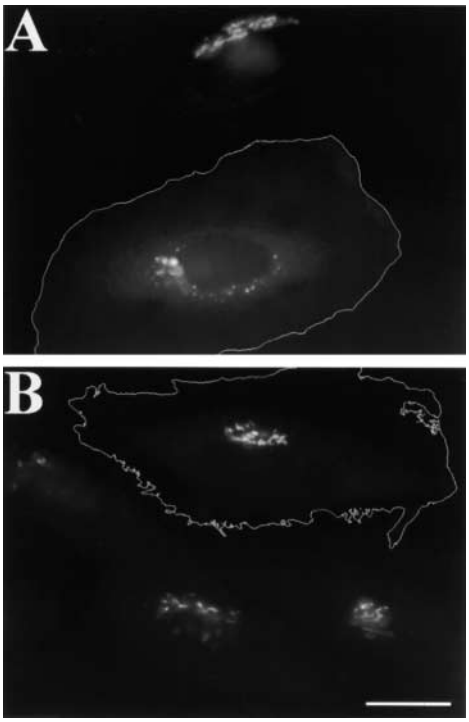


Figure 4. **Anti-p115–induced Golgi breakdown is blocked by the COPI inhibitor CBM.** Cells were injected with anti-p115 antibodies, incubated for 3 h in the absence (A) or presence (B) of 2 mM CBM, and stained for the Golgi marker GPP130. Injected cells are outlined. Although CBM itself slightly perturbed Golgi morphology in uninjected cells, its presence blocked Golgi breakdown in injected cells. Bar, 10 μm.

We also considered the possibility that p115 absence actually causes Golgi apparatus disassembly by recycling Golgi proteins through the ER, which leads to their accumulation in a post-ER compartment such as COPII vesicles or the ERGIC. Costaining of anti-p115–injected cells for the Golgi marker, giantin (Fig. 5 A), and the ERGIC marker, ERGIC-53 (Fig. 5 B), revealed that the disassembled Golgi apparatus was almost entirely distinct from the ERGIC and that the ERGIC maintained its normal punctate pattern. Because ERGIC-53 rapidly cycles between the ER and ERGIC, mostly bypassing the Golgi apparatus (Hauri et al., 2000, and references therein), and is redistributed to the ER under conditions of ER export blockade (Shima et al., 1998; Lee and Linstedt, 1999), the normal ERGIC pattern implies that ER export and ERGIC formation/maintenance continue in the absence of p115. If ER to ERGIC transport persists in the absence of p115, then the lack of abundant Golgi staining in the ERGIC argues against a disassembly pathway involving the ER. To directly demonstrate ER to ERGIC transport, we microinjected BFA-treated cells with anti-p115 antibodies and then performed a BFA washout. In this case, giantin (Fig. 5 C) moved from its redistributed position in the ER into structures that costained with ERGIC-53 (Fig. 5 D). Similar results were obtained for the Golgi marker GPP130. These observations indicate that anti-p115–induced Golgi breakdown is primarily direct.

Antigiantin antibody microinjection induces giantin degradation but not Golgi breakdown

Our finding that loss of p115 led to Golgi breakdown confirms a previous report that p115 is required for maintenance of interphase Golgi structure (Alvarez et al., 1999).

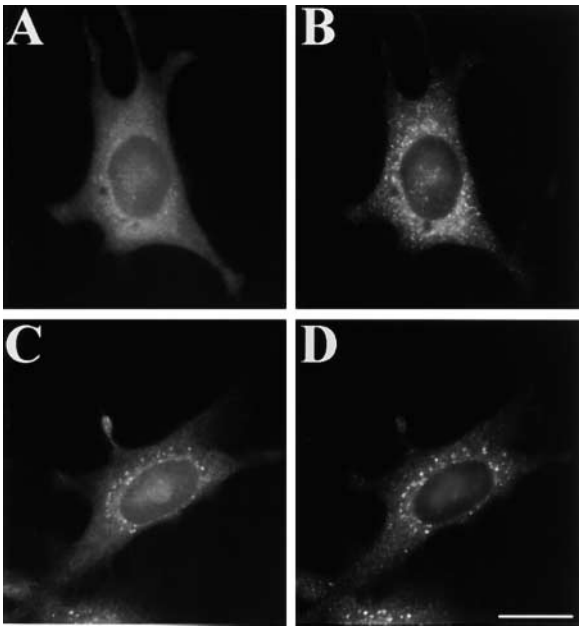


Figure 5. **Evidence for direct Golgi disassembly in cells lacking p115.** Cells were injected with anti-p115 antibodies, incubated for 6 h, and then costained directly for giantin with FITC-coupled anti-giantin pAb (A) and indirectly for ERGIC-53 with a mAb (B). Cells were also treated with BFA to collapse the Golgi into the ER, injected with anti-p115, washed to remove the BFA, incubated in the absence of BFA for 4 h, and then costained directly for giantin (C) and indirectly for ERGIC-53 (D). All cells shown were injected. Note the lack of Golgi staining in the ERGIC after breakdown and its presence after BFA washout. Bar, 10 μm.

Further, because the breakdown involved an apparent direct vesiculation, it suggests that p115 plays a role in the docking of Golgi-derived vesicles. Therefore, we next tested whether the role of p115 in Golgi structure maintenance requires binding to either of the putative tether components, giantin or GM130. Polyclonal antibodies were generated against a peptide containing the mapped p115 binding site in the NH_2 terminus of giantin. Upon microinjection into HeLa cells, the antigiantin antibodies induced degradation of giantin with a time course that was similar to the anti-p115–induced degradation of p115. Thus, early time points exhibited clustered giantin staining (unpublished data), and 2 h after injection giantin staining was undetectable (Fig. 6 A). The degradation was specific to giantin as other marker proteins did not exhibit any reductions in staining (see below), and giantin degradation did not occur upon injection of control anti-GST antibodies (unpublished data). The anti-giantin-induced giantin degradation, but not the antibody-induced giantin clustering, was blocked in microinjected cells incubated in the presence of the proteasome inhibitor MG132 (Fig. 6 B). This result indicated that, similar to the anti-p115–induced p115 degradation, the giantin degradation was proteasome mediated. Further, the absence of detectable giantin persisted for at least 48 h after injection with no apparent loss in cell viability, which allowed us to perform relatively long experiments with cells lacking giantin.

Surprisingly, the Golgi apparatus appeared morphologically and functionally normal in the microinjected cells lacking detectable giantin. Even 16 h after microinjection, a situation where the cells remained without detectable giantin staining for at least 14 h, there was no apparent defect in the GPP130 (Fig. 6 C), p115 (Fig. 6 D), or KDEL receptor (unpublished data) staining patterns based on comparison to adjacent, uninjected cells. This strongly suggests that giantin is not required for processes essential for maintenance of Golgi structure, and in the absence of giantin, the Golgi complex still mediates KDEL receptor recycling. Furthermore, p115 mediates a process essential for Golgi structure (see above and Alvarez et al., 1999), which implies that, in this case, p115 acts independently of giantin.

In light of these observations, we tested whether cells lacking detectable giantin retained the capability to undergo Golgi reassembly after either BFA-induced Golgi redistribution to the ER or mitosis-induced Golgi vesiculation. Indeed, the entire cycle of BFA-induced Golgi–ER fusion and Golgi re-emergence upon BFA washout was indistinguishable in microinjected and adjacent, uninjected cells, even though the experiments were carried out under conditions that reduced giantin levels below detection (unpublished data). Furthermore, cells lacking detectable giantin were able to divide (Fig. 6 E) and exhibited normal Golgi patterns (Fig. 6 F). For these experiments, isolated single cells on each coverslip were injected, incubated for 20 h, and analyzed. At the end of the incubation, each injected cell had doubled indicating passage through mitosis after injection. Normal Golgi reassembly was also evident in microinjected cells that were costained for microtubules (Fig. 6 G) and the Golgi complex (Fig. 6 H), and postmitotic pairs were identified by the short microtubule bundle at the site of cytokinesis. Therefore, cells lacking detectable giantin were viable, exhibited a normal Golgi staining pattern, and underwent cycles of Golgi disassembly/reassembly.

Microinjection of anti-GM130 antibodies that prevent GM130–p115 complex formation

Polyclonal antibodies were also generated against the NH_2-terminal domain of GM130, which contains its p115 binding site. Microinjection of these antibodies with subsequent incubations up to 16 h had no apparent effect on the staining patterns for giantin (Fig. 7 A), p115 (Fig. 7 B), GPP130 (unpublished data), and KDEL receptor (unpublished data). Not only was the interphase Golgi structure normal in anti-GM130–injected cells, but BFA-induced Golgi–ER fusion and Golgi reemergence upon BFA washout was indistinguishable in microinjected and adjacent, uninjected cells (unpublished data). Also, when isolated single cells on each coverslip were injected and incubated for 20 h, the cells divided normally and the daughter cells reassembled a normal Golgi apparatus as indicated by staining for GPP130 (Fig. 7 C).

In contrast to the unexpected antibody-induced antigen degradation described above for anti-p115 and antigiantin, microinjection of anti-GM130 had no apparent effect on GM130 abundance. This was evident from the Golgi staining pattern exhibited by the injected antibody from early time points through at least 16 h after injection; that is, the injected antibody remained bound to GM130 on the Golgi

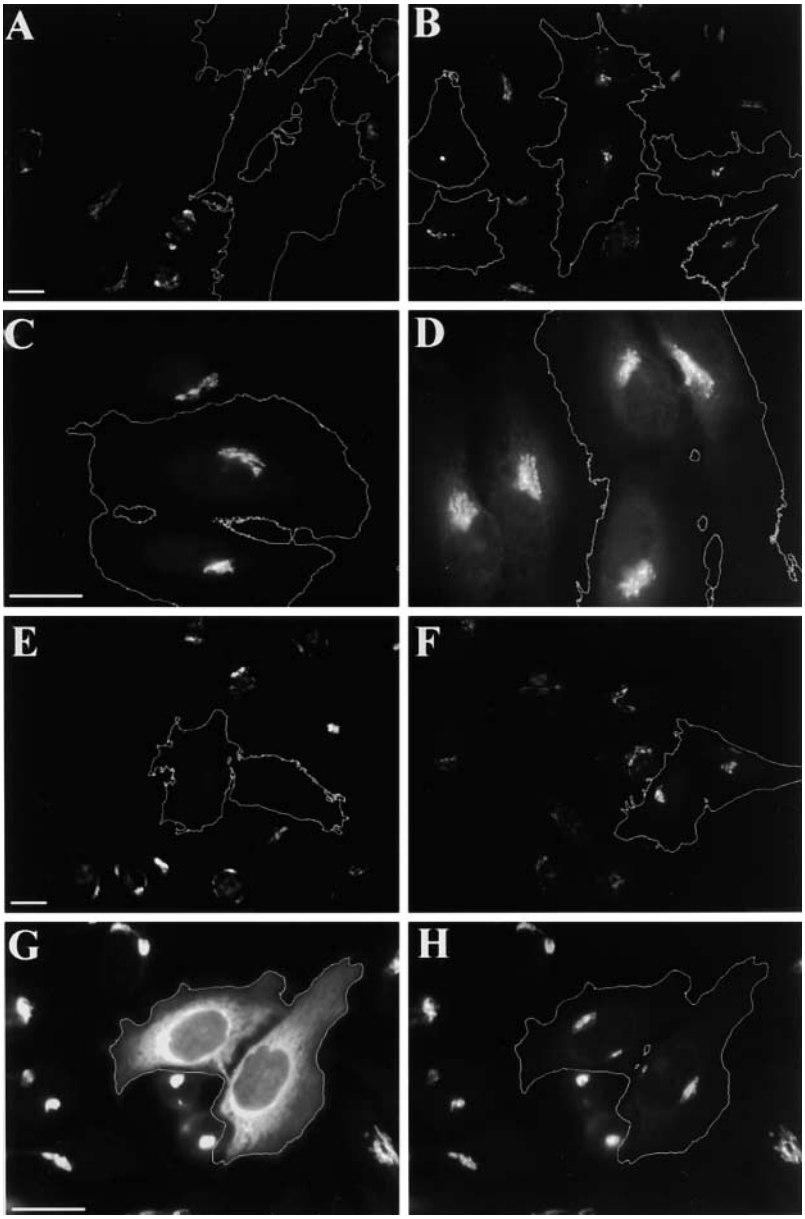


Figure 6. **Golgi maintenance and reassembly in the absence of detectable giantin.** Cells injected with rabbit antigiantin antibodies were incubated for 2 h in the absence (A) or 3 h in the presence (B) of 50 μM MG132 before staining with mouse antigiantin antibodies. Injected cells (outlined) lacked detectable giantin in the absence, but not the presence, of the proteasome inhibitor. Antigiantin-injected cells were incubated for 16 h and stained with anti-GPP130 (C) or anti-p115 (D). Despite giantin absence, injected cells exhibited normal staining patterns. Isolated single cells were injected, incubated for 20 h to allow cell division, and then stained for giantin (E), GPP130 (F), or costained for tubulin (G) and GPP130 (H). Note that daughter cells exhibited normal Golgi morphology in the absence of detectable giantin. Each injected cell divided in this experiment ($n = 20$). Images in A, B, E, and F were acquired with a 20× objective. Bar, 10 μm.

apparatus throughout the experiment with no apparent diminution. Furthermore, no reduction in staining or alteration in Golgi pattern was observed in microinjected cells costained for cellular GM130 with a monoclonal anti-GM130 antibody (unpublished data). Therefore, we performed binding experiments to confirm that the anti-GM130 antibodies actually inhibited p115–GM130 interaction.

In the first experiment, an immobilized GST–GM130 fusion protein containing its p115 binding site (Linstedt et al., 2000) was incubated with p115-containing cell extracts in the presence of anti-GM130 or control antibodies. Despite the relatively high concentration of GM130 present in the assay, the anti-GM130 antibody showed a concentration-dependent inhibition of p115 binding, whereas control anti-GST antibodies did not (Fig. 8 A). Thus, the anti-GM130 antibody had inhibitory activity against p115–GM130 complex formation, yet its microinjection did not displace p115 from the Golgi complex (Fig. 7 B). This observation is actually consistent with previous reports indicating that versions of p115 lacking the GM130 binding domain are still Golgi apparatus targeted (Nelson et al., 1998); that is, p115 appears to have at least one other Golgi-localized receptor. The receptor in question is not giantin because the giantin binding site in p115 maps to the same position as the GM130 binding site (Linstedt et al., 2000). Furthermore, co-injection of both antigiantin and anti-GM130 also did not displace p115 or cause any detectable Golgi abnormality (unpublished data). Therefore, to confirm that anti-GM130 antibodies disrupt p115–GM130 interaction without blocking p115 membrane binding, we performed a second binding experiment.

In this experiment, membrane fractions containing bound p115 were incubated with anti-GM130 antibodies and then subjected to either a co-immunoprecipitation assay to test for p115–GM130 complexes or to a centrifugation assay to test for p115 membrane association. The co-immunoprecip-

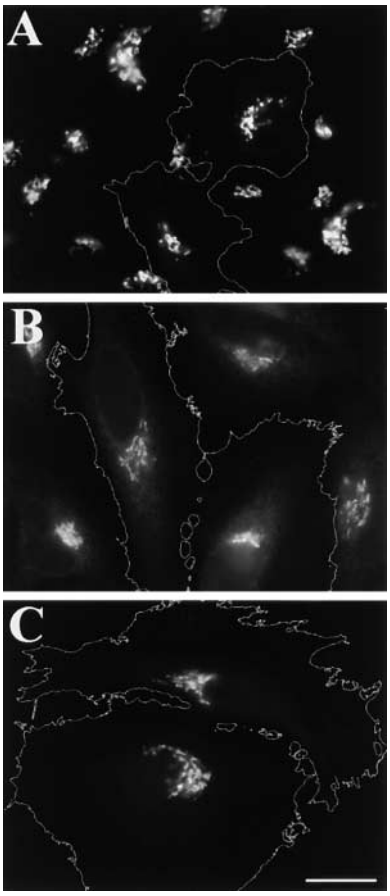


Figure 7. **Golgi maintenance and reassembly in anti-GM130–injected cells.** Cells injected with rabbit anti-GM130 were incubated for 16 h and stained using mouse antigiantin (A), or mouse anti-p115 (B). Isolated single cells were also injected, incubated for 20 h to allow cell division, and then stained for GPP130 (C). Note that injected cells (outlined) exhibited normal patterns. Bar, 10 μm.

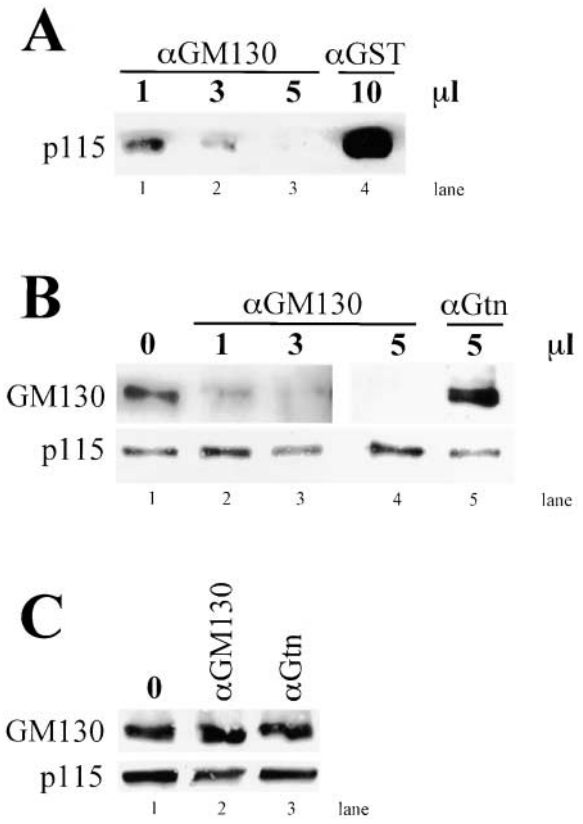


Figure 8. **Anti-GM130 blocks p115–GM130 complex formation without displacing membrane-associated p115.** (A) NRK cell lysates were added to bead-attached GST-GM130 that was preincubated with the amount of anti-GM130 (lanes 1–3) or anti-GST (lane 4) indicated. The amount of cellular p115 recovered on the beads was determined by immunoblotting. (B) NRK membrane fractions were pretreated in absence (lane 1) or the presence of the indicated amounts of anti-GM130 (lanes 2–4) or antigiantin (lane 5) before coimmunoprecipitation with anti-p115 covalently attached to beads. The amount of cellular GM130 and p115 recovered was determined by immunoblotting. (C) NRK membrane fractions were also pretreated without antibody (lane 1), with 5 μl anti-GM130 antibody (lane 2), or with 5 μl antigiantin (lane 3) and then collected by centrifugation. The recovery of membrane-associated p115 and GM130 was then determined by immunoblotting. Note that although anti-GM130 blocked p115 binding to GST-GM130, and it blocked recovery of p115–GM130 complexes from membranes; it did not block p115 membrane association. Bar, 10 μm.

itation assay indicated that anti-GM130 antibodies, but not control antibodies, disrupted p115–GM130 complexes present on the membranes (Fig. 8 B). In contrast, under the same conditions, recovery of membrane-associated p115 was not affected by either anti-GM130 antibodies or control antibodies (Fig. 8 C). Thus, disruption of p115–GM130 complex formation by anti-GM130 treatment of isolated membranes is not sufficient to cause membrane release of p115. Together, these experiments indicate that microinjection of antibodies that disrupt GM130–p115 complex formation in vitro, cause neither p115 membrane displacement in vitro or in vivo nor Golgi breakdown in vivo. This suggests that similar to the situation for giantin, the essential function of p115 in Golgi maintenance is independent of GM130.

Discussion

The model where COPI vesicles are tethered by a long giantin–p115–GM130–GRASP65 complex has important implications for both vesicle trafficking during interphase and Golgi breakdown at mitosis (Sönnichsen et al., 1998). During intra-Golgi trafficking, tethered vesicle transport may keep COPI vesicles physically linked to cisternae thereby preventing diffusion away from their donor and acceptor membranes. At the onset of mitosis, phosphorylation of subunits in the tether complex, such as GM130 (Nakamura et al., 1997), would prevent complex formation leading to release of COPI vesicles and their subsequent dispersal throughout the cytoplasm. If one of the normal functions of COPI vesicles is to retrieve Golgi-localized residents as cisternae mature, then at mitosis, COPI vesicle formation in the absence of docking and fusion would lead to accumulation of Golgi residents in COPI vesicles. Here, we used microinjection of antibodies directed against the binding sites in p115, giantin, and GM130, proteins implicated in tether formation, to study the role of these interactions in vivo. Surprisingly, anti-p115 and antigiantin antibodies caused an apparently specific degradation of their antigens. Consistent

with a key prediction of the model outlined above, loss of cellular p115 induced COPI-dependent Golgi vesiculation in interphase cells. However, cells lacking detectable giantin, or cells containing anti-GM130 antibodies that prevented GM130–p115 interactions, retained apparently normal Golgi structure and disassembly/reassembly properties. These observations strongly suggest that the putative giantin–p115–GM130–GRASP65 tether complex is not required for Golgi structural maintenance, nor is disruption of this complex sufficient to explain mitotic Golgi breakdown.

Although there was not an essential role for the tether complex, our observations confirmed that p115 is required for Golgi structural maintenance (Alvarez et al., 1999). This indicates that p115 acts in a pathway that is independent, or at least can function independently, of interactions with giantin and GM130. Absence of p115 induced apparent accumulation of Golgi vesicles suggesting that Golgi vesicle docking is in fact a key role of p115 (Waters et al., 1992). Anti-p115–induced Golgi vesiculation was prevented by CBM-mediated COPI displacement, pointing to a COPI origin for the accumulated vesicles. This is compatible with the in vitro requirement of p115 for binding of COPI-coated vesicles to isolated Golgi membranes (Sönnichsen et al., 1998).

Presumably, cessation of ER to Golgi transport in anti-p115–injected cells also contributed to loss of Golgi structure, as Golgi structure depends on input of membrane and other factors from the ER (Storrie et al., 1998), and p115 is required for ER to Golgi transport in semi-intact cells (Alvarez et al., 1999; Allan et al., 2000). There is also evidence that p115 is required for ER to Golgi transport in intact cells, as p115 is required for Golgi reassembly after BFA washout (Fig. 5 B; Alvarez et al., 1999), a reaction that involves transport of this type. Importantly, in semi-intact cells, inhibition of p115 blocks transport at the step where vesicular tubular clusters of the ERGIC would otherwise translocate from peripheral sites in toward the juxtanuclear Golgi complex (Alvarez et al., 1999). A role for p115 at this step would explain why peripheral ERGIC was maintained in the absence of p115 (Fig. 5 A) despite vesiculation of the Golgi complex. In fact, one possibility is that p115's sole required function is in the docking of Golgi-derived COPI-coated retrieval vesicles with the ERGIC. This reaction may contribute components that are essential to the translocation and maturation of pre-Golgi elements as they form new cis-cisternae. Components required for translocation and maturation might include receptors for cytoplasmic dynein and early Golgi enzymes, respectively. This view explains both COPI vesicle accumulation and arrest of vesicular tubular cluster translocation/maturation in the absence of p115. It is also consistent with recruitment of p115 into a SNARE complex during COPII formation (Allan et al., 2000), as this complex would then be used for the subsequent docking and fusion of COPI retrieval vesicles with ERGIC membranes. Furthermore, this reaction may be analogous to p115-dependent cisternal growth during in vitro Golgi reassembly (Shorter and Warren, 1999).

If p115's essential function for Golgi structure maintenance is to mediate COPI vesicle docking and fusion with the ERGIC, then this reaction apparently involves p115 binding partners other than giantin and GM130. Why then are these proteins required in p115-dependent reactions in vitro? One possibility is that giantin and GM130 do function together with p115 in intact cells, but that they function in transport steps other than COPI vesicle/ERGIC docking. Further, these other functions that involve p115, giantin, and GM130 must play a facilitating role rather than an essential role. This would fit with their description as tethers, because tethering is expected to be the outermost interaction in a series of interactions between vesicle and target membranes, which culminate in targeted membrane fusion. In the absence of tethering, downstream components, such as the interaction of cognate SNARE proteins, may suffice, albeit less efficiently. The situation could be dramatically different for in vitro assays. Because of dilution and disruption of spatial organizing features such as the cytoskeleton and membrane stacking, a requirement for tethering may be revealed. Thus, giantin and/or GM130 may function together with p115 in steps such as docking at the early Golgi complex (Alvarez et al., 1999; Allan et al., 2000), intra-Golgi COPI vesicle transport (Waters et al., 1992), and Golgi stack formation (Shorter and Warren, 1999); however not in a capacity required for Golgi biogenesis in vivo. It is also formally possible that GM130 and giantin are mutually redundant or that their respective functions are redundant with as yet unidentified proteins. However, the fact that co-injection of both antigiantin and anti-GM130 antibodies had no detectable effect on Golgi structure argues against mutual redundancy; and the lack of any identified proteins with meaningful sequence similarity in the human genome database argues against unidentified family members.

The idea that p115 plays both an essential role independent of giantin and GM130 as well as a nonessential role involving giantin and GM130 helps explain the noted lack of Golgi structural perturbation in cells where the GM130 NH_2 terminus was used to displace p115 from the Golgi apparatus (Seemann et al., 2000). Displacement of p115 from the Golgi complex, may block the nonessential role but have no effect on the essential role, as the essential role may involve, not Golgi-localized p115, but rather ERGIC-localized p115 initially recruited to membranes during COPII vesicle formation (Allan et al., 2000). This model could also account for the apparent sequential action of p115 relative to giantin and GM130 in semi-intact cell transport from the ER to the medial-Golgi complex (Alvarez et al., 2000), as the putative role of p115 in ERGIC/COPI docking is upstream of its putative role in docking at, or within, the Golgi complex. Also, the model is consistent with the fact that the *Saccharomyces cerevisiae* homologue of p115, Uso1p, is essential for viability (Nakajima et al., 1991), whereas this yeast does not have homologues for either giantin or GM130. Furthermore, the Uso1p acidic domain, which would be expected to mediate interaction with giantin or GM130 homologues, if they existed, is not required for growth (Seog et al., 1994).

Does tether inhibition at mitosis lead to Golgi vesiculation? Golgi breakdown in interphase cells in response to anti-p115–induced p115 degradation mimicked, to a first approximation, Golgi vesiculation in mitotic cells. This suggests that inhibition of p115 may play a major role in mi-

totic Golgi vesiculation. Indeed, there is evidence suggesting that at least the nonessential role of p115 is inhibited at mitosis, as p115's ability to interact with giantin and GM130 is reduced by dephosphorylation (Dirac-Svejstrup et al., 2000) and p115 becomes dephosphorylated at M-phase (Sohda et al., 1998). On the other hand, p115 inhibition is not likely to be the sole requirement for mitotic breakdown because the time course of the interphase breakdown ($t_{1/2} \approx 2$ h) was considerably slower than that which occurs from prophase to metaphase (<30 min), even after accounting for the rate of p115 degradation ($t_{1/2} \approx 1$ h). With regard to giantin and GM130, absence of detectable giantin (Fig. 6) and prevention of GM130–p115 interactions (Fig. 7) did not cause Golgi breakdown. This argues that mitotic phosphorylation of GM130 (Lowe et al., 1998), which prevents p115 binding (Nakamura et al., 1997), and mitotic phosphorylation of giantin (unpublished results) are not sufficient to cause Golgi vesiculation, although inhibition of these proteins by phosphorylation may still contribute to the vesiculation reaction. Further, these results suggest that one of the reactions that may be involved, in addition to p115 inhibition at mitosis, would be the inhibition of those transport factors that act immediately after the tethers in the docking and fusion of Golgi-derived vesicles.

Also noteworthy, was the phenomena of antibody-induced antigen degradation. To our knowledge this has not been previously observed, perhaps because many other studies have not stained for the corresponding antigen in antibody-injected cells. Because the loss of staining was time, temperature, and proteasome dependent, and because it followed an apparent clustering of the antigen, we suspect that antibody-induced cross-linking of the antigen somehow triggered binding and ubiquitination of the antigens by a ubiquitin ligase. In the case of giantin and the Golgi-localized fraction of p115, this was probably followed by proteasome recruitment to the Golgi membrane. The roles of cross-linking, ubiquitination, and proteasome localization are important subjects for future study. Giantin and p115 are long-lived proteins suggesting that targeted degradation does not normally play an important role in their regulation. Rather, the antibody-induced degradation of these antigens suggests that if the mechanism can be understood, it may be possible to engineer antibodies that cause degradation of any antigen of interest. This could be significant for further tests of in vivo function by creation of "knock-outs" without requiring targeted gene disruption or antisense RNA. In the cases of anti-p115–induced p115 degradation and antigiantin-induced giantin degradation examined here, this phenomena provided strong support for the hypothesis that the essential function of p115 in Golgi structure is independent of its hypothesized role in a giantin–p115–GM130–GRASP65 tether and sets the stage for further work into the roles of these proteins.

Materials and methods

Reagents and antibodies

Cyclohexanebis(methylamine) (CBM) (Sigma-Aldrich) was added directly to culture medium. High purity digitonin (Boehringer), brefeldin A (Sigma-Aldrich), and MG-132 (Calbiochem) were stored in DMSO at –20°C as 20, 10, and 10 mg/ml stocks, respectively. Antiglutathione *S*-transferase (GST), anti-p115, antigiantin, and anti-GM130 antibodies for microinjection were prepared as follows. GST-p115 (710–959), GST-giantin (1–469), and GST-GM130 (1–74) were generated (Linstedt et al., 2000) and used to immunize rabbits (Covance). High-titer bleeds were partially purified by ammonium sulfate precipitation. In the case of the anti-p115 sera, antibodies with anti-GST reactivity were first removed by affinity purification on GST-coupled beads that were prepared by allowing 2 ml of purified GST (5 mg/ml) to react with 0.5 ml Affi-gel beads (Bio-Rad Laboratories). These were used for control microinjections. The remaining antibodies with anti-p115 reactivity were then similarly purified on p115-coupled beads that were prepared by allowing 0.5 ml purified GST-p115 (2 mg/ml) to react with 0.2 ml Affi-gel beads. The resulting affinity purified antibodies were ammonium sulfate precipitated, dialyzed against KH buffer (125 mM potassium acetate, 25 mM Hepes), adjusted to 2 mg/ml, aliquoted, and snap-frozen. Antigiantin and anti-GM130 antibodies were dialyzed against KH buffer, aliquoted, and snap-frozen at 14 mg/ml. Antibodies used for immunofluorescence staining were against: GPP130 (A1–118; Linstedt et al., 1997), giantin (G1–133; Linstedt and Hauri, 1993; and FITC-labeled antigiantin pAb), β-COP (mAD; Sigma-Aldrich), p115 (7D1; provided by M.G. Waters, Princeton University, Princeton, NJ), GM130 (provided by G. Warren, Yale, New Haven, CT), ERGIC-53 (G1–93; provided by H.-P. Hauri, Biozentrum of the University of Basel, Basel, Switzerland), tubulin (ICN Biomedicals), and KDEL receptor (StressGen Biotechnologies).

Microinjection and analysis

HeLa cells were cultured on glass coverslips as described (Lee and Linstedt, 1999). For CBM treatments, the coverslips were coated with SuperFibronectin (Sigma-Aldrich). Before microinjection, the culture medium was adjusted to 25 mM Hepes-KOH (pH 7.4) and the thawed antibody aliquots were centrifuged at 100,000 rpm for 1 h in a TLA 100.3 rotor (Beckman Coulter). The cells were maintained at 37°C, injected using an Eppendorf microinjection system, and then were returned to the incubator. Staining and digital image acquisition were as previously described (Linstedt et al., 1997). Using the injected antibody staining pattern and the Trace Contour command in Adobe Photoshop, the injected cells were outlined. In certain cases, the staining of the injected antibody was omitted to exclude any possibility of bleedthrough. For digitonin release, each coverslip was incubated for 6 min at room temperature in 0.5 ml of 0.04 mg/ml digitonin in KHM (100 mM KCl, 25 mM Hepes-KOH 7.4, 2.5 mM magnesium acetate) before fixation. Mean cytoplasmic fluorescence was measured from 10,125 pixels in each cell, manually excluding the juxtanuclear area, using the Histogram function in Adobe Photoshop. Background, taken as the average pixel value outside the cells, was subtracted.

Binding experiments

For measuring binding of p115 to GST-GM130, normal rat kidney (NRK) cells were lysed in HKT (10 mM Hepes-KOH, pH 7.2, 100 mM KCl, 1 mM EDTA, 0.5% Triton X-100) and the lysate cleared by centrifugation at 100,000 rpm in the TLA 100.3 rotor. The cleared lysate (0.1 ml) was then added to a mixture of 5 μl GST-GM130–coated glutathione-agarose beads (~0.04 mg/ml) and the indicated amounts of the anti-GM130 or anti-GST polyclonal antibodies. After rotation for 60 min at 4°C, the beads were washed four times with 1 ml of HKT and eluted with 0.5 ml of 0.6 M KCl in HKT. Immunoblotting of the eluate was as described (Linstedt et al., 2000). To test for co-immunoprecipitation of p115–GM130 complexes from antibody-treated membranes, NRK cells were homogenized in KHM using a 25-gauge needle and centrifuged to obtain a postnuclear supernatant. The postnuclear supernatant was then underlayed with 10 μl of 80% sucrose and centrifuged at 16,000 *g* for 20 min at 4°C. The membranes were collected and incubated with various amounts of anti-GM130 or antigiantin polyclonal antibodies for 60 min on ice. The membranes were then solubilized with HKT and the lysate was centrifuged at 50,000 rpm in the TLA 100.3 rotor for 30 min. The cleared lysate was rotated at 4°C for 60 min with 20 μl of packed Affi-Gel beads that had been coupled to the anti-p115 polyclonal antibody (Bio-Rad Laboratories). Washing, elution, and detection were then performed as before. To assay membrane-associated p115 after anti-GM130 or antigiantin incubation, membranes were prepared and incubated with antibodies exactly as just described. The antibody-treated membranes were then adjusted to 1 ml KHM, underlayed with 10 μl of 80% sucrose, and centrifuged as before. After four such washes the amount of p115 and GM130 was determined by immunoblotting.

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