Cell, Vol. 118, 323–335, August 6, 2004, Copyright ©2004 by Cell Press

GMAP-210 Recruits γ-Tubulin Complexes to *cis*-Golgi Membranes and Is Required for Golgi Ribbon Formation

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Summary

Mammalian cells concentrate Golgi membranes around the centrosome in a microtubule-dependent manner. The mechanisms involved in generating a single Golgi ribbon in the periphery of the centrosome remain unknown. Here we show that GMAP-210, a *cis*-Golgi microtubule binding protein, recruits γ-tubulin-containing complexes to Golgi membranes even in conditions where microtubule polymerization is prevented and independently of Golgi apparatus localization within the cell. Under overexpression conditions, very short microtubules, or tubulin oligomers, are stabilized on Golgi membranes. GMAP-210 depletion by RNA interference results in extensive fragmentation of the Golgi apparatus, supporting a role for GMAP-210 in Golgi ribbon formation. Targeting of GMAP-210 or its C terminus to mitochondria induces the recruitment of γ-tubulin to their surface and redistribution of mitochondria to a pericentrosomal location. All our experiments suggest that GMAP-210 displays microtubule anchoring and membrane fusion activities, thus contributing to the assembly and maintenance of the Golgi ribbon around the centrosome.

Introduction

In interphase mammalian cells, the Golgi apparatus (GA) is a single structure that consists of stacks of flattened cisternae that are connected across noncompact regions. In many cells, the GA is localized at the cell center near the minus ends of microtubules (MTs). Long considered as the optimal localization for protein sorting and transport, the pericentriolar concentration of Golgi membranes has now been proposed to fulfill additional important roles (Colanzi et al., 2003; Rios and Bornens, 2003). Fragmentation and dispersal of the GA has been found to be required for mitotic progression (Sutterlin et al., 2002). Inhibition of Golgi fragmentation by Arf1 activation has been reported to induce gross defects in chromosome segregation and cytokinetic furrow ingression (Altan-Bonnet et al., 2003). The Golgi complex also appears to be capable of sensing stress and transducing apoptotic signals (Maag et al., 2003). Finally, several important signaling molecules, including Ras, have been now localized at the GA and shown to perform several biologically important functions (Bivona et al., 2003; Rios and Bornens, 2003).

The structural organization and position of the GA are thought to be dependent on golgin proteins and on cytoskeleton. All golgin proteins exhibit long coiled-coil regions that adopt rod-like structures. In addition, most of them are peripheral membrane proteins and bind to small GTPases (Barr and Short, 2003). A role of some golgin complexes in Golgi stack formation and membrane trafficking has been proposed (Barr et al., 1998; Barr and Short, 2003; Marra et al., 2001; Short et al., 2001). For instance, it has been shown that golgin-84 contributes to Golgi structure since its depletion by RNA interference results in the separation of Golgi stacks and their dispersal in the cytoplasm (Diao et al., 2003; Satoh et al., 2003).

It is also known that MTs and minus end-directed motors ensure the structural integrity and the central localization of the GA based on studies in which MTs were depolymerized or molecular motors were inactivated (Burkhardt et al., 1997; Grissom et al., 2002; Harada et al., 1998; Thyberg and Moskalewski, 1999; Xu et al., 2002). Under these conditions, the GA fragments and many Golgi elements are generated near ER exit sites. Scattered Golgi fragments are composed of short, stacked cisternae that resemble intact Golgi stacks (Harada et al., 1998; Thyberg and Moskalewski, 1999). These ministacks are capable of forming de novo upon export of Golgi proteins from the ER (Lippincott-Schwartz and Zaal, 2000), supporting the conclusion that Golgi stacks formation, i.e., transformation of ER-derived pleiomorphic tubulo-vesicular clusters into a regular array of flattened and stacked cisternae, is a MT-independent process. MTs are required, however, for linking together these mini Golgi stacks in order to generate a single Golgi ribbon at the cell center. Three-dimensional reconstructions of the Golgi region have revealed that interactions between the Golgi stacks and MTs occur mostly at the *cis*-Golgi (Marsh et al., 2001), suggesting a specific role of MTs in stabilizing the face where new Golgi cisternae are formed. In addition, it has been reported that the GA is involved in organizing a subset of stable MTs independently of the centrosome in interphase cells (Chabin-Brion et al., 2001).

In this work, we investigated the contribution of GMAP-210 to GA biogenesis in the periphery of the centrosome. GMAP-210 was identified as an autoantigen, peripherally associated to *cis*-Golgi membranes that remained in the so-called Golgi remnants after BFA treatment (Rios et al., 1994). Molecular characterization revealed that GMAP-210 is a long coiled-coil protein that binds to the GA via its N terminus (Infante et al., 1999). When overexpressed, GMAP-210 dramatically alters the structure and size of the GA that becomes enlarged and fragmented. Under these conditions, membrane transport into and out of the GA is blocked and

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the GA appears disassembled, suggesting a role of GMAP-210 in maintaining Golgi structure and in regulating membrane traffic (Pernet-Gallay et al., 2002). Due to all of these characteristics, GMAP-210 has been recently included in the golgin family (Barr and Short, 2003). However, GMAP-210 exhibits a unique characteristic: the presence of a highly basic, probably globular, C-terminal domain that binds to MT minus ends. Overexpression of a truncated mutant lacking C-terminal domain has no effect on GA structure, suggesting that perturbations of membrane transport and Golgi structure caused by GMAP-210 overexpression are linked to perturbations in MT network (Infante et al., 1999).

We show now that GMAP-210 interacts with γ-tubulin-containing complexes. γ-tubulin colocalizes with GMAP-210 at the GA and associates with Golgi membranes in a GMAP-210-dependent manner. Depletion of GMAP-210 by RNA interference causes fragmentation of the Golgi ribbon into small elements that contain proteins from all Golgi compartments. Finally, we show that when ectopically targeted to peripheral cytoplasmic organelles such as mitochondria, GMAP-210 induces the recruitment of mitochondria around the centrosome and a dramatic perturbation of the GA.

Results

GMAP-210 Binds to γ-Tubulin-Containing Complexes

As GMAP-210 was previously shown to bind MT minus ends (Infante et al., 1999), we tested the possibility that it could interact with γ-tubulin-containing complexes (γTCCs). We first performed reciprocal immunoprecipitation (IP) experiments using previously characterized anti-GMAP-210 (Infante et al., 1999) or anti-γ-tubulin (called herein γT4D; Tassin et al., 1998) polyclonal antibodies. Each protein coimmunoprecipitated specifically with the other (Figure 1A, left and middle panels). We next examined whether the association of GMAP-210 with γ-tubulin involved the other components of the nucleating complexes that directly bind to γ-tubulin, i.e., GCP2 and GCP3. NP-40-soluble cell extracts were immunoprecipitated with an anti-GCP3 antibody (Tassin et al., 1998; Figure 1A, right panel). GCP3 specifically pulled down both GMAP-210 and γ-tubulin, confirming the existence of GMAP-210/γ-tubulin/GCP3 complexes in vivo.

Co-IP experiments could not reveal, however, whether GMAP-210/γTCCs were associated or not with Golgi membranes since cytosolic pools of both γ-tubulin and GMAP-210 exist (Infante et al., 1999; Moudjou et al., 1996). To answer this question, we prepared Golgi membranes from HeLa cells and observed that γ-tubulin and GCP3 copurified with GMAP-210 (Figure 1B). Next, we performed fractionation experiments in order to separate cytosolic, membrane bound, and insoluble fractions of these proteins. Cos-7 cells were first extracted with saponin (or digitonin) and then treated with NP-40. Saponin-soluble, NP-40-soluble and -insoluble fractions were then analyzed by immunoblotting (Figure 1C). GM130, which has no cytosolic pool (Yoshimura et al., 2001), was used as a control of the fractionation procedure and as expected was almost undetectable in saponin-soluble fraction. On the contrary, significant amounts of GMAP-210, γ-tubulin, and GCP3 were present in saponin-soluble fraction. Indeed, when saponin-extracted cells were examined by immunofluorescence microscopy, cytosolic γ-tubulin labeling was no longer detectable (Figure 1D, compare middle panels). Instead, a conspicuous γ-tubulin pericentrosomal labeling largely overlapping with that of GM130 (not shown), or GMAP-210, became apparent (Figure 1D, bottom gallery). After NP-40 treatment, which extracted most Golgi membranes, γ-tubulin labeling of the GA was dramatically reduced and a prominent centrosome staining was observed (not shown). These results reveal that part of γ-tubulin is concentrated not only on the centrosome but also on Golgi membranes, an observation which, to our knowledge, has not yet been documented in situ. It must be noted that all of these experiments were carried out using γT4D (Tassin et al., 1998). This polyclonal antibody recognizes at least eight epitopes located throughout the entire sequence of human γ-tubulin as revealed by SPOT technique-based experiments (Figure 1E).

IP of GMAP-210 from NP-40-soluble fraction after saponin extraction revealed the presence of GMAP-210/γTCCs (Figure 1F). These complexes did not contain α-tubulin, suggesting that direct binding of GMAP-210 to MTs that we have previously observed in vitro (Infante et al., 1999) is not preserved under these conditions. Reciprocal experiments using γT4D provided identical results (not shown). Finally, when we performed sucrose density gradient centrifugation of this sample (Figure 1G), the major fractions of GMAP-210 and of γ-tubulin/GCP3 did not cosediment precisely. We thus carried out IP experiments using fractions corresponding to GMAP-210 peaks or to γ-tubulin/GCP3 peaks. GMAP-210/γ-tubulin complexes were mostly detected in γ-tubulin/GCP3-containing fraction (Figure 1H). Altogether these data demonstrate the existence of GMAP-210/γ-tubulin complexes in Golgi membranes.

GMAP-210 Recruits γ-Tubulin-Containing Complexes to Golgi Membranes

To further characterize the interaction between GMAP-210 and γTCCs, co-IP experiments were also carried out with cells transiently expressing full-length GMAP-210 or a truncated mutant lacking the C-terminal MT binding domain (ΔC-GMAP-210). As shown in Figure 2A, overexpressed full-length GMAP-210 coimmunoprecipitated with γ-tubulin with either antibody (left and middle panels) and GCP3 brought down both γ-tubulin and GMAP-210 (right panel). By contrast, no ΔC-GMAP-210/γ-tubulin complexes were detected when IP experiments were carried out from ΔC-GMAP-210-overexpressing cell extracts (Figure 2B, left and middle panels), and γ-tubulin/GCP3 complexes pulled down by anti-GCP3 antibody did not contain ΔC-GMAP-210 (right panel). However, a tiny band could be detected in both IP experiments, corresponding to endogenous GMAP-210/γ-tubulin complexes. Altogether, these results reveal an in vivo interaction between MT nucleating complexes, as monitored by both γ-tubulin and GCP3, and the C-terminal domain of GMAP-210.

To test whether GMAP-210/γ-tubulin interaction was

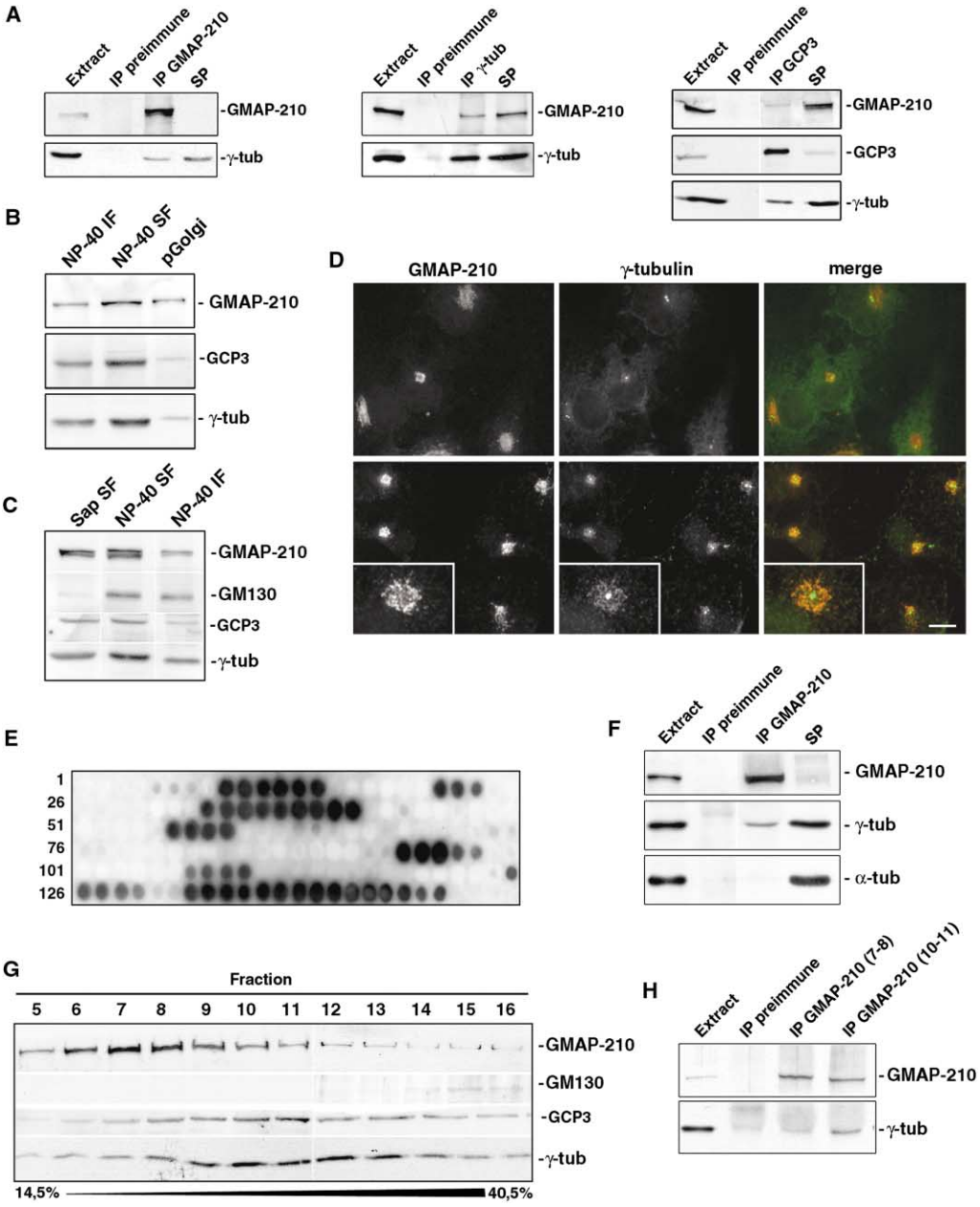

Figure 1. Interaction between GMAP-210 and γ-Tubulin/GCP3-Containing Complexes

(A) Western blotting of IP assays performed with anti-GMAP-210 (left panel), γT4D (middle panel), and anti-GCP3 antibodies (right panel) and probed for GMAP-210, γ-tubulin, and GCP3 as indicated at right. The input extract, IP with preimmune serum, and supernatant (SP) are also included.

(B) Insoluble (NP-40 IF), soluble (NP-40 SF), and purified Golgi membrane fractions (pGolgi) from HeLa cells were analyzed by immunoblotting with the indicated antibodies (right).

(C) Cos-7 cells were sequentially extracted with saponin and then with NP-40 as described in Experimental Procedures. Saponin-soluble (SAP SF), NP-40-soluble (NP-40 SF), and NP-40-insoluble (NP-40 IF) fractions were analyzed by immunoblotting with the indicated antibodies (right).

(D) Nonextracted (top gallery) or saponin-extracted (bottom gallery) Cos-7 cells were co-stained for GMAP-210 (left panels) and γ-tubulin (middle panels). In merged images at right, GMAP-210 is in red and γ-tubulin is in green. The Golgi-centrosome area of a single cell at higher magnification is shown. Bar, 10 μm.

(E) SPOT analysis of human γ-tubulin with the polyclonal antibody γT4D.

(F) Western blot analysis with the indicated antibodies (right) of a GMAP-210 IP assay performed on NP-40-soluble fractions obtained as described in (C).

(G) An identical NP-40-soluble fraction was fractionated by sucrose density. Then, the fractions were examined for the presence of GMAP-210, GM130, GCP3, and γ-tubulin.

(H) Western blot analysis with the indicated antibodies (right) of GMAP-210 IP assays performed on pooled fractions 7–8 and 10–11.

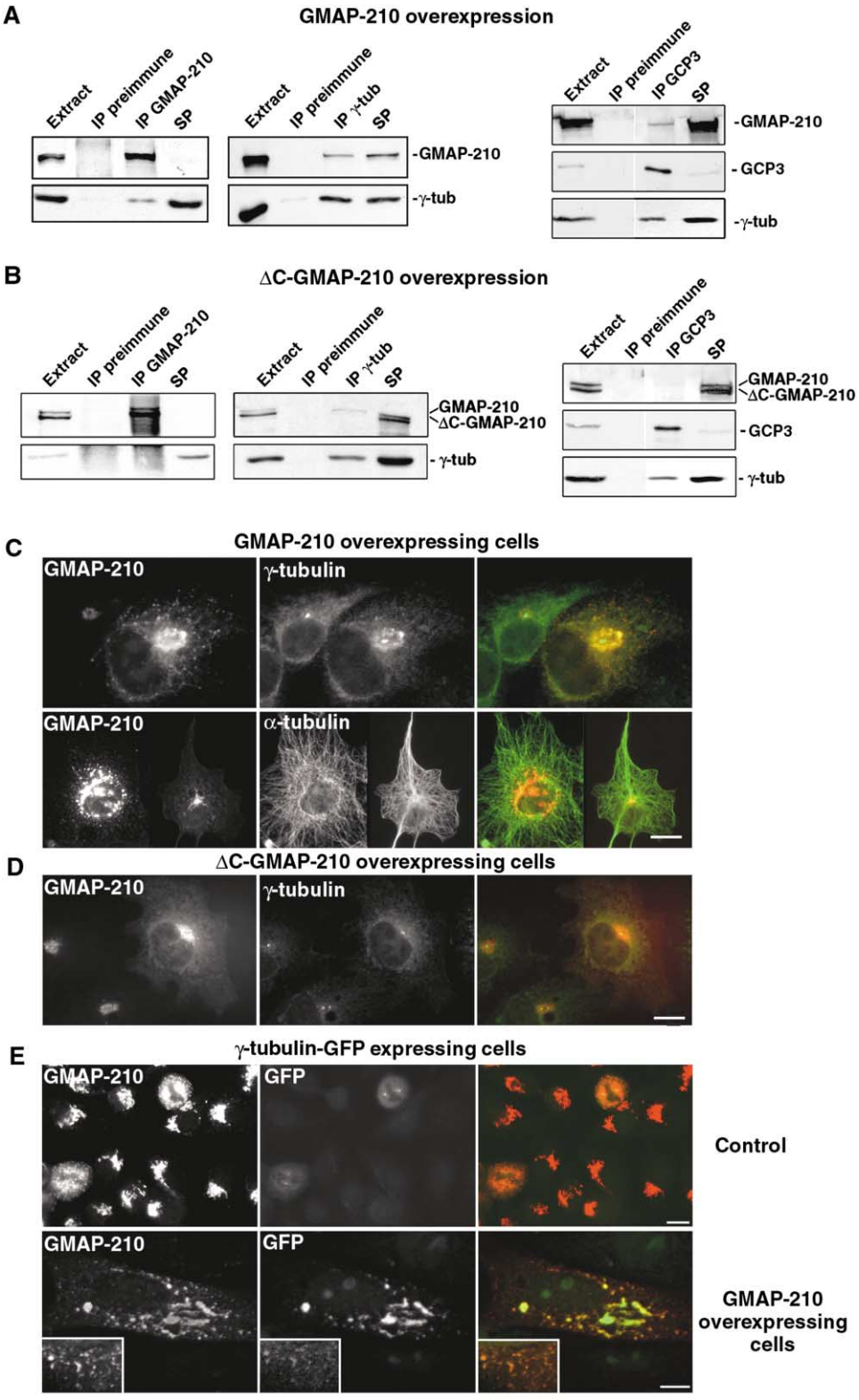

Figure 2. GMAP-210 Modulates the Amount of γ-Tubulin Bound to Golgi Membranes

(A–B) GMAP-210 (A) or ΔC-GMAP-210 (B) overexpressing cell extracts were immunoprecipitated as described above. Immunoprecipitates were analyzed by immunoblotting using anti-GMAP-210, anti-γ-tubulin, and anti-GCP3 antibodies as indicated.

(C–D) Cos-7 cells transiently transfected with vectors encoding GMAP-210 (C) or ΔC-GMAP-210 (D) were co-stained with antibodies against GMAP-210 and γ-tubulin or against GMAP-210 and α-tubulin as indicated and then analyzed by immunofluorescence. Colocalization of the two signals (GMAP-210 in red and γ-tubulin or α-tubulin in green) is shown in right panels. Bars, 10 μm.

(E) PK cells expressing γ-tubulin-GFP directly fixed in 2% PAF (top gallery), or previously transfected with a GMAP-210 encoding vector and then fixed (bottom gallery), were labeled with anti-GMAP-210 antibody.

direct, we performed in vitro transcription/translation reactions followed by reciprocal IPs with appropiate antibodies (not shown). Both γ-tubulin and GCP3 were shown to interact with GMAP-210. However, we could not conclude a direct interaction since we found that γTCCs were present in the reticulocyte lysate. It is possible that GMAP-210 interacts with the full nucleating complex as proposed for other γ-tubulin-interacting proteins (Terada et al., 2003; Usui et al., 2003).

We then asked whether increasing the cellular level of GMAP-210 by transfection would result in the recruitment of additional γ-tubulin to GMAP-210-enriched membranes. Consistent with our previous data, GMAP-210-overexpressing cells exhibited a dramatic enlargement and a fragmentation of the GA (Figure 2C, left panels) as well as a perturbation of the MT aster (compare transfected and nontransfected cells in middle panel). Under these conditions, a clear recruitment of γ-tubulin to membrane elements enriched in GMAP-210 could be seen in transfected cells, when compared to adjacent nontransfected cells (Figure 2C, top gallery). The intensity of γ-tubulin labeling strongly correlated with GMAP-210 expression level and with Golgi enlargement and fragmentation. As expected, no significant increase in γ-tubulin labeling was observed when ΔC-GMAP-210 was expressed (Figures 2D). To confirm these results, similar experiments were performed on PK and PTK cell lines stably expressing γ-tubulin-GFP (Figure 2E). These cells exhibited low centrosomal labeling and variable levels of cytoplasmic fluorescence. When fixed with 2% PAF, only mitotic cells could be clearly appreciated (Figure 2E, top gallery). After GMAP-210 overexpression, GFP fluorescence was observed to colocalize with Golgi elements in those cells expressing both GMAP-210 and γ-tubulin-GFP (Figure 2E, bottom gallery). Accumulation of γ-tubulin on Golgi membranes could be detected even at low GMAP-210 levels (insets in bottom gallery). From all of these experiments, we conclude that GMAP-210 controls the recruitment of γ-tubulin to Golgi membranes in living cells.

GMAP-210/γ-Tubulin Interaction Does Not Require Cytoplasmic MTs or the Pericentrosomal Location of GA

In order to better characterize the interaction between GMAP-210 and γTCCs in vivo, we modified both the integrity and localization of the GA by perturbing the MT network in GMAP-210-overexpressing cells. In GMAP-210-transfected cells treated with nocodazole (NZ), the GA fragmented into numerous scattered Golgi fragments (Figures 3A, left panels). Remarkably, γ-tubulin completely colocalized with GMAP-210 in scattered Golgi elements (Figures 3A, top gallery). Similar but weaker γ-tubulin labeling of Golgi elements could also be detected in adjacent nontransfected cells and in cells expressing γ-tubulin-GFP (not shown).

In order to investigate whether stable, NZ-resistant MTs remain on Golgi elements, we stained NZ-treated cells with antibodies against α-tubulin (Figure 3A, middle gallery) and detyrosinated α-tubulin, an isoform that accumulates in stable MTs. Detyrosinated tubulin colocalized with the GMAP-210-enriched elements (Figure 3A, bottom gallery). Since detyrosinated α-tubulin is rapidly retyrosinated upon MT disassembly into subunit dimers (Saoudi et al., 1998), this observation indicates that Golgi elements are not only enriched in γTCCs but are also associated with very short NZ-resistant MTs or tubulin oligomers. To verify that the GMAP-210/γTCCs interaction persisted after NZ treatment, nontransfected NZ-treated cells were extracted with saponin and NP-40 as described above (Figure 3B). Immunoblot analysis revealed that MT depolymerization did not significantly modify the membrane bound part of either γ-tubulin, GCP3, or GMAP-210 proteins (Figure 3B, top panel, compare to Figure 1C). When these membrane bound proteins were solubilized by NP-40 and used for IP experiments, GMAP-210/γTCCs interaction could be clearly demonstrated in cells lacking MTs (Figure 3B, bottom panel). Reciprocal experiments using γT4D provided identical results (not shown).

Colocalization of GMAP-210 and γ-tubulin was also preserved after treatment of GMAP-210-overexpressing cells with taxol, which promotes the complete polymerization of tubulin into noncentrosomal MT bundles. Under these conditions, the GA appeared fragmented and GMAP-210 and γ-tubulin colocalized with MT minus ends, mostly orientated toward the cell periphery (Figures 3C, top and middle galleries). On the other hand, anti-GM130 labeling revealed only partial colocalization with γ-tubulin (Figures 3C, bottom gallery). Taxol treatment induced partial segregation of GA proteins into different scattered Golgi elements (not shown), with only the GMAP-210-enriched membranes accumulating γ-tubulin. Sequential detergent extractions revealed the absence of cytosolic pools for GMAP-210, GCP3, or γ-tubulin in taxol-treated nontransfected cells. Instead, most of these proteins were NP40 soluble (Figure 3D, top panel). The presence of GMAP-210/GCP3/γ-tubulin complexes in membrane elements of taxol-treated cells was further demonstrated by IP experiments (Figure 3D, bottom panel). We finally expressed GMAP-210 together with p50/dynamitin in order to induce the fragmentation of the GA without MT depolymerization. Under these conditions, scattered Golgi elements exhibited a clear γ-tubulin labeling (Figure 3E). These results indicate that GMAP-210 is able to recruit γTCCs to Golgi membranes, no matter what the GA localization within the cell.

GMAP-210/γTCCs-Enriched Membranes Perturb MT Regrowth

The presence of GMAP-210/γTCCs at Golgi membranes in NZ-treated cells could explain why GMAP-210 overexpression perturbs the MT network. We thus examined MT repolymerization after NZ treatment in GMAP-210-overexpressing cells (Figure 4). Upon removal of the drug, MTs regrew rapidly from the centrosome in both transfected and untransfected cells (Figure 4A). Ten minutes after NZ washout, a well-defined MT aster was observed in most nontransfected cells and in almost half of the transfected cells (Figures 4A and 4B). At this time point, the GA was still fragmented and did not show signs of MT elongation, although a few MTs could be observed in areas where many Golgi membranes were concentrated. By 20 or 30 min, most of GMAP-210-overexpressing cells contained partially fragmented Golgi complexes and unfocused, aberrant MT networks

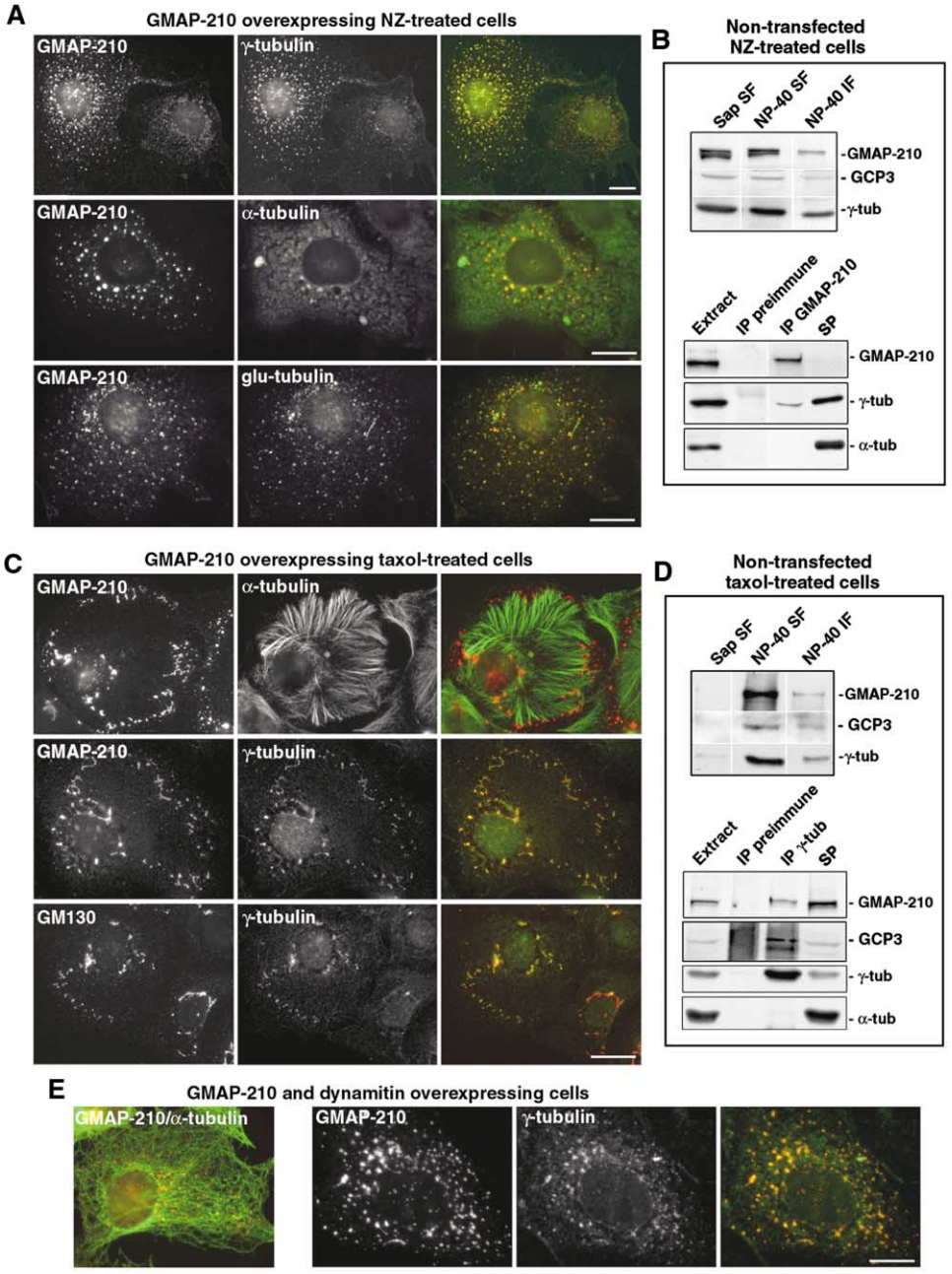

Figure 3. GMAP-210/γ-Tubulin Complexes Are Unaffected Either by MT Perturbation or Disruption of Dynactin-Dynein Complexes

(A) Cos-7 cells were transfected with GMAP-210 and treated with NZ for 5 hr. Cells were fixed and co-stained for GMAP-210 (left panels) and either γ-tubulin, α-tubulin, or detyrosinated (glu-) tubulin (middle panels). In the overlays at right, GMAP-210 is in red and α-tubulin, γ-tubulin, or glu-tubulin are in green. Bars, 10 μm.

(B) Western blot analysis with the indicated antibodies (right) of nontransfected NZ-treated cell extracts obtained by sequential extraction with saponin (SAP SF) and NP-40 (NP40 SF). Insoluble fraction (NP-40 IF) is also included (top panel). NP-40-soluble fraction from NZ-treated cells was immunoprecipitated with anti-GMAP-210 antibody and analyzed by immunoblotting with the indicated antibodies (right). The input extract, the preimmune serum, and the supernatant (SP) are also indicated (bottom panel).

(C) Similar experiments to those described in (A) but on cells treated with taxol for 5 hr. Cells were co-stained for GMAP-210 and α-tubulin or γ-tubulin, or for GM130 and γ-tubulin. In merged images (right panels), GMAP-210 or GM130 are in red and the others antibodies in green. Bar, 10 μm.

(D) Similar experiments to those described in (B) but performed on taxol-treated cells. NP-40-soluble fraction was used for immunoprecipitating GMAP-210/GCP3/γ-tubulin complexes using γT4D antibody.

(E) Finally, cells were cotransfected with vectors encoding p50/dynamitin and GMAP-210 and double stained with GMAP-210 and α-tubulin or GMAP-210 and γ-tubulin. In overlays, GMAP-210 is in red. Bar, 10 μm.

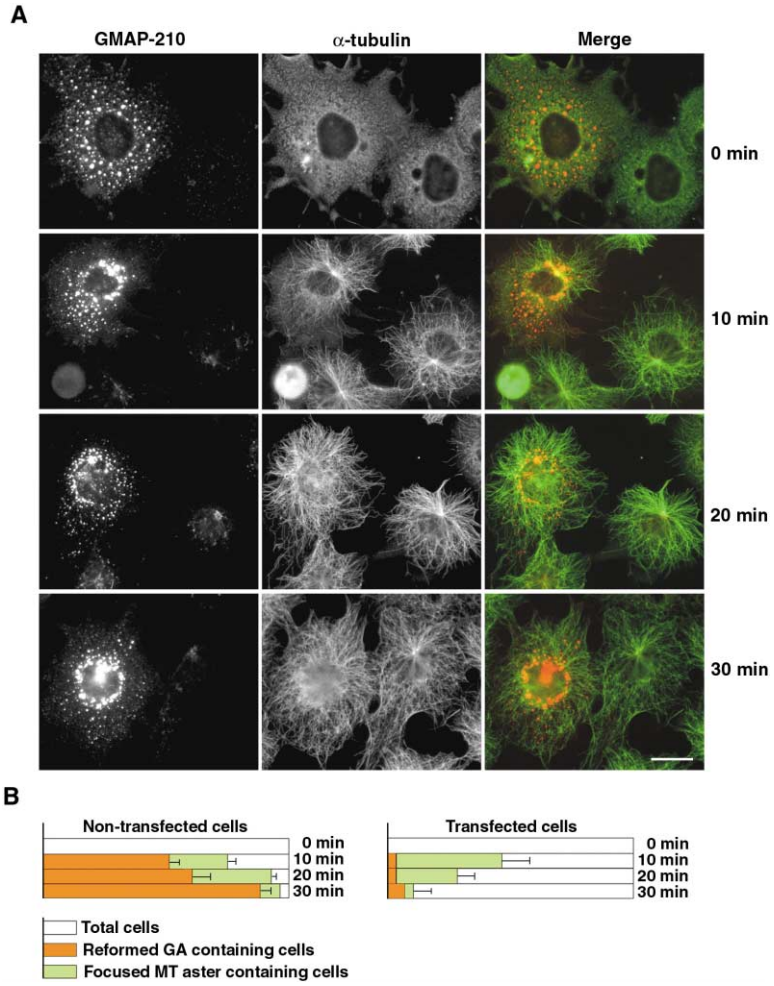

Figure 4. Effect of GMAP-210 Overexpression on MT Regrowth and Golgi Reformation after NZ Removal

(A) NZ-treated GMAP-210-overexpressing cells were washed twice with cold culture medium and left to recover in NZ-free medium for 0, 10, 20, and 30 min at room temperature. After fixation, cells were stained with anti-GMAP-210 and anti-α-tubulin antibodies. Merged images are also shown with GMAP-210 in red and α-tubulin in green. Bar, 10 μm.

(B) Quantitation of perturbation of MT network and Golgi fragmentation in NZ-washout experiments in GMAP-210-transfected or -nontransfected cells. The data represent the average of two different experiments.

as in steady-state conditions (compare transfected and nontransfected cells). This phenotype could be due either to late MT nucleation or to MT anchoring of preassembled MT seeds. We favor the latter possibility based on regrowth kinetics: the number of cells containing unfocused asters increased with time, suggesting a competition for MT anchoring between the centrosome and the GA. We conclude that if nucleation occurs at the Golgi surface, it is either different from bona fide nucleation taking place at the centrosome or nucleated MTs cannot elongate (see Discussion).

Reducing the Level of GMAP-210 Dramatically Fragments the Golgi Ribbon

To further analyze the functional significance of GMAP-210/γTCCs interaction, we lowered the expression level of GMAP-210 by RNA interference (RNAi). Two non-overlapping siRNAs were synthesized and separately transfected on HeLa cells. In both cases, a reduction in GMAP-210 level of 50%–60% was detected after 72 hr (Figure 5A). After correction for transfection efficiency that was calculated by counting GM130-positive/GMAP-210-negative cells, we estimated that expression of GMAP-210 per cell was reduced by more than 80%. This reduction was specific because all other Golgi markers tested remained unchanged (see below). Immunofluorescence of RNAi-treated cells with antibodies to GM130, giantin, CTR433, and galactosyl-transferase (GalTf) revealed that all Golgi compartments were drastically perturbed in the absence of GMAP-210 (Figure 5B). The Golgi ribbon appeared broken in fragments that remained mostly located near the nucleus and contained markers of the *cis*- (GM130 and giantin), medial- (CTR433), and *trans*-Golgi (GalTf), suggesting that some degree of Golgi organization is retained in these fragments. The extent of Golgi fragmentation correlated well with the reduction of GMAP-210 expression. When no GMAP-210 labeling could be appreciated, fragments were small and all of the same size (see insets in Figures 5D and 5E).

Remarkably, in the absence of GMAP-210, ER exit sites detected by Sec31 marker were less clustered around the GA. Partial colocalization of giantin-containing Golgi elements with ER exit sites was maintained although never at the cell periphery (Figure 5B). Finally, double labeling for γ-tubulin and GM130 revealed that Golgi fragments were no longer in a pericentrosomal location although they were mostly located near the nucleus.

Next, we analyzed the behavior of the fragmented GA when GMAP-210-depleted cells were subjected to different treatments. The response of Golgi proteins to BFA was seemingly normal. CTR433 (Figure 5C) and anti-GalTf (not shown), two Golgi membrane proteins

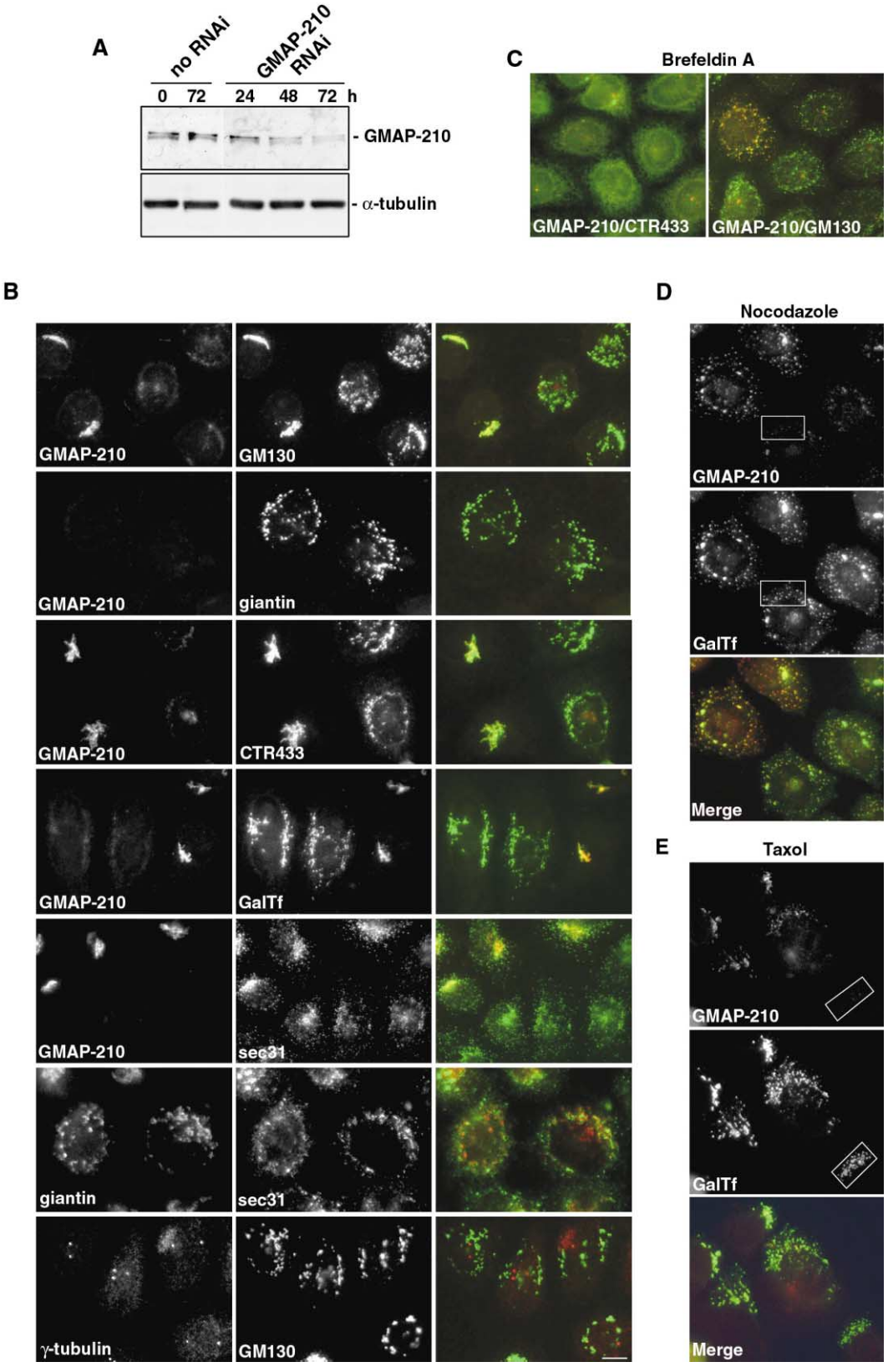

Figure 5. Depletion of GMAP-210 by RNAi Fragments and Mislocalizes the Golgi Apparatus

(A) HeLa cells were either mock transfected (no RNAi) or transfected with duplex RNA to target GMAP-210 and after 24, 48, and 72 hr, were subjected to Western blotting with antibodies to GMAP-210 or α-tubulin (loading control).

(B) RNAi-treated HeLa cells were fixed and double labeled with antibodies to GMAP-210, GM130, giantin, CTR433, galactosyl-transferase (GalTf), Sec31, and γ-tubulin as indicated. In the overlays, markers shown in left panels appear in red and those shown in middle ones in green.

(C–E) Cells with knocked-down GMAP-210 were incubated with BFA for 1 hr (C), or with NZ (D) or taxol (E) for 5 hr, fixed and double stained for GMAP-210 and CTR433, GM130, or GalTf as indicated. In merged images, GMAP-210 is in red and the rest of markers in green. Bar, 10 μm.

markers, redistributed to the ER (Figure 5C, left panel) whereas GM130 (and GMAP-210 in nondepleted cells; Figure 5C, right panel) ended up in the so-called BFA remnants. In NZ-treated cells, scattering of Golgi elements throughout the cytoplasm was extensive (Figure 5D). In GMAP-210-depleted cells incubated with taxol, Golgi fragments were clustered on one end of MT bundles (Figure 5E). It should be pointed out that after washout of BFA or NZ, the GA morphology and distribution in GMAP-210-lacking cells recovered that of nontreated cells, i.e., the Golgi fragments appeared located near the nucleus but not concentrated around the centrosome.

From these results, we conclude that GMAP-210 is required for the assembly of the Golgi ribbon around the centrosome.

Expression of Mitochondria-Targeted GMAP-210 Leads to Mitochondrial Clustering around the Centrosome

Finally we have targeted GFP-tagged versions of GMAP-210 or of its C terminus to the mitochondrial surface by addition of the sequence coding for the hydrophobic membrane anchor of the ActA protein from *L. monocytogenes* (Pistor et al., 1994). The mitochondrial localization of products was confirmed using anti-cytochrome-c-oxidase antibody or MitoTracker™ as indicated (Figure 6). A remarkable pericentrosomal clustering of mitochondria was observed in GFP-GMAP-210$_{mito}$ (Figure 6A) or GFP-Cter$_{mito}$ (Figure 6A) expressing cells, contrasting with the random distribution of mitochondria in nontransfected cells. Identical results were obtained with constructs containing an N-terminal myc tag instead of GFP (Figure 6A, bottom gallery).

The GA was profoundly disrupted in GFP-GMAP-210$_{mito}$-transfected cells. Golgi proteins such as GM130 showed an unusual spotty distribution within a huge cluster of mitochondria (Figure 6B). Pericentrosomal accumulation of mitochondria was not dependent on endogenous GMAP-210 since it occurs even in the presence of BFA (Figure 6B). On the contrary, GA morphology appeared normal in GFP-Cter$_{mito}$-transfected cells although surrounded by clustered mitochondria (Figure 6B). This clustering is maintained in the presence of BFA. Expression of GFP-GMAP-210$_{mito}$ induced changes of the MT network similar to those produced by overexpression of full-length GMAP-210 at the Golgi surface (Figure 6A, bottom gallery). Remarkably, there was a correlative effect on viability: cell survival was much better for GFP-Cter$_{mito}$-transfected cells than for GFP-GMAP-210$_{mito}$-transfected cells (not shown). Finally, the existence of GMAP-210 C terminus/γ-tubulin complexes on the mitochondrial surface was demonstrated by IP with either anti-c-*myc* or anti-γ-tubulin antibodies on cells expressing c-*myc*-Cter$_{mito}$ (Figure 6C).

Perturbation of MT network with either NZ (Figure 6D, left panel) or taxol (Figure 6D, middle panel) led to a redistribution of mitochondria throughout the cytoplasm in both GFP-GMAP-210$_{mito}$ (Figure 6D) and GFP-Cter$_{mito}$-transfected cells (not shown). In NZ-washout experiments, mitochondria returned to the cell center in transfected cells; this return indicates that the integrity of MT system is responsible for mitochondrial clustering. However, at high expression levels of mitochondria-targeted GMAP-210 (Figure 6D, right panel) or C-terminal variants, a significant number of mitochondria remained in a pericentrosomal location. We conclude that GMAP-210 contains the information to localize Golgi membranes around the centrosome.

Discussion

In this work, we report that the *cis*-Golgi network-associated protein GMAP-210 recruits γ-tubulin to Golgi membranes and is required for proper positioning and biogenesis of the GA. Our data demonstrate that, in addition to the centrosome, Golgi membranes represent in vivo docking sites for γTCCs. These Golgi-associated γTCCs control the assembly or stabilization of a subset of MTs that could be used to facilitate Golgi ribbon formation at the periphery of the centrosome (see below). These membrane-associated MTs could be either assembled from newly generated MT seeds that are released from the centrosome (Abal et al., 2002) and captured on the Golgi membranes, as suggested by MT regrowth experiments in GMAP-210-transfected cells, or directly nucleated on membranes.

GMAP-210 Is Required for Golgi Ribbon Formation

The process by which incoming membranes are incorporated into the *cis*-Golgi or undergo homotypic fusion to generate the first Golgi cisternae only begin to be understood. To our knowledge, the only matrix protein that contributes at this early and critical step of Golgi biogenesis is golgin-84 (Diao et al., 2003). Our present and previous data indicate that GMAP-210 plays an important role in the formation of the Golgi ribbon. We previously showed that overexpression of GMAP-210 enlarges and fragments the Golgi ribbon, completely disassembles the stacked structure of the GA, and blocks both anterograde and retrograde transport from the *cis*-Golgi (Infante et al., 1999; Pernet-Gallay et al., 2002). We now report that depletion of GMAP-210 results in extensive breakdown of the Golgi ribbon into small elements that contain Golgi resident proteins and remain loosely packed at the cell center, close to the nucleus. This phenotype, i.e. a fragmentation of the Golgi ribbon without extensive dispersion of elements to the cell periphery, indicates that the role of GMAP-210 must be expected in both the maintaining of membrane elements clustered around the centrosome and in their fusion once they are there (see Figure 7). Noteworthy, cells inactivated for GMAP-210 respond to BFA but are then unable to reform a Golgi ribbon after BFA washout. Notably also, GA from cells without GMAP-210 do respond to MT perturbation by taxol or NZ; thus dynein can translocate membranes without GMAP-210 and the recycling of membranes through the RE can also take place in the absence of GMAP-210. Altogether these data strongly support a role for GMAP-210 in promoting the fusion of incoming membrane elements to the *cis*-Golgi or to each other for generating the first cisternae of the *cis*-Golgi.

Comparing phenotypes induced by dynein inactivation, or MT disassembly, and by GMAP-210 depletion suggests that MTs participate in two separate mechanisms for Golgi biogenesis: (1) a dynein-dynactin-depen-

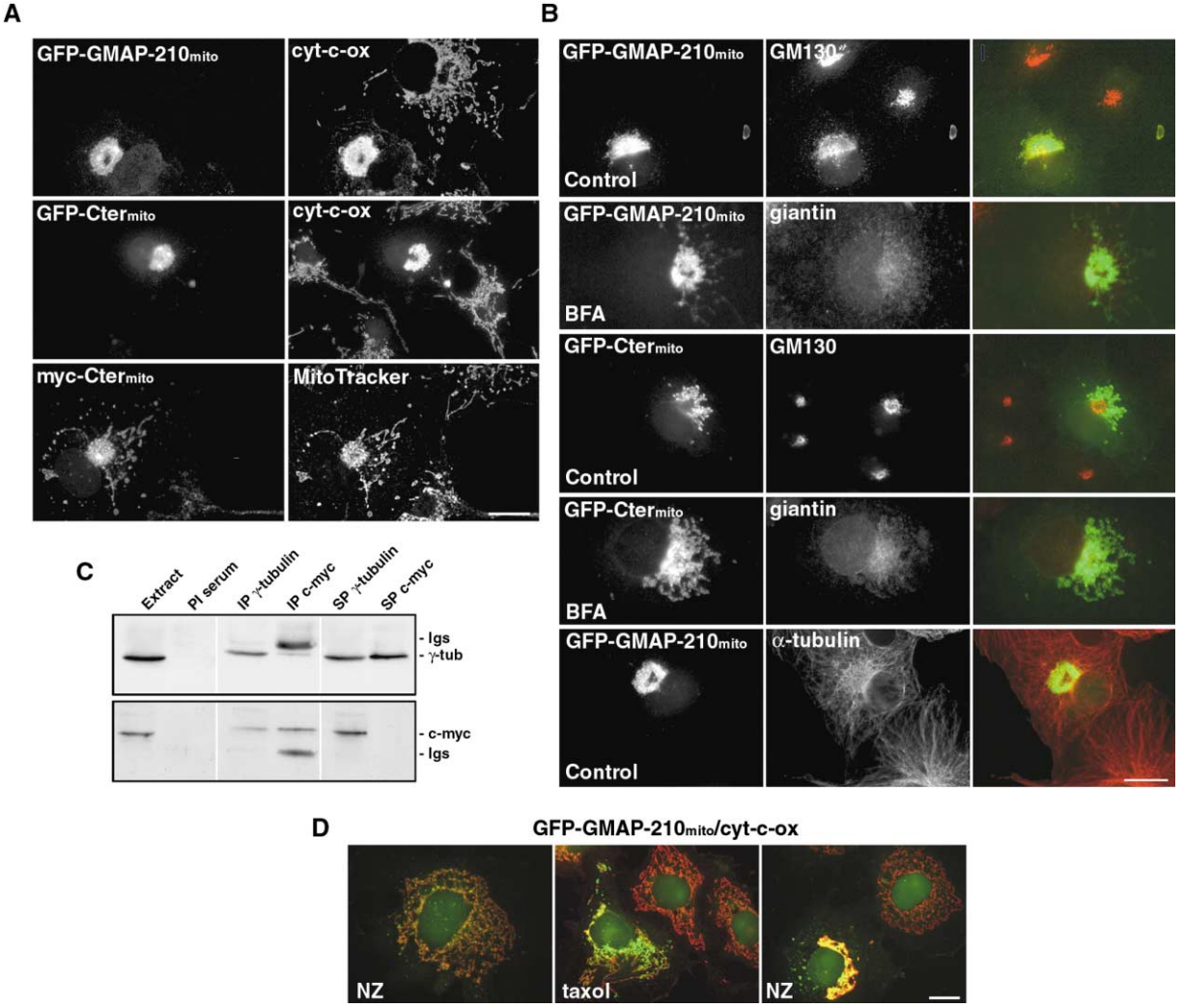

Figure 6. GMAP-210 Participates in the Pericentrosomal Localization of the GA via Its C-Terminal Domain

(A) Full-length GMAP-210 or its C-terminal domain were cloned in fusion with the ActA hydrophobic membrane anchor and tagged with GFP (GFP-GMAP-210$_{mito}$ or GFP-Cter$_{mito}$) or c-*myc* (myc-Cter$_{mito}$). Cells transiently transfected with GFP-GMAP-210$_{mito}$ or GFP-Cter$_{mito}$ were stained with anti-cytochrome-c-oxidase antibody as a mitochondrial marker. In bottom gallery, cells transfected with a vector encoding for myc-Cter$_{mito}$ were double stained with anti-c-*myc* antibody and MitoTracker™ for visualizing mitochondria. Bar, 10 μm.

(B) GFP-GMAP-210$_{mito}$ or GFP-Cter$_{mito}$ overexpressing cells, treated or not with BFA, and stained with the Golgi markers GM130 or giantin as indicated. Merged images are shown at right. A single cell overexpressing GFP-GMAP-210$_{mito}$ stained for α-tubulin is shown in the bottom gallery. Note the disaparition of the MT aster. Bar, 10 μm.

(C) Lysates from Cos-7 cells expressing myc-Cter$_{mito}$ were immunoprecipitated with a preimmune serum, a polyclonal anti-γ-tubulin antibody, or a monoclonal anti-c-*myc* antibody as indicated. After SDS-PAGE, Western blots were probed with monoclonal anti-γ-tubulin and anti-c-*myc* antibodies. The input lysate (Extract) and supernatants (SP) are indicated.

(D) GFP-GMAP-210$_{mito}$ overexpressing cells treated with NZ or with taxol and stained with anti-cytochrome-c-oxidase. Bar, 10 μm.

dent translocation of pre-Golgi elements from the cell periphery toward the cell center involving centrosome-anchored astral MTs and (2) GMAP-210-dependent fusion of these Golgi elements, involving another subset of MTs and generating a single ribbon around the centrosome (see Figure 7).

GMAP-210 Ensures the Pericentrosomal Location of the GA

A striking result of this work arose from mitochondrial targeting experiments, which indicate that the GMAP-210/γ-tubulin complex is involved in the centrosomal location of the GA. Mitochondria covered with GMAP-210 accumulated γ-tubulin and localized around the centrosome in a MT-dependent and Golgi-independent manner. The 20 kDa C terminus of GMAP-210 was apparently sufficient to induce perinuclear clustering of mitochondria.

MT-dependent movement of mitochondria is achieved by kinesin-like motors and by dynein. Perinuclear clustering of mitochondria has been described in several conditions in which kinesin-like motor activity is inhibited so the minus end-directed transport predominates (De Vos et al., 2000; Rodionov et al., 1993; Tanaka et al., 1998). Interestingly, overexpression of the MT binding protein Tau also caused mitochondrial collapse (Ebneth

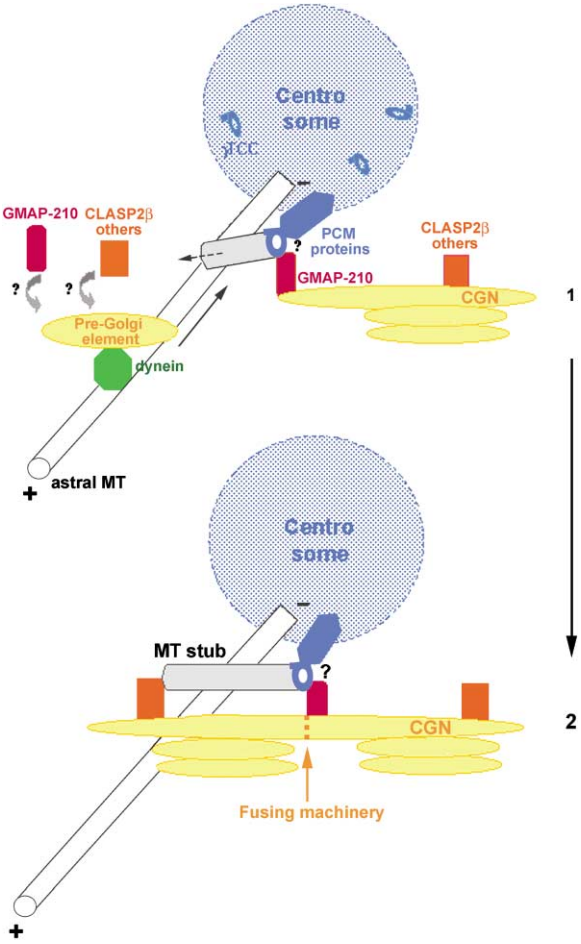

Figure 7. GMAP-210 Links *cis*-Golgi Biogenesis to MT Stabilization

A working model on how the coordinated activities of dynein-dynactin and GMAP-210 could control the Golgi ribbon formation around the centrosome: (1) Pre-Golgi structures are translocated toward the cell center using astral MTs and the dynein-dynactin complex. CGN-associated GMAP-210 would directly or indirectly (via γTCCs) bind to the pericentriolar material. (2) Close to the centrosome, where MT seeds are formed and released, GMAP-210 could capture them and facilitate the local growth of MTs, which would then favor *cis*-Golgi ribbon formation at the cell center by bridging pre-Golgi elements to membranes. GA-associated MT plus end binding proteins, such as CLASP2β, would contribute to plus ends stabilization of Golgi-anchored MTs and so to the generation of a subpopulation of stable and short MTs associated to Golgi membranes.

et al., 1998) indicating that, in addition to motors activities, organelle transport involves regulation of static MT binding activities that could act as brakes or anchors. The GMAP-210-mediated binding of mitochondria to MT minus ends could indeed impair their plus end-directed transport to the cell periphery. At the Golgi surface, a similar static link could interfere with retrograde membrane movements powered by kinesin contributing to the maintenance of the Golgi structure and to the pericentrosomal steady-state localization of the GA.

GMAP-210 Links Golgi Biogenesis with Pericentrosomal Localization

A picture emerges in which biogenesis of the Golgi ribbon at the cell center is accomplished by the coordinated activities of dynein-dynactin and GMAP-210 (Figure 7). Pre-Golgi structures are translocated toward the centrosome using astral MTs and the dynein-dynactin complex. CGN-associated GMAP-210 could bind to the pericentriolar material either directly or indirectly via γTCC since some PCM proteins such as pericentrin or AKAP450 are themselves γTCC-interacting proteins (Dictenberg et al., 1998; Takahashi et al., 2002). Close to the centrosome, where MT seeds are formed and released, GMAP-210 could capture them and facilitate the local growth of MTs that would then favor *cis*-Golgi ribbon formation at the cell center by bridging pre-Golgi elements to membranes. GA-associated MT plus end binding proteins, such as CLASP2β (Akhmanova et al., 2001), would contribute to plus ends stabilization of Golgi-anchored MTs and so to the generation of a subpopulation of stable and short MTs associated to Golgi membranes (Marsh et al., 2001).

Whether GMAP-210 participates directly in membrane fusion, through its long coiled-coil that would confer properties similar to those exhibited by some golgins, will deserve further study. Supporting this possibility is our observation that mitochondria carrying GMAP-210 in their surface, but not those carrying a truncated mutant lacking the long coiled-coil domain, profoundly perturbed the GA morphology as well as markers distribution and markedly impaired cell viability. We have attempted to analyze the behavior of ΔC-GMAP-210 targeted to mitochondria. Unfortunately, this construct led to mitochondria aggregates that precluded definitive conclusions. Future studies will aim at elucidating how molecular machineries controlling membrane fusion and MT anchoring are coordinated in order to ensure the correct structure and localization of the GA.

Finally, the range of signaling events occurring at both the GA and the centrosome is quickly increasing. A recent work reveals that spindle pole body-associated γ-tubulin plays an essential role in the coordination of mitotic events in *A. Nidulans* in addition to its role in MT nucleation (Prigozhina et al., 2004). One can speculate that Golgi-associated γ-tubulin could play a similar role. Early in mitosis, association of the GA with MT minus ends is lost and the GA disassembles. Inhibition of GA fragmentation has been reported to block mitotic progression (Sutterlin et al., 2002) or to produce gross defects in chromosome segregation and cytokinetic furrow ingression (Altan-Bonnet et al., 2003). It should be also rewarding to look at the role of GMAP-210 in postmitotic reformation of the GA.

In conclusion, the long-described subset of stable MTs present in the Golgi area, distinct from the aster of cytoplasmic MTs, would be directly involved in GA biogenesis and activity. By providing Golgi membranes with a mechanism that anchors MT minus ends at the centrosome boundary, GMAP-210 appears as a key protein for linking the biogenesis of the Golgi ribbon with its location at the cell center.

Experimental Procedures

Cell Culture, Drug Treatments, and Transfection

HeLa and Cos-7 cells were cultured in DMEM containing 10% FCS. PtK and PK γ-tubulin-GFP expressing cells were cultured as described (Khodjakov and Rieder, 1999). Cells were treated with either 10 μM NZ (Sigma) or 5 μM Paclitaxel™ (Sigma) for 2–5 hr at 37°C

before fixation. BFA (Sigma) treatment was with 5 μg/ml for 1 hr at 37°C. Cos-7 cells were transfected by electroporation as described (Infante et al., 1999). PK and PtK cells were transfected with Lipofectamine2000 (Life Technologies) according to manufacturer's instructions. SDS-PAGE, immunoblotting, cell lysis, and IP were performed as described (Infante et al., 1999).

Antibodies and SPOT Analysis

Rabbit anti-GMAP-210, anti-detyrosinated tubulin, anti-γ-tubulin γT4D, and anti-GCP3 antibodies have been described (Infante et al., 1999; Kreis, 1987; Tassin et al., 1998). For SPOT technique-based experiments, a total of 146 overlapping 15-mer amino acid peptides frameshifted by three residues and representing the complete γ-tubulin sequence were synthesized and probed with γT4D antibody as described (Llanos et al., 1999).

Monoclonal anti-α-tubulin, anti-γ-tubulin (clone GTU-88), and anti-c-*myc* (clone 9E10) antibodies were purchased from Sigma. Monoclonal anti-GM130, anti-GMAP-210, and anti-cytochrome-c-oxidase were from BD Biosciences. Anti-HA monoclonal antibody was from Boehringer Mannheim Corp. (USA) and anti-GFP monoclonal antibody from Clonthec. Monoclonal anti-giantin antibody was a gift of H.P. Hauri (Switzerland). The medial Golgi marker CTR433 has been previously characterized (Jasmin et al., 1989). Specific antibodies for GalTf and Sec31 were generous gifts of E. Berger and W. Hong. Anti-IgG peroxidase-labeled secondary antibodies were from Amersham. Secondary antibodies conjugated to Alexa fluorophores were from Molecular Probes.

Generation of Constructs

pECE-GMAP-210 and pECE-ΔC-GMAP-210 (ΔC1778) were as described (Infante et al., 1999). GMAP-210 and ΔC-GMAP-210 were also subcloned into pGEM1 and pEGFP-C1 vectors. C-terminal domain of GMAP-210 (aa 1778–1979) was subcloned in pEGFP-C1 (GFP-Cter), in pECE containing an HA tag (HA-Cter), and in pcDNA3 with upstream c-*myc* tag (c-*myc*-Cter) vectors. For mitochondria targeting, the sequence encoding the C-terminal hydrophobic anchor of ActA (a gift of P. Cossart, France) was added at the C terminus of both GFP-GMAP-210 and GFP-Cter. pCB6-HA-dynamitin was a gift of Dr. T. Schroer.

Cell Extraction and Golgi Purification

For immunofluorescence experiments, Cos-7 cells grown in coverslips were extracted with 50 μg/ml digitonin in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl for 5 min at 37°C, or with 0,02% saponin in PHEM buffer containing 0,02% BSA for 5–15 min at 37°C. After washing in the same buffer without detergent, extracted cells were processed by IF or re-extracted with NP-40 solution (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40) for 1 min at 37°C before fixation. For biochemical experiments, 2×10^7 cells were incubated with 1 ml of digitonin or saponin prepared as described above but containing protease inhibitors (1 mM PMSF and 1 μg ml^{-1} of pepstatin, leupeptin, and aprotinin) for 5 min at room temperature. Saponin- or digitonin-soluble fractions were pooled and extracted cells were washed with buffer alone. Then extracted cells were incubated with 1 ml of NP-40 buffer containing protease inhibitors. NP-40-insoluble fraction was solubilized with Laemmli sample buffer.

Fractions enriched in Golgi membranes were isolated from HeLa cells by flotation in a sucrose gradient as described (Infante et al., 1999).

Sucrose Gradient Analysis

Six hundred microliters of NP-40-soluble fraction obtained from saponin-extracted cells was loaded on the top of a 2.4 ml 15%–40% continuous sucrose gradient. The gradient was run at 100,000 g for 16 hr at 4°C. Two hundred microliter fractions were collected from the top. An aliquot of each fraction was diluted in sample buffer and boiled. The rest of the fraction was frozen.

RNA Interference

RNAi was performed on HeLa cells using Oligofectamine (Life Technologies) with duplex RNA oligos (Proligo) for 1–3 days as described by Elbashir et al. (2001). GMAP-210 was targeted with the sequences AAGATGCAACAATTAGAACTC (oligo4077) or AAGAATGTCTTGCT GGTAACA (oligo2079). GMAP-210 levels were quantitated from Western blots as described (Pernet-Gallay et al., 2002).

Fluorescence Microscopy

Cells were grown in coverslips and fixed in 100% methanol at −20°C for 6 min. For GFP fluorescence, cells were fixed in 2% or 4% paraformaldehyde for 10 min at room temperature and permeabilized with either acetone at −30°C for 3 min or 0.15% saponin in PBS containing 10% FCS. Where necessary, 500 nM MitoTracker™ Green (Molecular Probes) was added to culture medium for 30 min to stain mitochondria. Epifluorescence microscopy was performed using a Leica microscope and a chilled CCD camera.

Acknowledgments

We thank H.P. Hauri, E. Berger, and W. Hong for antibodies, P. Cossart and T.A. Schroer for ActA and HA-dynamitin constructs, and A. Khodjakov for γ-tubulin-GFP-expressing cell lines. We also thank D. Job for collaborative work on SPOT experiments, R. Jimenez for technical support, F. Perez and B. Goud for ongoing discussion, and P. Chavrier and B. Oakley for critical reading of the manuscript. This work was supported by grants from the Ministerio de Ciencia y Tecnología, Spain to C.F. (CICYT PM99-0143) and to R.M.R. (CICYT BMC2003-00800) and from the Human Frontier Science Program Organization RG0319/1999-M to M.B. R.M.R. is financed by the Ramon y Cajal program from the MCYT, Spain.

Received: December 2, 2003
Revised: May 28, 2004
Accepted: June 1, 2004
Published: August 5, 2004

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