Golgi structural stability and biogenesis depend on associated PKA activity

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Summary

The mammalian Golgi complex consists of stacks of cisternae linked laterally into a continuous perinuclear ribbon structure. Protein kinase A is stably associated with the Golgi complex during interphase. To analyze its role in Golgi structural maintenance cells were depleted of protein kinase A regulatory subunits using small interfering RNAs. Under these conditions, the catalytic subunits redistributed to the cytosol and the entire Golgi complex underwent disassembly into multiple juxtanuclear fragments. A similar effect took place following pharmacological inhibition or redistribution of the complete holoenzyme to the cytosol. Golgi fragments maintained their polarization and competence for anterograde protein trafficking. By electron microscopy, they were identified as whorl-like structures composed of concentrically arrayed cisternae. To test a possible role of protein kinase A in Golgi biogenesis we analyzed its involvement during Golgi reassembly from the endoplasmic reticulum. In cells incubated with protein kinase A inhibitors, Golgi reconstruction was arrested at a late step of the reassembly process. This is consistent with the stage of enzyme recruitment from cytosol to emerging Golgi membranes during the reassembly process. We conclude that protein kinase A activity plays a relevant role in the assembly and maintenance of a continuous Golgi ribbon from separated membrane stacks.

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Key words: Golgi complex, Protein kinase A, AKAP

Introduction

The unique compartmentalized organization of the Golgi complex in mammalian cells is thought to provide optimal structural support for the ordered, sequential processing of molecules along the exocytic pathway. The main part of the Golgi consists of stacks of flat cisternae whose membranes are crowded with enzymes and receptors. During their journey across the stacked cisternae, newly synthesized proteins and lipids are subjected to extensive modifications and are finally sorted for delivery to different cellular destinations (Dunphy and Rothman, 1985). Membrane-associated coiled-coil proteins form a crosslinked matrix that is responsible of cisternae stacking (Barr and Short, 2003). In addition, the stacks of cisternae are laterally linked by tubular connections and this gives the Golgi complex the appearance of a continuous juxtanuclear ribbon (Ladinsky et al., 1999).

Golgi structural integrity depends on export from the endoplasmic reticulum (ER). During their life span, Golgi transmembrane proteins such as glycosyltransferases and receptors continuously travel to the ER and re-emerge to enter the Golgi again. The extent of ER recycling varies among proteins. Receptors and components of the transport machinery continuously cycle between the ER and the Golgi as required by their functional activity (Lee et al., 2004). Processing enzymes, however, constitutively cycle at a low rate (Storrie et al., 1998). The functional significance of their cycling is not so obvious but it may be important for both Golgi remodeling during interphase and partitioning of this organelle during mitosis (Altan-Bonnet et al., 2004; Altan-Bonnet et al., 2006; Ward et al., 2001). The existence of this pathway, however, is highlighted by experiments in which ER export is blocked by either pharmacological (i.e. brefeldin A, BFA) treatment or interfering with the assembly of the export machinery (Lippincott-Schwartz et al., 1989; Ward et al., 2001). In both cases, Golgi components redistribute to the ER as expected from the continuous Golgi-ER retrograde traffic. Export from the ER occurs at specialized subdomains known as ER-export sites whose organization depends on the association of the COPII coat transport complex to the ER membrane (Bannykh et al., 1996). They are considered to play an essential role in Golgi reassembly from the ER (Bevis et al., 2003). Thus, both Golgi transmembrane proteins and peripherally associated proteins such as components of the Golgi matrix become concentrated at the ER exit sites during Golgi outgrowth from the ER (Puri and Linstedt, 2003; Ward et al., 2001).

In addition to its established role in protein processing and sorting, the Golgi complex is increasingly recognized as a center for signal reception and transmission. This view considers the Golgi complex as a structural scaffold where different signal transduction pathways converge and are propagated to effectors located elsewhere in the cell (Donaldson and Lippincott-Schwartz, 2000). It would explain the presence of a significant number of regulatory molecules associated with the Golgi membranes such as G proteins, phospholipid and protein kinases, phospholipases, cytoskeleton motor proteins, etc. (reviewed by Altan-Bonnet

et al., 2004). Protein kinase A (PKA) is a well-known constituent of the Golgi complex (Nigg et al., 1985b). This tetrameric enzyme is associated with the cytosolic surface of Golgi membranes through the interaction of the dimer of regulatory RIIα subunits with a putative anchor protein, whereas the two catalytic Cα subunits are bound to RIIα. Previous work has revealed that Golgi-associated PKA activity regulates protein transport, particularly the formation of membrane intermediates for the anterograde and retrograde transport routes (Cabrera et al., 2003; Martín et al., 2000; Muñiz et al., 1996; Muñiz et al., 1997). Nevertheless, the functional significance of PKA association with the Golgi membranes is mostly unknown. For instance, although pioneer work by Nigg et al. (Nigg et al., 1985a) indicated that Golgi-associated PKA participates in the control of gene expression, its involvement in a particular signal transduction pathway has not yet been reported. Also, the role of PKA association in Golgi structural organization and biogenesis has not been investigated.

In this study, we have examined the effects derived from PKA enzymatic inhibition or displacement on Golgi structure. Our data indicate that Golgi maintenance depends on the associated PKA activity. Furthermore, we report that PKA is recruited from the cytosol during Golgi reassembly from the ER and that PKA inhibition interferes with Golgi reconstruction.

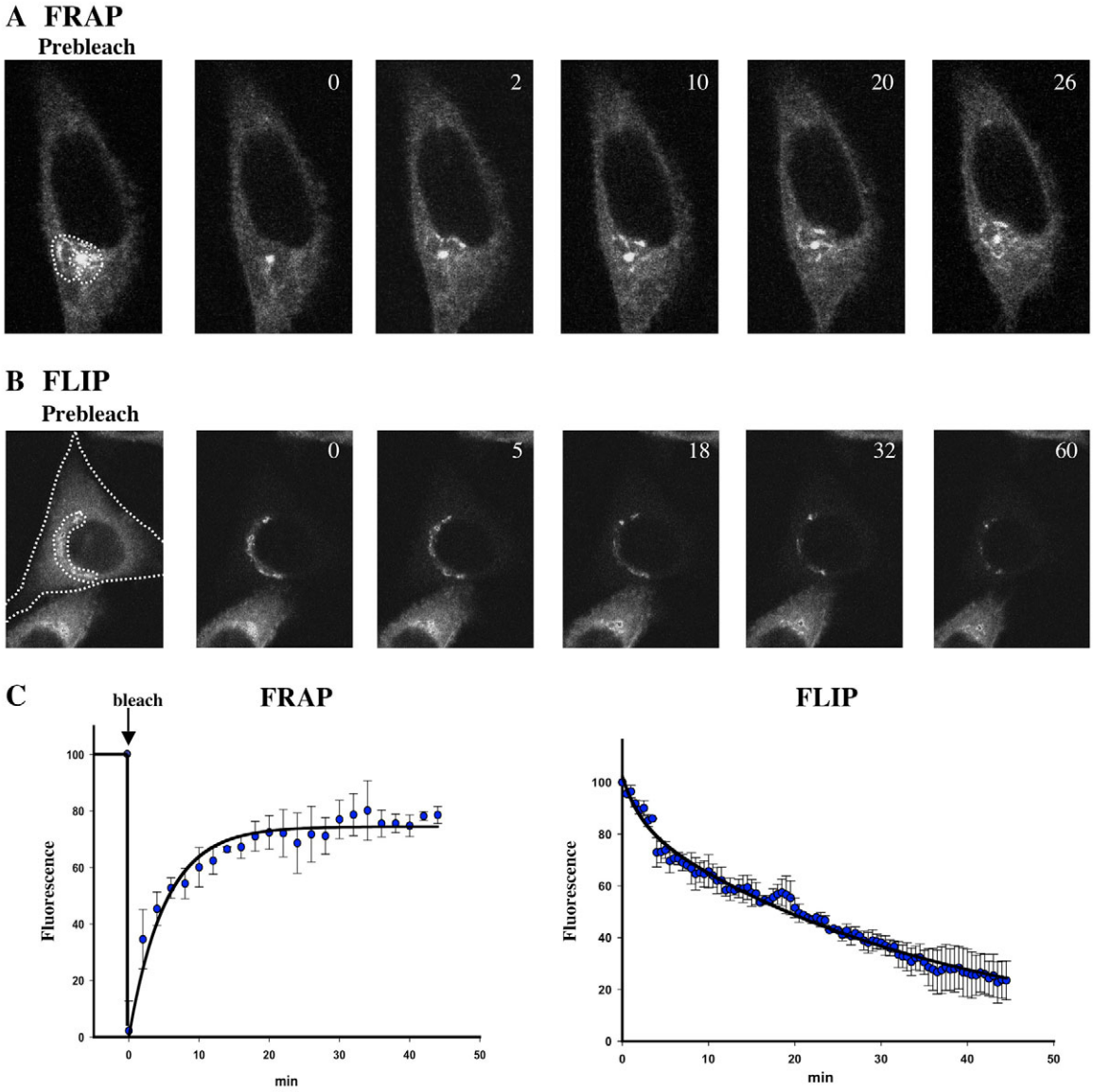

Fig. 1. Kinetics of RIIα binding to and dissociation from the Golgi. Cells stably expressing RIIα-GFP were photobleached with a high-intensity laser light and then the recovery of fluorescence was monitored by taking images at the indicated time points (in minutes). (A) The Golgi complex (outlined region) was subjected to a single bleach and recovery was analyzed by FRAP. Note that the centrosome was excluded from the bleached area. (B) The cytoplasm (area between the two encircled structures) was bleached repeatedly (every 4 minutes) to analyze RIIα-GFP loss from the Golgi by FLIP. (C) Time-course of the evolution of Golgi-associated fluorescence during both FRAP and FLIP. Values are the mean ± s.e.m. of three different determinations.

Results

Dynamics of Golgi-associated PKA

Living HeLa cells stably expressing RIIα-GFP were used in photobleaching experiments to determine the dynamics of Golgi-associated PKA. In these cells, RIIα-GFP was concentrated in the Golgi complex and the centrosome, and also dispersed throughout the cytoplasm, mirroring the localization pattern of the endogenous protein (Martin et al., 1999). PKA recruitment from cytosol to the Golgi membranes was analyzed by FRAP (Fig. 1A). Golgi fluorescence recovery occurred with a half-time of 3.65 minutes. This is in contrast with FRAP data obtained with γ-COP-YFP. Under similar experimental conditions this coat protein associated rapidly (half-time 35.47 seconds) to the surface of the Golgi membranes. This is in agreement with previous reports on coatomer recruitment (Presley et al., 2002). In the case of RIIα-GFP, Golgi fluorescence recovery was rather incomplete because only 73-80% of the initial, prebleach value was reached after prolonged incubations (>1 hour) (Fig. 1C). This suggested the existence of a significant (estimated to be 26%) immobile fraction of this protein permanently associated with the Golgi membranes. Furthermore, FLIP determinations also supported this view (Fig. 1B). PKA dissociation from the Golgi to the cytosol took place with a half-time of 21.3±0.3 minutes (Fig. 1C), which is also a high value for a peripheral Golgi protein. Taken together, these measurements indicate that many of the PKA molecules associated with the Golgi complex are locked at this location and did not exchange rapidly with the population of molecules that diffuse freely throughout the cytosol.

Effect of RIIα depletion on Golgi organization

To examine the effect of PKA displacement on Golgi structural organization we incubated cells with siRNA designed to specifically inhibit RIIα biosynthesis. We used two siRNAs corresponding to non-overlapping sequences in RIIα with similar efficiency in both cases. Results obtained with one of them (siRNA1) are shown in Fig. 2 and subsequent figures. Golgi depletion was effective after 72-80 hours and affected 70-90% of the cells as judged by immunofluorescence detection (Fig. 2A). Accordingly, during this treatment the total amount of RIIα decreased to 11% of control, untreated cells without significant decrease in the amount of the catalytic Cα subunit (Fig. 2B). In comparison to the Golgi complex, it took longer, at least 96 hours, for the centrosome to be completely deprived of RIIα. In RIIα-depleted cells, the Cα subunit was dissociated from the Golgi and appeared dispersed throughout the cytoplasm (Fig. 2A). Indeed, the amount of Cα associated with total microsomal membranes was reduced compared with untreated cells (Fig. 2C). No depletion was obtained when cells were incubated with a control siRNA designed to target the rodent form of RIIα (Fig. 2A). Therefore, incubation with siRNA specific for human RIIα induced the redistribution of PKA catalytic activity from the Golgi complex to cytosol.

To evaluate the effect of RIIα depletion on Golgi organization we used cells stably expressing a truncated version of galactosyltransferase tagged with CFP. In preliminary experiments, this construct was shown to exhibit a widely extended localization pattern across the Golgi stack. Thus, significant colocalization was observed with endogenous protein markers from both the *cis*- (giantin, GM130) and *trans*-Golgi network (TGN46) compartments (Fig. 3A). Therefore the galactosyltransferase-CFP fluorescent pattern allowed us to examine the fate of most Golgi membranes after treatment with siRNA. In contrast to the normal Golgi ribbon seen in cells transfected with a control siRNA, the Golgi complex of cells depleted of RIIα by transfection with the appropriate siRNA appeared fragmented into multiple discrete elements (Fig. 3A). For the most part these fragments remained clustered in the perinuclear region of the cytoplasm. Fragmentation affected all Golgi compartments and occurred in >90% of RIIα-depleted cells whereas a similar effect was observed in <3% of non-depleted cells. Quantitative image analysis indicated that although total Golgi fluorescence was comparable in RIIα-depleted and non-depleted cells, the average number of Golgi fragments per cell increased fivefold in the former (Fig. 3B).

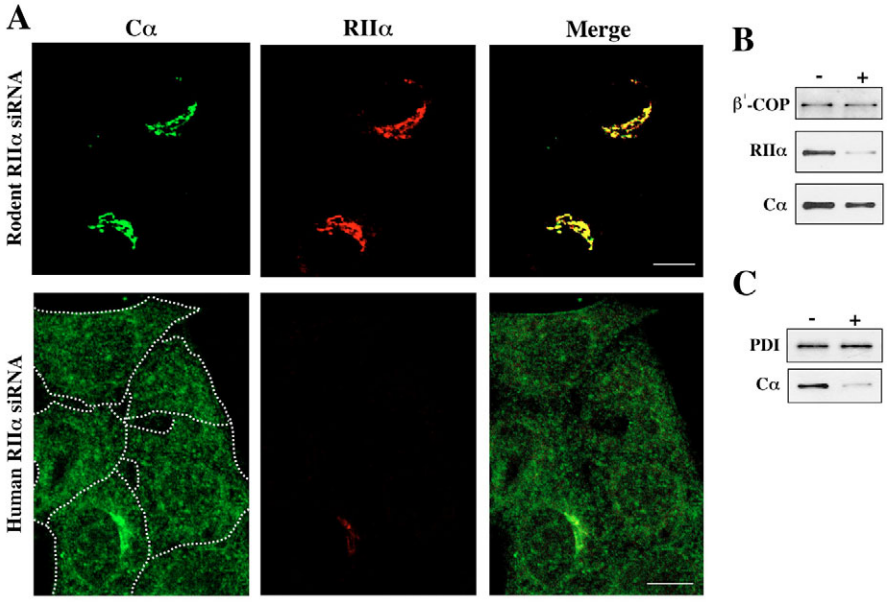

Fig. 2. RIIα depletion by siRNA treatment. HeLa cells were incubated (+) or not (–) for 72 hours with an siRNA sequence specific for human RIIα. Alternatively, they were incubated with a control siRNA specific for the rodent form of this protein. (A) Cells were fixed and processed for immunofluorescence with antibodies specific for RIIα and Cα. Cells incubated with siRNA specific for human RIIα were outlined in the image corresponding to Cα immunostaining. Bars, 10 μm. (B) Cells were lysed and processed for immunoblotting to detect both RIIα and Cα. β′-COP was detected to normalize the amount of protein loaded. (C) Cells were homogenized and total microsomal membranes were prepared. Membranes were lysed and processed for immunoblotting to detect Cα. In this case, protein disulfide isomerase (PDI) was used for normalization.

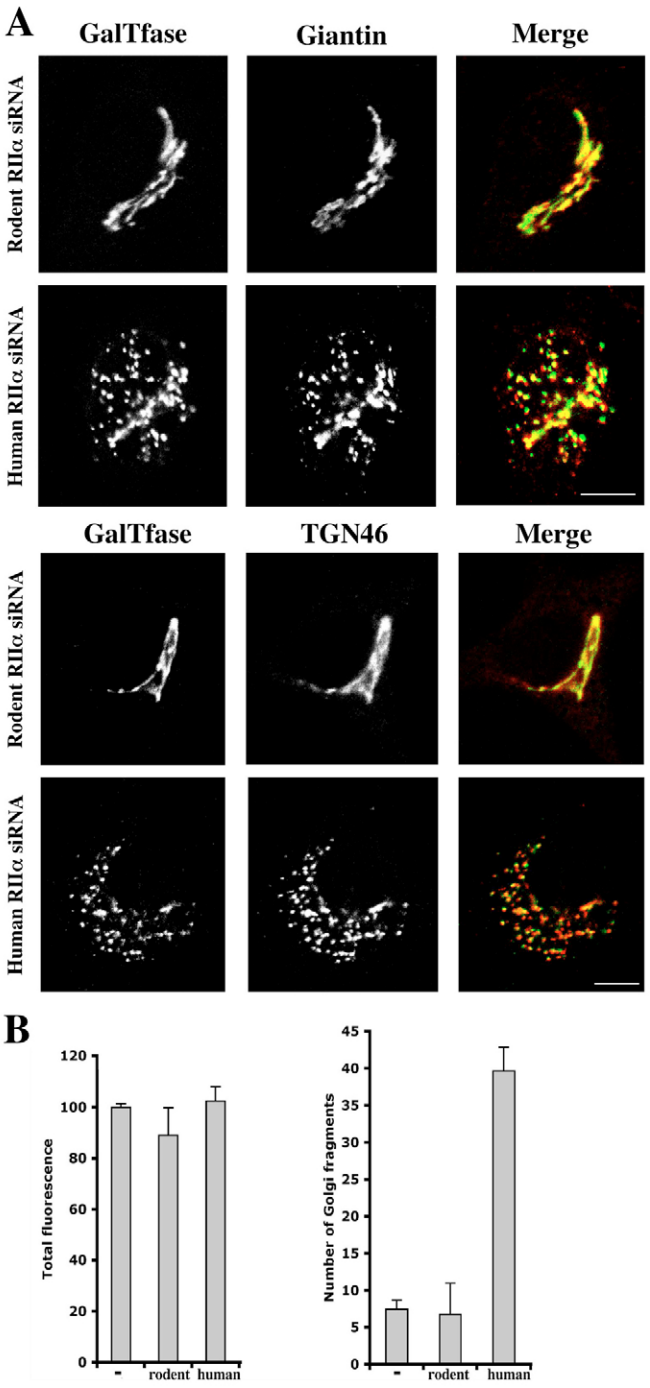

Fig. 3. Golgi disassembly induced by RIIα depletion. HeLa cells stably expressing galactosyltransferase-CFP (GalTfase) were incubated for 80 hours with siRNA designed to inhibit the synthesis of either the human or the rodent form of RIIα. (A) Cells were fixed and processed for indirect immunofluorescence with antibodies against the indicated Golgi proteins. Bars, 10 μm. (B) Golgi-associated fluorescence and the average number of Golgi fragments per cell was determined from cells (*n*=12) expressing GalTfase-CFP and incubated or not (–) with either a control siRNA against the rodent form of RIIα or, alternatively, an siRNA against the human version of this protein.

Staining with an antibody against α-tubulin showed that the microtubule network was mostly unaffected in RIIα-depleted cells (not shown). Furthermore, cells were viable. Particularly, no indication of apoptosis could be appreciated by DNA (Hoechst 33342) staining. Cell adhesion, however, seemed to be hindered because many cells became rounded and detached from the substratum during siRNA treatment.

The nature of Golgi fragments originated during RIIα depletion was investigated by electron microscopy. The typical organization of the Golgi complex in interphase cells consisting of stacks of flattened, elongated cisternae and associated vesicles was replaced in RIIα-depleted cells by an array of globular membranous structures (Fig. 4A). Most of them were found concentrated in the perinuclear region of the cell but apparently they were not connected to each other. Each of these structures consisted of tightly packed curved and partly swollen cisternae and associated vesicles (Fig. 4B). Quite often they show a whorl appearance, with different cisternae arranged concentrically around a centre occupied by vesicles and tubulovesicular structures (Fig. 4C,D). Some of these membranes were found to be positive for HPA-HRP staining indicating that they were derived from the *cis*-Golgi compartment (not shown). By contrast, the structural organization of other organelles such as the ER, mitochondria, and nuclear envelope was not affected extensively by RIIα depletion (Fig. 4A).

Polarization of Golgi fragments, on the other hand, persisted after disassembly. Thus, not only were protein markers from the different Golgi compartments present in the fragments but they also remained relatively segregated. For instance, the degree of overlapping between *cis*-Golgi (giantin) and TGN (TGN46) markers seen in the Golgi fragments of RIIα-depleted cells was comparable to that of the intact organelle in control, untreated cells (Fig. 5). Indeed, most fragments showed both *cis* and *trans* sides with little or no overlapping (Fig. 5, inset). Additionally, Golgi fragments remained functional in terms of protein transport to the plasma membrane. Thus, the intracellular transport of an anterograde cargo such as the G glycoprotein of the envelope of the vesicular stomatitis virus (VSV-G) was not affected. In particular, this protein reached the plasma membrane with a similar kinetics in both control and RIIα-depleted cells (supplementary material Fig. S1).

Effect of PKA displacement on Golgi organization

In principle, Golgi disassembly would be triggered by either the absence of RIIα or Cα redistribution. To distinguish between these two possibilities we analyzed the effect of expressing a soluble protein designed to bind RIIα with high affinity. We expected such a protein would trap RIIα and cause redistribution of the complete PKA holoenzyme from the Golgi to cytosol. In eukaryotic cells, PKA targeting to different subcellular structures including the Golgi complex occurs throughout interaction of the RII regulatory subunits with A-kinase anchoring proteins (AKAPs) (Wong and Scott, 2004). Ht31 is a peptide sequence that contains the RII-binding motif of AKAP-Lbc and has been previously used as an effective antagonist of any AKAP-RII interaction (Carr et al., 1992; Rosenmund et al., 1994). As a probe, we used a fusion protein formed by the Ht31 sequence fused to GFP (Alto et al., 2003).

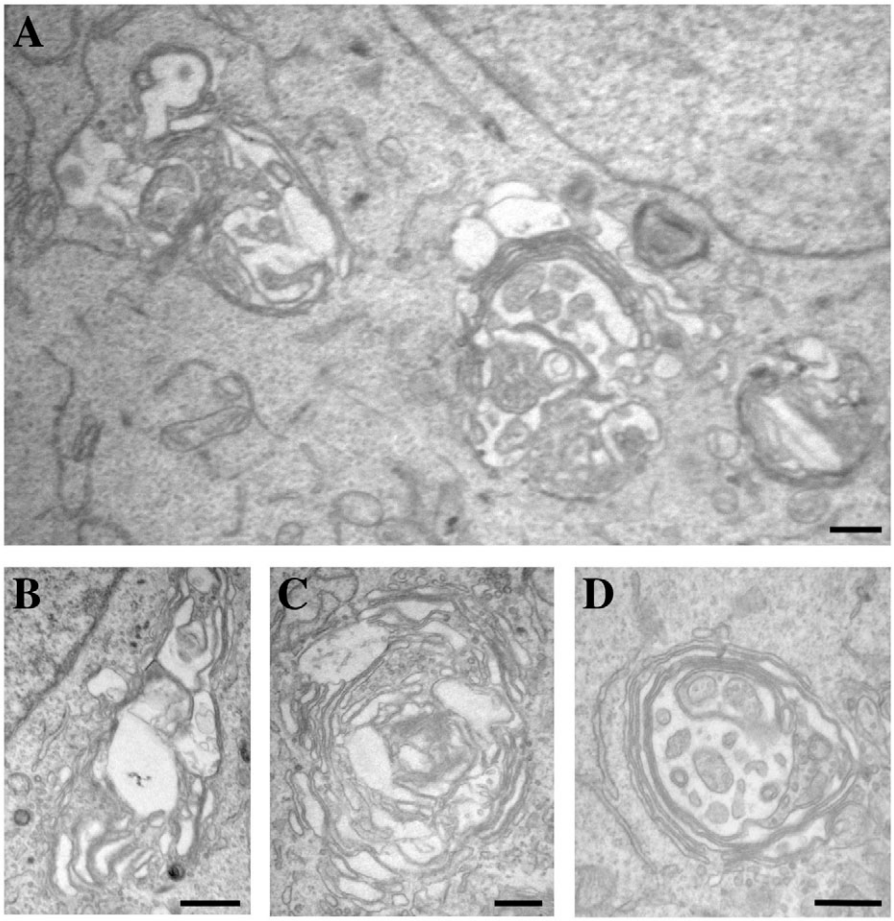

Fig. 4. Ultrastructural changes in Golgi organization during RIIα depletion. HeLa cells were plated on a dish containing a single coverslip and incubated for 80 hours with siRNA specific for human RIIα. The efficiency of depletion in the culture was first evaluated by immunofluorescence detection of RIIα in cells attached to the coverslip. The rest of the cells were then fixed and processed for transmission electron microscopy. (A) General view of the cytoplasm of RIIα-depleted cells. (B-D) Different Golgi stacks are shown. They appear as whorls composed of multiple concentrically arrayed cisternae. Cisternae appear dilated and fragmented. Bars, 0.5 μm.

As anticipated, cells expressing this construct showed most PKA redistributed to the cytosol (Fig. 6A). However, depending on the level of expression of Ht31-GFP, the Golgi complex was more or less deprived of RIIα. Yet this organelle underwent collapse and appeared fragmented in most (72%, *n*=87) cells. As in the case of RII-depleted cells, Golgi fragments (average 33 per cell, Fig. 6B) remained clustered in the perinuclear area and contained protein markers from all the Golgi compartments.

It was previously reported that interaction of Ht31 with RIIα could be abolished by preventing amphipathic helix formation, which can be achieved by proline substitution of a crucial isoleucine residue (Hausken et al., 1994). Therefore, as a control, we used the corresponding mutated form of the GFP fusion protein (designated Ht31P-GFP) in our experiments. Irrespective of the level of expression of this construct, RIIα remained associated with the Golgi complex. Furthermore, no change in Golgi structural

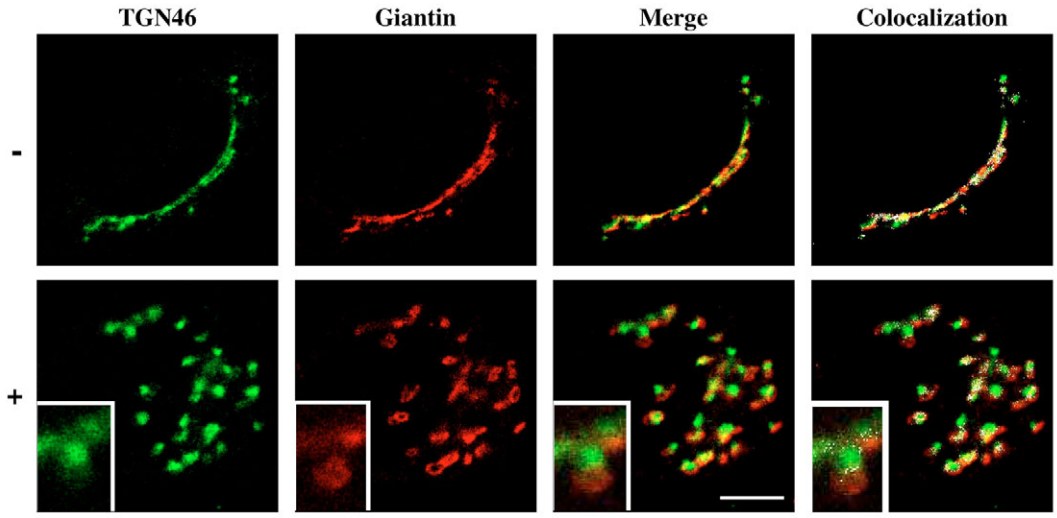

Fig. 5. Assessment of Golgi polarization after disassembly. Non-transfected HeLa cells were incubated (+) or not (–) for 80 hours with siRNA designed to inhibit the synthesis of RIIα. They were fixed and processed for indirect immunofluorescence with antibodies against the indicated endogenous Golgi proteins: giantin to label the *cis*-Golgi compartment and the TGN marker TGN46. Areas of colocalization (<10% total pixels) are white in the colocalization image. Bar, 10 μm.

organization was noted in cells overexpressing it (Fig. 6A). In summary, these results indicated that Golgi breakdown is the consequence of PKA catalytic activity redistribution rather than RIIα depletion.

Golgi disassembly induced by PKA inhibition

We next tested whether PKA inhibition would induce a similar effect to that caused by enzyme redistribution. We incubated cells with inhibitors of PKA catalytic activity and looked at the structure of the Golgi complex by immunofluorescence. As shown in Fig. 7A, treatment with either H89 or myristoyl-PKI induced Golgi disassembly. The former is a broad-range kinase inhibitor although at low dose it shows selectivity towards PKA. By contrast, PKI is a potent and specific peptide inhibitor of PKA catalytic subunit. Cells incubated with a low (5-20 μM) concentration of either inhibitor exhibited moderate Golgi fragmentation corresponding to 17-20 fragments per cell. Extensive Golgi fragmentation (35-47 fragments per cell) took place in cells exposed to an elevated (30 μM or higher) concentration of H89 whereas no further increase in Golgi fragmentation was observed with PKI (Fig. 7B). In order to strictly evaluate the role of PKA in the process, both inhibitors

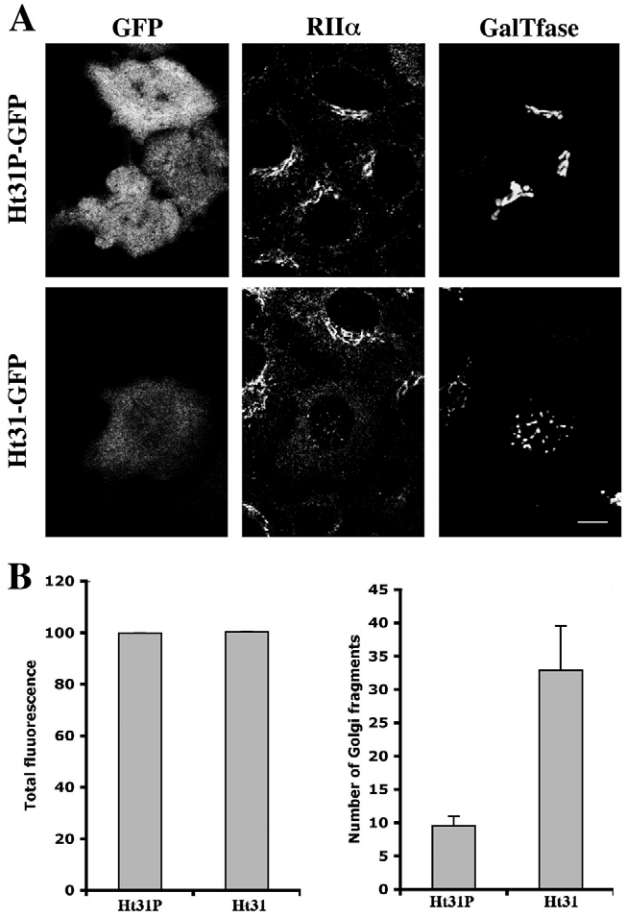

Fig. 6. Golgi disruption caused by PKA displacement. (A) Cells stably expressing galactosyltransferase-CFP (GalTfase) were transfected with cDNA encoding either a wild-type Ht31-GFP construct or the mutated form Ht31P-GFP. 72 hours post transfection, they were fixed and processed for immunofluorescence with anti-RIIα antibody. Bar, 10 μm. (B) Cells expressing the indicated Ht31-GFP construct were fixed and processed for immunofluorescence with anti-giantin antibody. Golgi-associated fluorescence and the average number (± s.e.m.) of Golgi fragments per cell were determined (*n*=12).

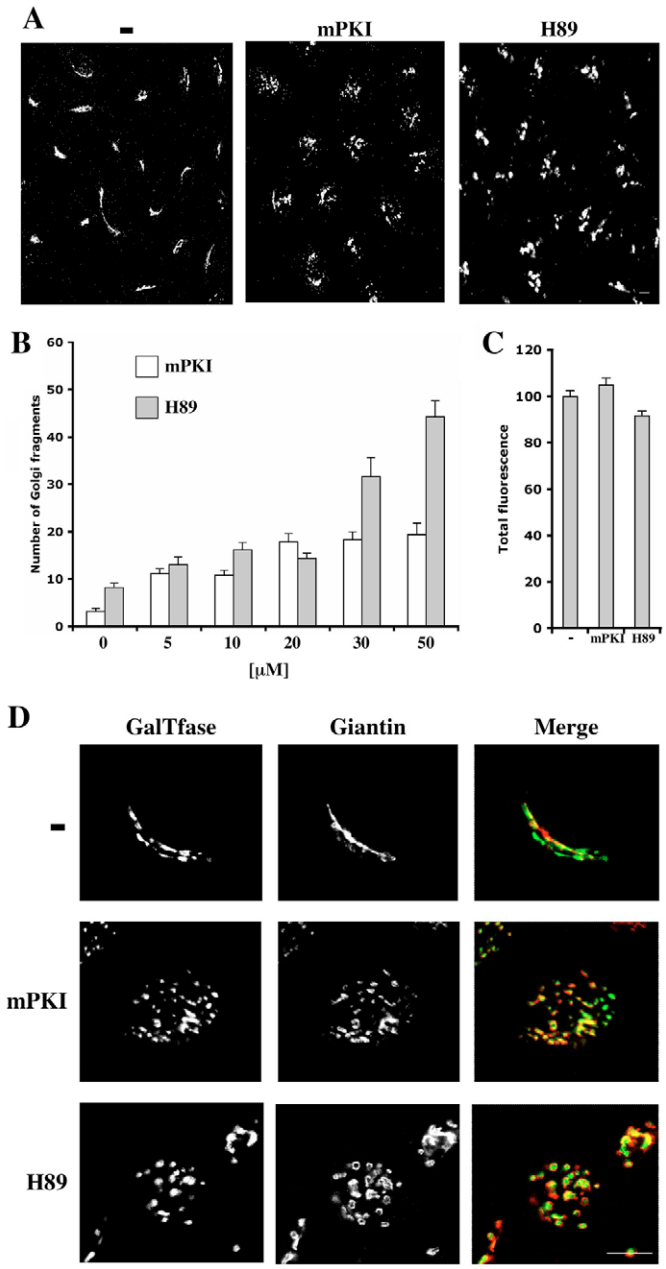

Fig. 7. Golgi disruption caused by treatment with PKA inhibitors. (A) Non-transfected cells were incubated or not (–) with 20 μM of either H89 or myristoyl-PKI (mPKI) for 2 hours at 37°C. They were fixed and processed for immunofluorescence with anti-giantin antibody. (B) The average number (± s.e.m.) of Golgi fragments per cell (*n*=25) was determined from non-transfected cells incubated for 2 hours at 37°C with the indicated concentrations of each inhibitor and stained for immunofluorescence with anti-giantin antibody. (C) Golgi-associated fluorescence was determined from cells (*n*=20) incubated or not (–) for 2 hours at 37°C with 20 μM of each inhibitor. (D) Cells stably expressing GalTfase-CFP were similarly incubated, fixed and processed for immunofluorescence with anti-giantin antibody. Bars, 10 μm.

were used at 20 μM. Under these conditions, the continuous Golgi ribbon typical of control, untreated cells was replaced by a set of clustered fragments in cells incubated with either inhibitor (Fig. 7D). The time course of Golgi disassembly was also analyzed. Whereas the number of Golgi fragments per cell did not vary significantly from 30 minutes to 2 hours of incubation in the presence of PKI, a slight increase from 10 to 19 fragments per cell occurred during the same time period in cells exposed to H89. Golgi-associated fluorescence, on the other hand, did not vary with either inhibitor (Fig. 7C). The structural features of Golgi fragments generated during PKI or H89 incubations resembled those generated during RIIα depletion by siRNA treatment. In particular, Golgi stacks became globular structures formed by concentrically arrayed, curved, swollen cisternae (Fig. 8). Together, these results indicated that Golgi-associated PKA activity plays a relevant role in the maintenance of the structural organization of this organelle. We concluded that either prolonged inhibition of the PKA catalytic activity or displacement from the Golgi gives rise to disruption of the Golgi ribbon. We next asked whether it would be also involved in the assembly of this structure.

Role of PKA activity in Golgi biogenesis

We first examined the subcellular localization of PKA subunits under conditions of Golgi redistribution into the ER. For instance, transient expression of dominant-negative mutated forms of the COPII component Sar1p interferes with the function of this coat complex and this blocks protein export from the ER (Kuge et al., 1994; Ward et al., 2001). Since the Golgi-ER recycling pathway persists under these conditions, all the Golgi transmembrane proteins are finally relocated to the ER. The exact point of transport block differs, however, depending on the particular mutated version being expressed. Expression of a GDP-restricted form of Sar1p, designated T39N, prevents the assembly of the COPII complex and this causes disassembly of the ER exit sites (Kuge et al., 1994; Stroud et al., 2003; Ward et al., 2001). By contrast, expression of a GTP-restricted form of Sar1p, designated H79G, inhibits export from the ER exit sites which otherwise assemble normally (Miles et al., 2001; Ward et al., 2001). We found that both PKA subunits were distributed dispersed throughout the cytosol in cells expressing the constitutively inactive T39N form of Sar1p. By contrast, in cells expressing the permanently active H79G mutant form of Sar1p PKA subunits were concentrated at the ER exit sites (Fig. 9A). PKA association with the centrosome remained unaltered in both cases. These findings raised the possibility that PKA activity could regulate protein export from the ER by influencing the assembly and organization of the ER exit sites. To test this, we examined the distribution of sec13, another component of the COPII complex and a marker of the ER exit sites (Tang et al., 1997). No difference in the punctate staining pattern of sec13 was noted in cells depleted of RIIα by siRNA treatment in comparison to control, non-depleted cells (Fig. 9B). A similar result was obtained with cells expressing Ht31-GFP or

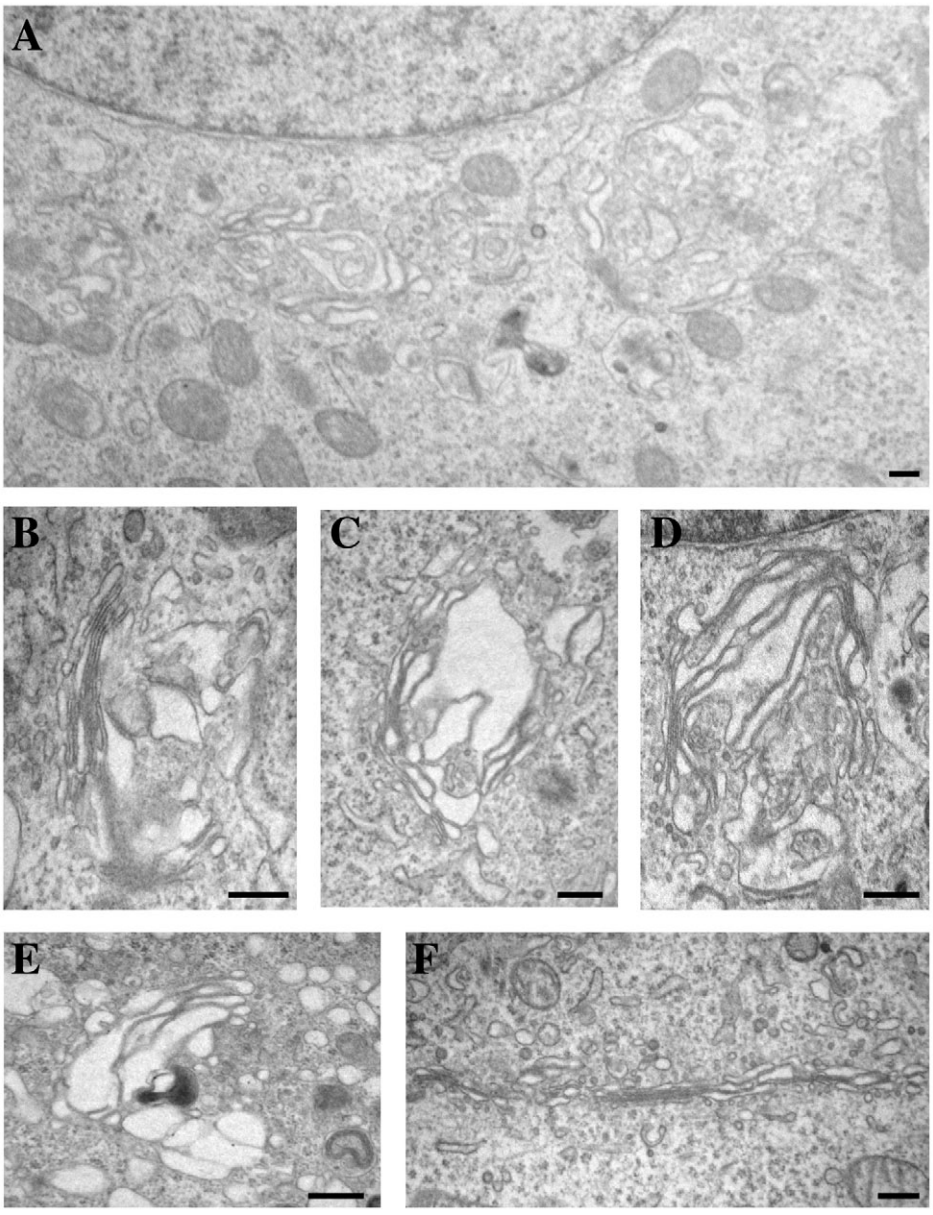

Fig. 8. Ultrastructural changes in Golgi organization following PKA inhibition. Cells were incubated at 37°C with 20 μM of either H89 (A-D) or myristoyl-PKI (E) for 2 hours before fixation and processing for transmission electron microscopy. (F) The Golgi ribbon of a control, untreated cell is shown for comparison. Bars, 0.5 μm.

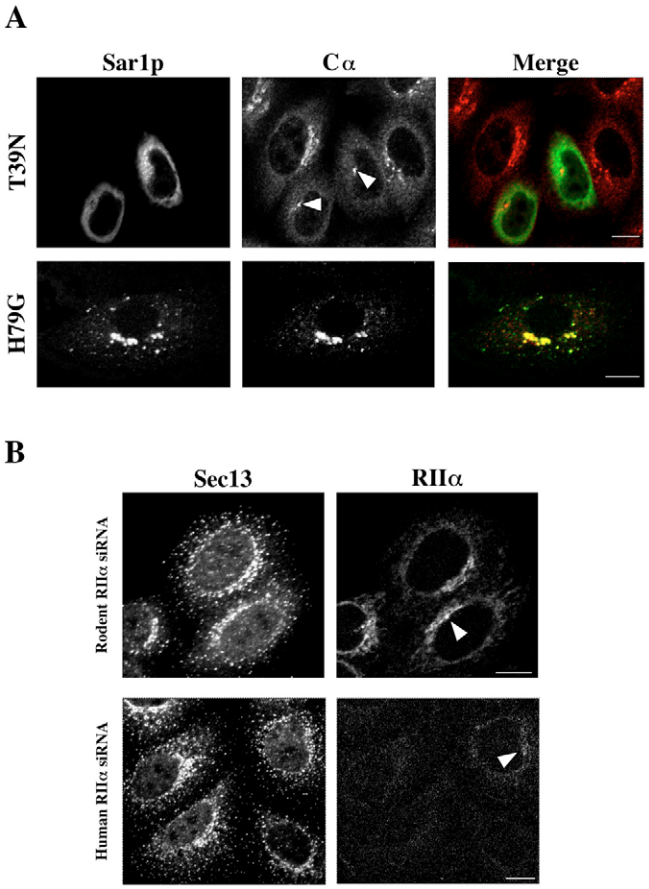

Fig. 9. Role of PKA in the organization of ER exit sites.
(A) Presence of PKA at the ER exit sites. Cells overexpressing CFP-tagged forms of the indicated mutated versions of Sar1p were fixed and processed for immunofluorescence with antibodies to Cα.
(B) PKA displacement does not affect the distribution of ER exit sites. Cells incubated for 80 hours with siRNA to target either the human or the rodent form of RIIα were fixed and processed for immunofluorescence with antibodies to Sec13p. Staining of the centrosome is indicated with arrowheads. Bars, 10 μm.

incubated with PKA inhibitors (not shown). Therefore, the number and distribution of the ER exit sites does not seem to be determined by the localization and activity of PKA.

In a different approach, we looked at the role of PKA activity in the Golgi reassembly process during reversal from brefeldin A (BFA) treatment (Fig. 10). In BFA-treated cells, PKA subunits were localized dispersed throughout the cytosol whereas Golgi transmembrane proteins (exemplified in Fig. 10 by the galactosyltransferase-CFP construct) showed a reticular fluorescent pattern characteristic of the ER. During early reversal times (5-10 minutes) Golgi proteins started to emerge from the ER at multiple peripheral sites. Subsequently (20 minutes) they clustered to the perinuclear region of the cell. With time these intermediates coalesced to form a continuous Golgi ribbon, which became evident by 40-60 minutes of recovery. Interestingly, recruitment of the PKA holoenzyme to the Golgi membranes occurred late in the reassembly process. Thus, most of the Golgi fluorescent spots detected at early reversal times did not contain associated PKA (Fig. 10, 10-20 minutes). Indeed, even at late time points, newly emerging Golgi membranes as opposed to the reassembled Golgi ribbon lacked associated PKA (Fig. 10, 40 minutes). These observations suggested that PKA activity might play a role in a final stage of the reassembly process. To analyze this further we studied the effect of PKA inhibition on Golgi reconstruction. Cells expressing galactosyltransferase-CFP were treated with H89 during recovery from BFA incubation (Fig. 11). In the continuous presence of H89 Golgi membranes emerged from the ER at much lower rate than in cells not incubated with this agent. The sequence of events was, however, similar. Initially, Golgi components exited the ER at multiple peripheral sites (Fig. 11A, 30 minutes) and later on (1-2 hours) they became clustered in the juxtanuclear region. The reassembly process seemed to be arrested at this stage because complete reconstruction of the Golgi complex did not occur even after prolonged incubations (Fig. 11A, 3 hours). In particular, no Golgi ribbon was formed in the presence of H89. Instead, a number of disconnected elements remained clustered in the perinuclear region. These were characterized by electron microscopy as whorl-like membranous structures composed of concentrically arrayed cisternae (Fig. 11C). Apparently, they were final intermediates in the reassembly process because the continuity of the Golgi ribbon was readily re-established upon H89 removal (Fig. 11A, 3 hours + 1 hour). These data, therefore, indicated that PKA catalytic activity is required at a final step of the Golgi reassembly process, that is, the formation of a continuous ribbon structure from separate Golgi stacks. PKA requirement, on the other hand, corresponded to the recruitment of the holoenzyme from the cytosol to the Golgi membranes during reassembly.

Discussion

The functional significance of PKA association with the Golgi complex of mammalian cells is a mystery. Since its subcellular localization was first reported two decades ago (Nigg et al., 1985b) this kinase has been recognized as an outstanding member of the set of signaling and regulatory molecules associated to the Golgi under steady-state conditions. With the exception of a role in the regulation of transport activities (Cabrera et al., 2003; Martín et al., 2000; Muñiz et al., 1996; Muñiz et al., 1997), however, there is no clear indication on the implication of Golgi-associated PKA in any relevant event. In this report, we provide data indicating that Golgi-associated PKA activity plays a relevant role in the assembly and maintenance of the continuous ribbon structure, a characteristic feature of the Golgi organization in mammalian cells. Recently, this ribbon-like architecture was shown to determine the efficiency of glycoprotein processing by allowing even distribution of glycosyltransferases among different Golgi stacks (Puthenveedu et al., 2006). Golgi-associated PKA could therefore contribute to the structural integrity and functional uniformity of this organelle.

A structural role for Golgi-associated PKA activity is suggested from data derived from the application of photobleaching techniques to living cells. The analysis of the dynamics of association to and dissociation from the Golgi of RIIα indicated that this protein is stably associated with the cytosolic surface of Golgi membranes and, in contrast to other Golgi peripheral proteins, does not exchange rapidly with the population of soluble molecules. Indeed, a significant fraction of Golgi-associated RIIα did not exchange at all and behaved

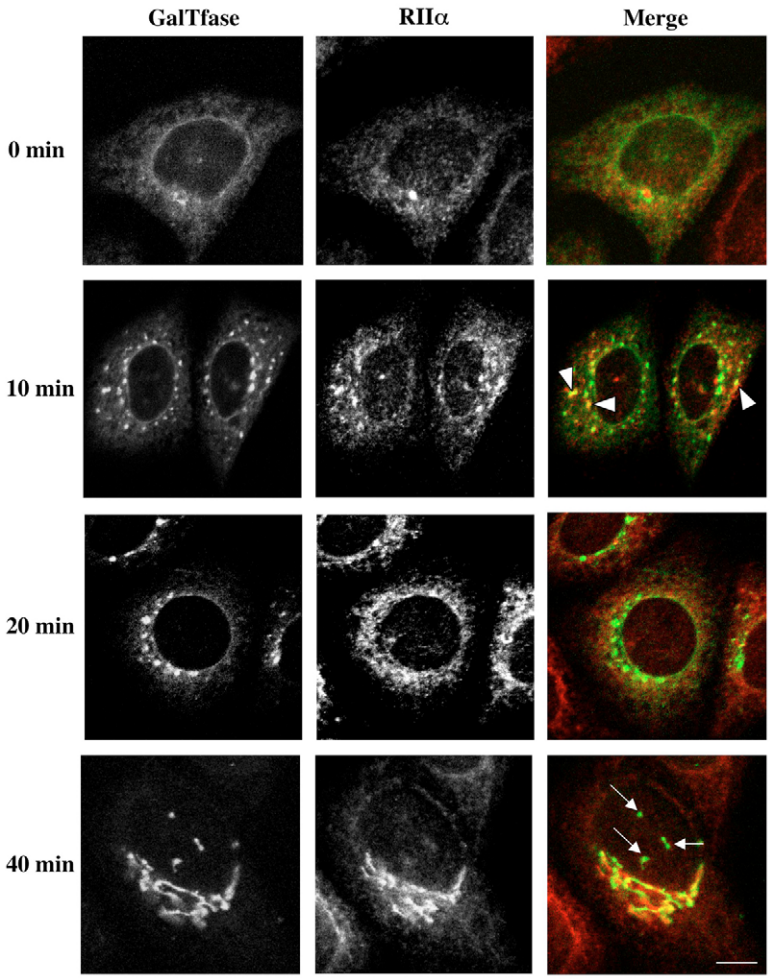

Fig. 10. PKA recruitment during Golgi reconstruction. Cells stably expressing GalTfase-CFP were incubated with 2 μg/ml BFA for 1 hour at 37°C and then allowed to recover for the indicated time periods. They were fixed and processed for immunofluorescence with anti-RIIα antibody. Newly formed Golgi fragments containing (arrowheads) or not (arrows) associated RIIα are indicated. Bar, 10 μm.

as immobile molecules. To reveal such a structural role, three experimental strategies were used. First, cells were depleted of RIIα by siRNA treatment. PKA targeting to different subcellular structures including the Golgi complex is achieved through association of the regulatory RII subunits to AKAPs (Michel and Scott, 2002; Wong and Scott, 2004). The catalytic C subunits, in turn, are RII bound. Accordingly, RIIα depletion by siRNA treatment gave rise to Cα redistribution from the Golgi complex to the cytosol. Second, cells were transfected with a plasmid encoding Ht31-GFP. This construct interferes with the association of RIIα to AKAPs and in our study caused cytosol redistribution of the complete PKA holoenzyme allowing us to evaluate the consequences of PKA displacement without affecting enzyme expression. Third, and most significantly, cells were incubated with either H89 or myristoyl-PKI to inhibit PKA catalytic activity. Results were similar in all three cases and showed that the structural organization of the Golgi complex depends on PKA association. Thus, following PKA pharmacological inhibition or its displacement from the Golgi, this organelle appeared disrupted into a number of fragments that remained clustered in the perinuclear area of the cell. We conclude that the integrity of the Golgi ribbon requires both proper location and enzymatic activity of the PKA holoenzyme.

Golgi fragments were polarized as judged by the distribution of both cis and trans (TGN) markers. This suggests that the mechanism responsible for Golgi disassembly might involve unlinking of the continuous Golgi ribbon into separate stacks. For instance, PKA phosphorylation of a structural protein could be required in order to maintain Golgi ribbon organization. Golgi unlinking would then occur whenever this modification is somehow abrogated. Alternatively, the Golgi complex could undergo fragmentation as the result of a block in ongoing protein trafficking. This, however, does not seem to be the case because Golgi fragments maintain at least their competence to transport VSV-G to the plasma membrane. Also, accumulation of transport vesicles was not observed in either RIIα-depleted cells or cells treated with PKA inhibitors. The effect caused by either PKA inhibition or mislocalization on Golgi structure would therefore be similar to that caused by GM130 knockdown (Puthenveedu et al., 2006). Further studies will be needed to characterize the Golgi proteins that are substrates of PKA activity and to determine how this modification affects Golgi organization.

The involvement of PKA activity in Golgi biogenesis was established by studying the organelle reassembly during recovery from BFA treatment. PKA subunits were recruited from the cytosol to the Golgi membranes as these emerged from the ER and fused to form a readily identifiable Golgi ribbon. The time of association, however, was clearly delayed in comparison to the time of re-emergence from the ER. In agreement with previous studies (Bannykh et al., 2005; Puri and Linstedt, 2003), Golgi membranes exited the ER at multiple peripheral sites that with time converged to the juxtanuclear region where they fused and reconstructed the ribbon organization. PKA association was coincident with ribbon formation. Thus, most Golgi intermediates detected at early times of the reassembly process lacked associated PKA. Furthermore, PKA inhibition during reversal from BFA treatment blocked ribbon formation by preventing the fusion of Golgi elements previously clustered in the perinuclear area. These elements corresponded to globular Golgi stacks composed of multiple concentrically arranged cisternae. Interestingly, similar structures have been recently reported to occur in cells recovering from BFA treatment in the absence of any other additional agent (Bannykh et al., 2005). This suggests that they might represent transient intermediates in the normal process of Golgi reassembly from the ER. Golgi fragments generated upon PKA displacement or inhibition also showed similar morphology. Therefore, the available evidence indicates that PKA activity is involved in the assembly and maintenance of the ribbon architecture typical of the mammalian Golgi complex.

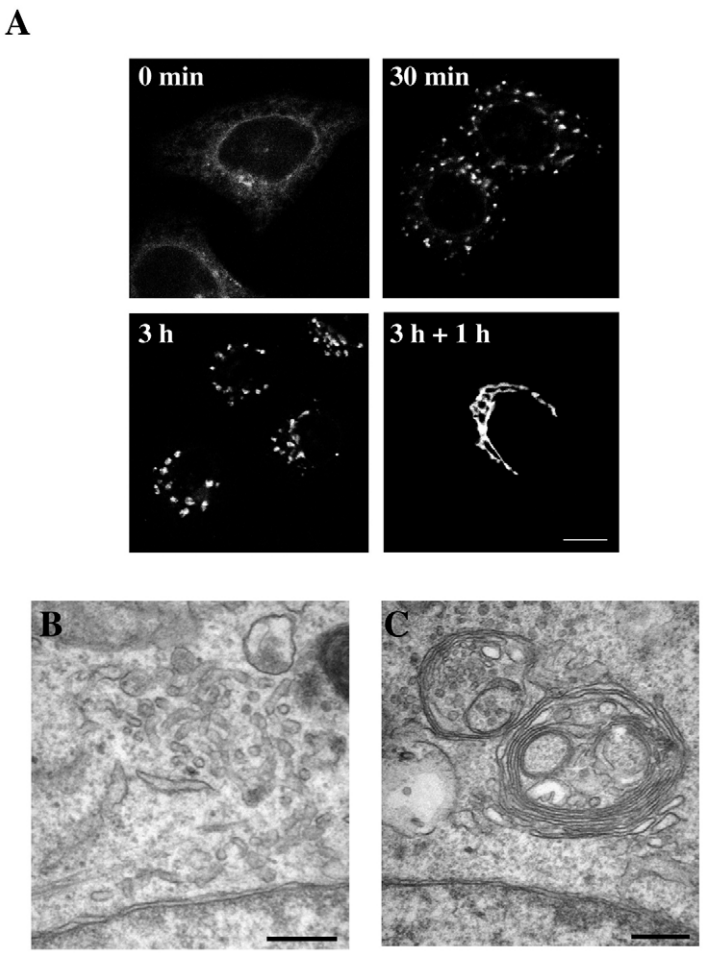

Fig. 11. Golgi reconstruction abrogated by PKA inhibition. (A) Cells stably expressing GalTfase-CFP were incubated with 2 μg/ml BFA for 1 hour at 37°C. During the last 20 minutes of BFA treatment 20 μM H89 was added to the medium. Incubation continued in the presence of H89 but in the absence of BFA for the indicated time periods. Additionally, after 3 hours of BFA reversal in the presence of H89 cells were incubated in medium with no inhibitor for 1 hour (3 h + 1 h). Bars, 10 μm. (B) Non-transfected cells were incubated with 2 μg/ml BFA for 1 hour at 37°C before fixation and processing for electron microscopy. A vesicular-tubular cluster believed to represent the starting structure for Golgi reassembly (Bannykh et al., 2005) is shown. (C) Cells were allowed to recover from BFA treatment for 3 hours at 37°C in the continuous presence of 20 μM H89 before fixation and processing. In this case, whorl-like structures accumulate in the perinuclear region. Bars, 0.5 μm.

The isoquinolinesulfonamide H89 has been extensively used to block reversibly protein export from the ER. Used at moderate concentration (50 μM or higher) H89 abolishes assembly of the COPII coat complexes at the ER exit sites by preventing Sar1p recruitment to the membrane (Aridor and Balch, 2000). A protocol consisting of the sequential incubation of the cells with first BFA and then H89 has been proposed as a way to achieve both complete redistribution of all Golgi components and dismantling of the ER exit sites leaving no Golgi remnant structure in the cytoplasm (Puri and Linstedt, 2003). Since no similar effect was obtained with other PKA inhibitors, the molecular target of H89 action was assumed to be a different protein kinase (Aridor and Balch, 2000; Lee and Linstedt, 2000). In principle, our observation that PKA is recruited to the ER exit sites in cells expressing a constitutively active, GTP-restricted mutant form of Sar1p could argue against the above interpretation. However, additional data indicate that the assembly of COPII coat complex at the ER exit sites does not require PKA activity. Thus, PKA is not normally localized at the ER export sites in both control, untreated cells and cells incubated with BFA. As indicated by the sec13 staining pattern the organization and distribution of these sites was not affected by either PKA inhibition or displacement. Also, Golgi membranes were able to slowly exit the ER in the presence of a low (20 μM) concentration of H89 sufficient to inactivate PKA. The inhibitory effects caused by a high dose of this agent on both COPII assembly and constitutive ER export might therefore be explained by the inhibition of some other protein kinase different to PKA. For instance, a novel PCTAIRE kinase has recently reported to interact with components of the COPII complex (Palmer et al., 2005).

Whereas compelling evidences indicate that PKA is not the protein kinase involved in the recruitment and assembly of the COPII coat complex and therefore cannot control constitutive protein export from the ER, the possibility that PKA activity might regulate transport of particular cargo proteins cannot be ruled out. For instance, both PKA and PKC-dependent phosphorylation of the NR1 NMDA receptor determines its export from the ER (Scott et al., 2003). On the other hand, the preferential association of PKA to the Golgi complex in mammalian cells suggests that this kinase is primarily involved in the modulation of Golgi activities. In particular, the data presented here indicate that PKA activity regulates Golgi structural organization.

Materials and Methods

Plasmids, antibodies and reagents

A bacteria expression plasmid containing the full-length sequence of murine RIIα was provided by S. Taylor (UCSD, San Diego, CA). To make the RIIα-GFP construct, the coding sequence was subcloned into pEGFPN2 (BD Biosciences Clontech, Palo Alto, CA) using *Sac*I and *Sma*I restriction sites. Similarly, the coding sequence of γ-COP provided by F. Wieland (BZH, Heidelberg, Germany) was transplanted from a prokaryotic expression plasmid to pEYFPN1 (BD Biosciences) using previously created *Sal*I and *Hin*dIII sites. The cDNA encoding galactosyltransferase-CFP was generated from pEYFP-Golgi by switching the fluorescence protein-coding region for that of CFP taken from pECFPN1 (BD Biosciences). Plasmids coding the Sar1p mutant forms Sar1 [H79G] and Sar1[T39N] were provided by R. Pepperkok (EMBL, Heidelberg, Germany) and J. Lippincott-Schwartz (National Institutes of Health, Bethesda, MD), respectively. To obtain CFP-tagged versions of these proteins, the corresponding coding sequence was subcloned into pECFPC1 (BD Biosciences). Plasmids encoding either Ht31-GFP or the mutated form Ht31P-GFP were provided by J. D. Scott (Vollum Institute, Portland, OR) (Alto et al., 2003). Mouse monoclonal antibody against giantin was donated by H. P. Hauri (Biozentrum, Basel, Switzerland) (Linstedt and Hauri, 1993). Rabbit polyclonal to Sec13p was a kind gift of W. Hong (IMCB, Singapore) (Tang et al., 1997). 8G5F11 mouse monoclonal antibody against the extracytoplasmic domain of vesicular stomatitis virus G glycoprotein (VSV-G) was provided by D. S. Lyles (Wake Forest University, NC) (Le François and Lyles, 1982). Additional primary antibodies used were: rabbit polyclonals to PKA subunits RIIα and Cα (Santa Cruz Biotech, Santa Cruz, CA), mouse monoclonal to PKA RIIα subunit (BD Biosciences), sheep polyclonal to TGN46 (Serotec, Oxford, UK), rabbit polyclonal to β′-COP (provided by F. Wieland), rabbit polyclonal to protein disulfide isomerase (PDI, Stressgen Biotech., Victoria, Canada). The following goat anti-mouse and anti-rabbit IgG secondary antibodies were used: labelled with Alexa Fluor 488 from Molecular Probes (Eugene, OR), conjugated to Cy3 from Amersham Biosciences (Piscataway, NJ), and conjugated

to HRP from Biosource (Camarillo, CA). Also, Cy3-conjugated and Alexa Fluor 488-labelled donkey anti-sheep IgG were from Chemicon (Temecula, CA) and Molecular Probes respectively. H89 was from Calbiochem (La Jolla, CA), myristoyl-PKI from Biomol International (Plymouth Meeting, PA), and BFA from Epicentre Technologies (Madison, WI).

Cell culture and treatments

HeLa cells (ATCC CCL-2) were maintained in culture in DME medium containing 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C with 5% CO_2. Pharmacological treatments were carried out in serum-free medium.

RNA interference and cell transfection

To knock down RIIα, cells were transfected using Oligofectamine (Invitrogen, Carlsbad, CA) with duplex siRNA (Dharmacon Research, Lafayette, CO) over 72-80 hours according to Elbashir et al. (Elbashir et al., 2001). Human RIIα was targeted using one of two siRNA oligonucleotides directed against the sequences TTCGCAGTGGAGTACTTCA (siRNA1) and AAGTTCTCGATGCCATGTTTG (siRNA2). The mouse version of the latter sequence differing in one single nucleotide was used to design a negative-control siRNA. Transient transfections with cDNA were performed using FuGENE 6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Cells stably expressing either RIIα-GFP or galactosyltransferase-CFP were obtained by transient transfection followed by selection with 1 mg/ml G418.

Immunofluorescence, photobleaching and image analysis

Indirect immunofluorescence was performed with cells cultured on poly-L-lysine-coated glass coverslips. Cells were fixed for 10 minutes in either ice-cold methanol or 4% paraformaldehyde in phosphate-buffered saline (PBS). They were rinsed thoroughly with PBS and incubated for 10 minutes with PBS containing 0.5% BSA and 0.05% saponin. Cells were incubated for 30 minutes at 37°C with primary antibody diluted in PBS-BSA-saponin, rinsed, and similarly incubated with secondary antibody. In the case of VSV-infected cells, they were fixed with paraformaldehyde and sequentially incubated with primary antibody against VSV-G and secondary antibody (both diluted in PBS-BSA) before permeabilization with PBS-BSA-saponin and staining with anti-RIIα and goat anti-rabbit IgG secondary antibody. After immunostaining, cells were rinsed with PBS and mounted for microscopy using Mowiol 4-88 (Calbiochem). All images were acquired using a Leica TCS SP2 confocal microscope. For quantitative analysis, a stack of serial images (0.2 μm thickness each) was obtained by scanning the cells in the *z* direction and used to generate a maximum intensity projection. After applying a fixed threshold to all images, total Golgi-associated fluorescence and the number of Golgi-derived fluorescent fragments per cell were determined using the 'Analyze Particles' function of the NIH Image software.

Photobleaching experiments were performed on living cells expressing RIIα-GFP and cultured on glass bottom 35 mm culture dishes (MatTek, Ashland, MA). They were imaged with the same confocal system mentioned above using the 488 nm line of an argon laser. During imaging, cells were maintained in MEM without Phenol Red and supplemented with both sodium bicarbonate and HEPES on a stage preheated to 37°C. Non-saturated images were taken with a 63×1.4 NA objective and a pinhole diameter equivalent to 4-5 Airy units at 512×512 pixels. Before bleaching, three to four images at low-intensity illumination were taken to establish the fluorescence baseline. Selective photobleaching was performed at 80-100% laser power and recovery was monitored by taking images at low power in a time-lapse sequence. In FRAP experiments, images were taken every 2 minutes. During FLIP experiments, the selected area was repeatedly bleached every 4 minutes and images were taken at intervals of 30 seconds. Fluorescence intensities were measured using the Leica confocal software. Background fluorescence outside the cell was subtracted from total cellular fluorescence whereas to assess Golgi-associated fluorescence, the cytoplasm overlap was similarly subtracted. FRAP quantification was performed as described (Liu et al., 2005). Briefly, the Golgi-associated fluorescence was represented as the ratio of Golgi-to-total cellular fluorescence at each time point divided by the initial ratio before bleaching (prebleach situation).

Transmission electron microscopy

Cells grown on 35 mm culture dishes were used. They were fixed at room temperature for 1 hour in 2.5% glutaraldehyde in 0.1 M sodium cacodylate-HCl, pH 7.3. Cells were postfixed for 1 hour with 1% osmium tetroxide, 1% potassium ferrocyanide in buffer and stained overnight with 1% uranyl acetate aqueous solution before dehydration and resin embedding. Cell monolayers were cut en face and sections were stained with lead citrate.

Biochemical procedures

Cell monolayers were rinsed three times with PBS. Cells were scraped from the dish and resuspended in 0.5 ml of PBS containing protease inhibitors (1 mM PMSF, 5 mM benzamidine, 100 μg/ml soybean trypsin inhibitor, 20 μg/ml aprotinin, and 10 μg/ml each pepstatin A, leupeptin, antipain). They were sedimented by centrifugation at 12,000 ***g*** for 10 minutes at 4°C. Cell pellets were incubated on ice for 30 minutes with lysis buffer (50 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 5 mM EDTA, 1% Triton X-100) containing protease inhibitors. Cell lysates were centrifuged at 4°C for 10 minutes at 12,000 ***g*** and the supernatants combined with an equal volume of 2× electrophoresis sample buffer.

Total microsomal membranes were prepared from cells previously rinsed with PBS and scraped from the dish. They were homogenized in cold PBS containing protease inhibitors using a ball-bearing homogenizer. The postnuclear supernatant was centrifuged at 100,000 ***g*** for 1 hour at 4°C. The microsomal pellet was rinsed with 10 mM Tris-HCl, pH 8.0, and resuspended in electrophoresis sample buffer.

Electrophoresis and immunoblotting was performed as described previously (Martin et al., 1999).

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