The Golgi-associated COPI-coated buds and vesicles contain β/γ-actin

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It has been shown previously that the morphology and subcellular positioning of the Golgi complex is controlled by actin microfilaments. To further characterize the association between actin microfilaments and the Golgi complex, we have used the Clostridium botulinum toxins C2 and C3, which specifically inhibit actin polymerization and cause depolymerization of F-actin in intact cells by the ADP ribosylation of G-actin monomers and the Rho small GTP-binding protein, respectively. Normal rat kidney cells treated with C2 showed that disruption of the actin and the collapse of the Golgi complex occurred concomitantly. However, when cells were treated with C3, the actin disassembly was observed without any change in the organization of the Golgi complex. The absence of the involvement of Rho was further confirmed by the treatment with cytochalasin D, which causes actin polymerization with the constitutively activated form of RhoA, both of which induced the stress fiber formation without affecting the Golgi complex. Immunogold electron microscopy in normal rat kidney cells revealed that β- and γ-actin isoforms were found in Golgi-associated COPI-coated buds and vesicles. Taken together, the results suggest that the Rho signaling pathway does not directly regulate Golgi-associated actin microfilaments, and that β- and γ-actins might be involved in the formation and/or transport of Golgi-derived vesicular or tubular intermediates.

Cytoplasmic and submembrane actin microfilaments are organized in linear bundles, two-dimensional nets, and three-dimensional gels. Actin isoforms segregate to functional regions of the cell in vertebrates and show slight functional differences in vitro. In particular, α isoforms are found in skeletal, cardiac, and vascular smooth muscles, γ isoform is found in enteric smooth muscle, and β and γ isoforms are present in the cytoplasm of non-muscle cells (ref. 1 and references therein). In addition, through actin cytoskeleton rearrangement, members of the Rho family Cdc42, Rac, and Rho proteins regulate the formation of filopodia, ruffles, and stress fibers, respectively (2).

Recently, actin and actin-associated proteins have been implicated in regulating the membrane dynamics of the Golgi complex (3–20). Actin-disrupting drugs have been used widely to show the involvement of actin in the endocytic pathway (ref. 20 and references therein). However, the role of actin in the secretory pathway is controversial. For example, treatment with cytochalasin D does not affect the Golgi-to-plasma membrane transport of either the VSVG protein, when monitored by fluorescence-activated cell sorter analysis (10), or the glycosaminoglycans release in the culture medium (20). However, cytochalasin B alters the in vivo formation of VSVG-GFP transport intermediates from Golgi membranes (21). Recently, using in vitro assays, diverse isoforms of actin-binding proteins have been resolved biochemically in Golgi enriched fractions. It has also been shown that the newly budded and coated vesicles from Golgi membranes can bind to actin in vitro (18).

Clostridium botulinum toxins C2 and C3 depolymerize F-actin by ADP ribosylation of G-actin monomers and the small GTP-binding proteins Rho, respectively (22–24). We have found that the treatment with C2 toxin in normal rat kidney cells (NRK) led to a simultaneous disassembly of actin microfilaments and the collapse of the Golgi complex. The collapse of the Golgi complex is defined as the loss of the characteristic reticular and perinuclear morphology into a circular and more compact structure located in the centriolar region (ref. 10; see Fig. 1G). This criterion is used to define the reorganization of the Golgi complex on depolymerization of actin by C2, C3, on the other hand, caused actin disassembly without affecting Golgi complex. This suggests that, although actin is involved in the organization of the Golgi complex, this regulation is independent of Rho. Additionally, immunogold electron microscopy of intact cells revealed the presence of β- and γ-actin isoforms in Golgi-derived COPI-coated buds and vesicles. The significance of these findings is described below.

Materials and Methods

Materials. DMEM and FCS were from GIBCO/BRL Life Technologies (Paisley, Scotland); secondary tetramethylrhodamine B isothiocyanate (TRITC) or FITC F(ab′)2 fragments were from Boehringer Mannheim; phallolidin-TRITC, cytochalasin D, and lysophosphatidic acid were from Sigma. [32P]NAD was purchased from Amersham Pharmacia. C3 exoenzyme was from Cytoskeleton (Denver). All other chemicals, if not otherwise stated were from Sigma and/or Merck.

Cell Culture. NRK cells were cultured in DMEM supplemented with 10% FCS. The cells were grown at 37°C in a humidified atmosphere of 5% CO2.

Starvation Culture Conditions. Unlike Swiss 3T3 (25), NRK cells are resistant to clarifying of the cytoplasm of stress fibers in serum-deprived culture medium. Consequently, we thus developed the following protocol: Cells were prepared by seeding onto glass coverslips in tissue culture wells or Petri dishes containing DMEM/10% FCS. After the cell attachment, cells were washed three times in DMEM with NaHCO3 without FCS (starvation medium) and were incubated for 48 h. Thereafter, the medium was removed, and cells were briefly incubated in the starvation medium containing cytochalasin D (5 μM, final concentration; 5 min, 37°C). To remove cytochalasin D, three washes were performed in starvation medium, and cells were

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Abbreviations: TRITC, tetramethylrhodamine B isothiocyanate; NRK cell, normal rat kidney cell; Man II, mannosidase II.

F.V. and A.L. contributed equally to this study.

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further incubated in freshly prepared starvation medium for 1–3 days.

**Incubation and Microinjection of C2 and C3 Toxins.** Cell culture experiments: Botulinum toxin C2 (100 ng/ml of the catalytic subunit and 200 ng/ml of the binding component, final concentrations) or botulinum toxin C3 (20 μg/ml, final concentration) were added to NRK cells cultured in DMEM supplemented with 10% FCS at 37°C. Microinjection experiments: NRK cells were grown to 50–70% confluence on Eppendorf Celllocate Coverslips. The coverslips were transferred to a Petri dish containing culture medium buffered with 25 mM Hepes. C3 toxin was diluted in microinjection buffer (100 mM KCl/5 mM Hepes, pH 7.25) and was centrifuged at 14,000 × g at 4°C for 10 min. Cells were microinjected with an Automated Microinjection System (model 5242, Eppendorf). The coverslips were then transferred to a Petri dish containing fresh culture medium and were returned to the incubator for 10 min. Coverslips were then processed for immunofluorescence as described below.

**Immunofluorescence.** Toxin-treated and nontreated NRK cells were quickly washed in PBS (0.01M phosphate buffer/0.15 M NaCl, pH 7.2) and were fixed in freshly prepared paraformaldehyde (4% in PBS) at room temperature for 15 min. Cells were then washed 3 × 5 min, in PBS, and then in PBS containing ammonium chloride. The cells were then rinsed in PBS and were permeabilized with PBS containing 0.1% saponin and 0.1% BSA for 15 min. Cells were further processed for double-label immunofluorescence by using the rabbit polyclonal anti-mannosidase II (Man II) (1:4,000) and TRITC-phalloidin (diluted 1:250 from a stock solution of 0.2 mg/ml) antibodies followed by protein A-gold (26) (1561). Gold particles were counted and ascribed to one of the following categories: COP-coated buds, lateral rims (lateral portions of the cisterna showing the characteristic dilatation), or flattened central portion of the cisternae. Samples were visualized in a Zeiss EM10 electron microscope. Statistical analysis was performed with the Student’s t test.

**Fluorescent Phalloidin Binding Assay.** Control and poisoned NRK cells were incubated with 4% paraformaldehyde in PBS for 15–30 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After 3 rinses in PBS, cells were incubated with TRITC-phalloidin (1:1,000 from a stock solution of 0.2 mg/ml) in PBS for 15 min, washed 3 times in PBS, and extracted with methanol for 25 min at room temperature. The fluorescence intensity of the supernatants was measured in a Kontron Instruments fluorometer (SFM25) at 554 nm and 573 nm (excitation and emission).

[32P]ADP-Ribosylation Assay. NRK cells were incubated with C3 exoenzyme (20 μg/ml) for various times. Cells were washed twice by centrifugation (1,000 × g for 5 min) in complete medium and were lysed by periodic agitation for 15 min in ice-cold lysis buffer (50 mM Tris-HCl/120 mM NaCl/2.5 mM EDTA/1 mM DTT/0.5% Nonidet P-40, pH 7.4) containing protease inhibitors (200 mM phenylmethylsulfonyl fluoride and 5 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM β-glycerophosphate). After centrifugation (16,000 × g for 10 min) the supernatant (corresponding to 80 μg of protein) was incubated (30 with 53 μl of ribosylation buffer (150 mM Tris-HCl/30 mM nicotinamide/15 mM thymidine/15 mM DTT/7.5 mM MgCl2/10 μM [32P]NAD, pH 8.0) in the absence or presence of 7.5 ng of recombinant C3 exoenzyme. The samples were mixed with 14 μl of 5× sample buffer (125 mM Tris/4% SDS/20% glycerol/10% 2-mercaptoethanol/0.01% bromophenacyl blue, pH 6.8), were boiled for 5 min, and were subjected to SDS/PAGE using a 5% stacking gel and a 15% separating gel. The proteins were electrophoretically transferred to nitrocellulose membranes and subjected to autoradiography.

**Results and Discussion**

**C2 Treatment Induces Disassembly of Actin Microfilaments and the Collapse of the Golgi Complex.** C2 is an ADP ribosyltransferase that modifies monomeric G-actin but not polymerized F-actin (35). It enters the cells by receptor-mediated endocytosis (30). A time course of treatment with C2 toxin added in the culture medium showed a correlation between the depolymerization of actin and the collapse of the Golgi complex (Fig. 1). For example, after 2 h of treatment, the onset of the Golgi collapse (Fig. 1C) was observed simultaneously with a slight loss of F-actin filament integrity (Fig. 1D). After 4 h, the morphological alterations were more pronounced. In particular, the Golgi complex was more compact (Fig. 1F), and there was an extensive disassembly of actin stress fibers (Fig. 1F). After 6 h, the actin filaments were severely disassembled (Fig. 1H), and Golgi complex was found as a tightly collapse morphology (Fig. 1G). Notably, the Golgi complex collapse was produced without significant changes in the cellular shape (Fig. 1F and H). A quantitative analysis of the alterations in the Golgi complex and F-actin content after C2 treatment was performed (Fig. 2A). The percentage of the Golgi complex with its characteristic reticular and perinuclear morphology (Fig. 1A) diminished in parallel to the F-actin content (Fig. 2A). When C2 toxin was microinjected into the cytoplasm, we observed a concomitant collapse of the Golgi complex (Fig. 4A, small arrows) and the disruption of actin (Fig. 4B, small arrows).
arrows). Together, these results show an interaction between actin microfilaments and the Golgi complex. This is consistent with our previous observations (10, 11, 20).

**In Situ Immunolocalization of \(\beta\)- and \(\gamma\)-Actin Isoforms to Golgi-Associated COP-Coated Buds and Vesicles.** C2 transferase alters the non-muscle \(\beta/\gamma\) and not the \(\alpha\)-G-actin isoform (31, 32). Thus, only actin microfilaments containing \(\beta/\gamma\) isoforms should be perturbed by C2, and, therefore, C2 treatment should affect the subcellular structures linked only to these isoforms. Our findings with C2 toxin suggest that the Golgi complex is associated with the \(\beta/\gamma\) actin microfilaments. Staining with \(\beta\)- and \(\gamma\)-actin antibodies at the fluorescence microscopy level did not show a clear Golgi staining. This is because these actin isoforms are abundant cytoplasmic proteins and may mask the small but specific interaction to Golgi membranes. We, therefore, examined the subcellular localization of \(\beta\)- and \(\gamma\)-actin isoforms in NRK cells by cryoimmunoelectron microscopy. We describe and illustrate the immunolabeling for \(\beta\)-actin only, although similar results were also obtained with the \(\gamma\)-actin antibodies (data not shown). Anti-\(\beta\) actin antibodies strongly labeled cortical actin bundles (Fig. 3A, arrows) and non-membrane-associated structures (Fig. 3C and D; Table 1), the latter probably reflecting the cytoplasmic network of actin microfilaments. More importantly, gold labeling was visualized in the Golgi cisternae and associated structures (Fig. 3B–E; Table 3). Gold particles were also visualized in both COP-coated vesicles and budding profiles emerging from rims of the cisternae (Fig. 3C and E, arrows; Table 2). COP-coated structures were initially identified by the shape and size of the coat that is clearly distinguishable from the clathrin coat (refs. 28 and 29; Fig. 3D, arrowhead; Table 2). Double immunogold labeling experiments confirmed that \(\beta\)-actin colocalized in COPI-coated membranes (Fig. 3D and E, arrows). The quantitative analysis of the gold labeling in the Golgi stack demonstrated a preferential association of \(\beta/\gamma\) actins in the lateral rims and buds of Golgi cisternae, representing both together the 80% of the total gold labeling for \(\beta\)-actin (Table 3).

In a recent report (18), it has been shown by immunoblotting techniques that fractions of Golgi membranes and vesicles contain \(\beta\)-actin. Although this is a good indication of the presence of actin in Golgi membranes and derived structures, the possibility of another membrane compartments in the Golgi-enriched fractions cannot be ruled out. We state this because (i) Golgi enriched fractions also contain endosomes and plasma membrane, and (ii) COPI coats are also found in endosomes (33, 34). Interestingly, there was a significant labeling of noncoated vesicles and/or tubules (Table 2; Fig. 3C and D). Because the COPI coat is quickly lost after vesicle formation, these uncoated structures most probably represent naked COPI-coated membrane transport intermediates. Therefore, the results with C2 toxin and immunolocalization of \(\beta/\gamma\) actin to Golgi complex and Golgi-derived structures in intact cells are conclusive of the association of actin microfilaments with the Golgi complex.

**C3 Induces the Disassembly of Actin Stress Fibers but Does Not Alter the Reticular and Perinuclear Morphology of the Golgi Complex.** The small GTP-binding protein Rho controls the stress fiber forma-
C3 exoenzyme inactivates Rho by ADP ribosylation (36). To examine whether Rho regulates the Golgi-associated actin microfilaments, we incubated NRK cells with C3 and, as described previously for C2, monitored changes in the morphology of the Golgi complex and F-actin integrity (Fig. 5). In C3-treated cells, the Golgi complex retained its characteristic reticular and perinuclear morphology (Fig. 5 C, E, and G). In contrast, the stress fibers were progressively disassembled (Fig. 3). C3 exoenzyme inactivates Rho by ADP ribosylation (35).

Table 1. Subcellular distribution of β-actin in the Golgi complex: Golgi area

<table>
<thead>
<tr>
<th>Location</th>
<th>β-actin Distribution</th>
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<tr>
<td>Golgi stack</td>
<td>29.3 ± 5.6</td>
</tr>
<tr>
<td>Vesicles</td>
<td>44.5 ± 6.6*</td>
</tr>
<tr>
<td>Non-membrane bound</td>
<td>26.2 ± 5.7*</td>
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</table>

Numbers represent the percentages (mean ± SEM) of the total labeling in distinct locations in the Golgi area. *, P < 0.03.
5 D, F, and H). However, the time required for the C3 toxin to exert its effect was much longer than that for C2. C3 enters the cell by an unknown mechanism, and this lag in the toxic action may be attributable to delayed binding and the subsequent internalization. This is not the case when C3 is directly microinjected into the cytoplasm, where the toxic effect is produced in minutes (25). Consequently, we microinjected C3 toxin, and results obtained were identical (Fig. 4 C and D) to those observed when the toxin was added to culture medium (Fig. 5).

A quantitative analysis showed that the decrease in F-actin content by C3-treatment (Fig. 2 B) was not accompanied by a decrease in the percentage of the Golgi complex with normal morphology (Fig. 5 A and C).

The biological effects of C3 exoenzyme correlate well with the extent of ADP ribosylation of Rho in vivo (37, 38). Consequently, the ability of C3 exoenzyme to ADP ribosylate Rho in vivo was also tested (Fig. 6) in parallel to immunocytochemical experi-

Table 2. Subcellular distribution of β-actin in the Golgi complex:

<table>
<thead>
<tr>
<th>Clathrin-coated vesicles</th>
<th>COP-coated vesicles</th>
<th>Non-coated vesicles</th>
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<tbody>
<tr>
<td>5.2 ± 1.4*</td>
<td>31.1 ± 1.9**</td>
<td>63.6 ± 1.1*</td>
</tr>
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</table>

Numbers represent the percentages (mean ± SEM) of each type of vesicles labeled for β-actin. The type of vesicle was defined by using morphological criteria on the basis of thickness and appearance of the coat (18 nm and 10 nm for clathrin and COP, respectively). *, P < 0.0004. †, P < 0.0001.

Table 3. Subcellular distribution of β-actin in the Golgi complex:

<table>
<thead>
<tr>
<th>Coated-buds</th>
<th>Lateral rims</th>
<th>Central portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>36.0 ± 6.1</td>
<td>44.0 ± 2.3*</td>
<td>20.0 ± 4.0*</td>
</tr>
</tbody>
</table>

Numbers represent the percentages (mean ± SEM) of gold particles in each subcompartment (see Material and Methods). *, P < 0.006.
ments (Fig. 5). The amount of Rho ADP ribosylated in vivo was determined by in vitro ADP ribosylation of lysates of treated cells with [32P]NAD. Once Rho is ADP ribosylated, it can no longer serve as a substrate for the in vitro ADP ribosylation reaction (39). The exposure of NRK cells to C3 exoenzyme caused a progressive ADP ribosylation of Rho in vivo (Fig. 6). After 48 h of incubation with C3, when the disassembly of actin was morphologically complete (Fig. 5H) and the F-actin content was at its lowest level (Fig. 3B), 86% of Rho was ADP ribosylated in vivo. It is important to note that >80% of ADP ribosylation of Rho is required to observe its biological effect (39). Taken together, the results suggest that Rho does not directly regulate the Golgi complex (data not shown). Taken together, results with C3 toxin, treatment with lysophosphatidic acid in starved NRK cells were supported by a Comisión Interministerial de Ciencia y Tecnología grant to G.E. (SAF 97-0016).

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