DEPARTAMENTO DE BIOLOGÍA CEL·LULAR I ANATOMIA PATOLÒGICA
FACULTAT DE MEDICINA

DINÁMICA DE LA ACTINA Y TRÁFICO DE MEMBRANAS ASOCIADO AL COMPLEJO DE GOLGI: PAPEL REGULADOR DE RHOA, RAC1 Y CDC42

Tesis presentada por Olga B. Matas Guadix y
dirigida por el Dr. Gustavo Egea Guri
para optar al grado de Doctora en Bioquímica

Barcelona, Mayo del 2005

IX. ANEXO

Molecular Biology of the Cell
Vol. 13, 866–879, March 2002

Regulation of Protein Transport from the Golgi Complex to the Endoplasmic Reticulum by CDC42 and N-WASP

Ana Luna,*† Olga B. Matas,* José Angel Martínez-Menárguez,‡ Eugenia Mato,* Juan M. Durán,* José Ballesta,‡ Michael Way,§ and Gustavo Egea*‖

*Departament de Biologia Cel.lular i Anatomia Patològica, Facultat de Medicina, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, E-08036 Barcelona, Spain; ‡Departamento de Biología Celular, Facultad de Medicina, Universidad de Murcia, E-30071 Murcia, Spain; and §Imperial Cancer Research Fund, London Wc2A 3PX, England

Submitted October 30, 2001; Revised December 12, 2001; Accepted January 4, 2002
Monitoring Editor: Vivek Malhotra

Actin is involved in the organization of the Golgi complex and Golgi-to-ER protein transport in mammalian cells. Little, however, is known about the regulation of the Golgi-associated actin cytoskeleton. We provide evidence that Cdc42, a small GTPase that regulates actin dynamics, controls Golgi-to-ER protein transport. We located GFP-Cdc42 in the lateral portions of Golgi cisternae and in COPI-coated and noncoated Golgi-associated transport intermediates. Overexpression of Cdc42 and its activated form Cdc42V12 inhibited the retrograde transport of Shiga toxin from the Golgi complex to the ER, the redistribution of the KDEL receptor, and the ER accumulation of Golgi-resident proteins induced by the active GTP-bound mutant of Sar1 (Sar1[H79G]). Coexpression of wild-type or activated Cdc42 and N-WASP also inhibited Golgi-to-ER transport, but this was not the case in cells expressing Cdc42V12 and N-WASP(ΔWA), a mutant form of N-WASP that lacks Arp2/3 binding. Furthermore, Cdc42V12 recruited GFP-N-WASP to the Golgi complex. We therefore conclude that Cdc42 regulates Golgi-to-ER protein transport in an N-WASP–dependent manner.

INTRODUCTION

The involvement of microtubules in intracellular membrane trafficking is well established (Thyberg and Moskalewski, 1999 for review), and the role of actin in membrane traffic is under extensive investigation (DePina and Langford, 1999; Qualmann *et al.*, 2000; Apodaca, 2001 for reviews). In the secretory pathway, actin filaments are required to maintain the organization of the Golgi complex (Valderrama *et al.*, 1998; di Campli *et al.*, 1999) and for protein transport from the Golgi to the plasma membrane and the ER (Müsch *et al.*, 2001; Valderrama *et al.*, 2001). In addition, actin-binding protein spectrin/ankyrin isoforms and several myosins have been localized to the Golgi complex and implicated in transport functions (Beck *et al.*, 1994; Müsch *et al.*, 1997; Buss *et al.*, 1998; Godi *et al.*, 1998; Stow *et al.*, 1998; Heimann *et al.*, 1999).

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.01–12–0579. Article and publication date are at www.molbiolcell.org/cgi/doi/10.1091/mbc.01–12–0579.

‖ Corresponding author. E-mail address: egea@medicina.ub.es.
† Present address: DPC Dipesa, Dept. Técnico-Científico, 08029 Barcelon, Spain.

There is also increasing evidence implicating the Rho family of small GTPases in membrane trafficking (Ridley, 2001 for review), particularly Rho and Rac in endocytic processes (Lamaze *et al.*, 1996; Chimini and Chavrier, 2000; Garrett *et al.*, 2000; Jou *et al.*, 2000; West *et al.*, 2000; Ellis and Mellor, 2000 for review). With respect to the secretory pathway, our findings suggest that Rho does not regulate actin–Golgi interactions (Valderrama *et al.*, 2000). However, Cdc42 is reported to be associated with Golgi membranes, and it binds to the γ component of the coatomer (Erickson *et al.*, 1996; Wu *et al.*, 2000). Furthermore, Cdc42 is involved in cell polarity, regulating the generation of basolateral transport vesicles from the *trans*-Golgi network (TGN; Kroschewski *et al.*, 1999; Cohen *et al.*, 2001; Müsch *et al.*, 2001; Rojas *et al.*, 2001). Hence, Cdc42 seems to be involved in ER-to-Golgi and post-Golgi protein transport.

Because actin is implicated in the Golgi-to-ER protein transport (Valderrama *et al.*, 2001) and Cdc42 is located in the Golgi complex, we examined the possible regulatory role of Cdc42 in the ER/Golgi membrane dynamics in nonpolarized mammalian cells. We show that Cdc42 controls the Golgi-to-ER protein transport via N-WASP, a homologue of the Wiskott-Aldrich syndrome protein (WASP). These re-

sults are consistent with our proposal that Cdc42 exerts its effects on retrograde transport machinery by directly modulating actin dynamics at the ER/Golgi interface, probably through the Arp2/3 complex.

MATERIALS AND METHODS

Material and Expression Constructs

The GFP expression vectors for the wild-type N-WASP and N-WASP(ΔWA) forms were previously reported (Moreau *et al.*, 2000), and those for the wild-type and the dominant-positive and dominant-negative Cdc42 forms (del Pozo *et al.*, 1999) were kindly provided by Francisco Sánchez-Madrid (Hospital La Princesa, Madrid). Cy3-tagged native Shiga toxin fragment B was a gift from Ludger Johannes and Bruno Goud (Institute Curie, Paris). The dominant-negative Sar1 mutant (Sar1[H79G], $Sar1^{dn}$) expression vector (Kuge *et al.*, 1994) was supplied by Rainer Pepperkok (EMBL, Heidelberg). Polyclonal antibodies against KDEL receptor, Gal-T, giantin, Man II, and GFP were provided by H.-D. Soling (University of Götingen, Götingen), E. Berger (University of Zürich, Zürich), H.-P. Hauri (Biozentrum, Basel), K. Moremen (University of Georgia, Georgia), and D. Shima (Imperial Cancer Research Fund, London), respectively. Monoclonal P5D4 anti–VSV-G protein antibody was from Sigma Chemical Co. (St. Louis, MO). DMEM and fetal calf serum (FCS) were from Life Technologies/Brl Life Technologies (Paisley, UK); secondary TRITC or FITC $F(ab')_2$ fragments were from Boehringer Mannheim (Mannheim, Germany). Cascade blue dextran was from Molecular Probes (Eugene, OR) and Mowiol was from Calbiochem (Nottingham, UK). Unless otherwise stated, all other chemicals were from Sigma Chemical Co.

Cell Culture

HeLa and NRK cells were cultured in DMEM medium containing 10% FCS supplemented with 10 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown in a humidified incubator at 37°C and 5% CO_2.

Microinjection Experiments

For microinjection, HeLa and NRK cells were grown for 1–2 d on Eppendorf Cellocate coverslips (Hamburg, Germany) or on normal glass coverslips. For single- or double-microinjection experiments, the GFP-Cdc42 and GFP-N-WASP constructs and the recombinant $Sar1^{dn}$ construct were first diluted to 50–100 ng/ml and then microinjected into the nuclei with an Automated Microinjection System (Model 5242; Eppendorf). Cells for microinjection experiments were cultured in DMEM plus 10% FCS medium containing 25 mM HEPES and supplemented with penicillin, streptomycin, and glutamine. After microinjection, the coverslips were transferred to a Petri dish containing fresh culture medium and returned to the incubator for expression. For the control $Sar1^{dn}$ experiments, the nuclei of cells were microinjected with the cascade blue–conjugated dextran as a microinjection marker. For comicroinjection experiments with Cdc42 or N-WASP constructs, GFP-induced fluorescence was used to identify microinjected cells.

Transient Transfection Experiments

The transfection method used was FuGENE 6 (Roche Diagnostics Corporation, Mannheim, Germany). Briefly, the cells were plated 1 day before transfection in coverslips at 50–80% confluence and incubated overnight. To prepare FuGENE 6 reagent and DNA complex, FuGENE 6 and DNA were mixed in a 3:1 proportion (μl and μg, respectively) and serum-free medium was added to a final volume of 100 μl. The complex was gently mixed and incubated for 15 min at room temperature. Meanwhile, the coverslips were washed with serum-free medium, and the appropriate volume of serum-free medium was added. Finally, the FuGENE:DNA complex and a 5% of FCS were added to the cells for overnight incubation.

VSV-G and Shiga Toxin Transport Assays

Infection with the temperature-sensitive mutant ts045 VSV was performed as described elsewhere (Valderrama *et al.*, 1998). Indirect immunofluorescence transport of VSV-G from ER-to-Golgi complex was performed following Bonatti *et al.* (1989).

For the native ST-B transport experiments, HeLa cells were first incubated for 30 min in binding medium (FCS-free DMEM) and treated with Cy3-Shiga toxin B-fragment for 45 min at 4°C, and the unbound toxin was then washed for 5 min in ice-cold PBS. Thereafter, cells were incubated with DMEM at 20°C for 2 h to accumulate the internalized ST-B in early/recycling endosomes. They were then heated to 37°C to synchronize the ST-B transport to the ER via the Golgi complex.

Indirect Immunofluorescence

Indirect immunofluorescence was carried out as previously described (Valderrama *et al.*, 1998, 2000) with the following antibody dilutions: anti-KDELr, 1:1000; anti–Gal-T, 1:100; antigiantin, 1:500; anti–VSV-G, 1:50; and FITC/TRITC-conjugated secondary antibodies, 1:35. The coverslips were mounted on microscope slides using Mowiol (Calbiochem). Microscopy and imaging were performed with an Olympus BX60 epifluorescence microscope with a cooled Olympus CCD camera (Lake Success, NY) or a Leica TCS-NT confocal microscope (Heerbrugg, Switzerland). The images were processed on PCs computers using Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA).

Immunoelectron Microscopy

HeLa cells transfected with the GFP-Cdc42 expression vector constructs were processed for cryosectioning as described elsewhere (Martínez-Menárguez *et al.*, 1999). Briefly, cells were fixed overnight with 2% paraformaldehyde plus 0.2% glutaraldehyde in 0.1 M phosphate buffer, pelleted by centrifugation, embedded in 10% gelatin, and cut into small blocks. The blocks were infused with 2.3 M sucrose, frozen in liquid nitrogen, and stored for cryoultramicrotomy. Cryosections were single-immunolabeled with rabbit polyclonal antibodies against GFP followed by protein A-gold. Samples were visualized in a Philips Tecnai 12 electron microscope (Eindhoven, The Netherlands). To establish the relative distribution of GFP-Cdc42V12 and GFP-Cdc42N17 in the Golgi area, gold particles were counted and ascribed to one of the following categories: lateral (defined as the lateral zones of the Golgi cisterna showing its characteristic dilatation), flattened central portions of the Golgi cisternae, peri-Golgi transport intermediates, and nonmembrane structures. The number of the gold particles assigned to each category was expressed as a percentage of the total labeling in the Golgi area. A total of 25 randomly selected Golgi areas were analyzed. Statistical analysis was performed using the Student's *t* test.

RESULTS

Cdc42 Is Located in the Lateral Rims of the Golgi Cisternae and Golgi-associated Transport Intermediates

GFP-tagged Cdc42 proteins (wild-type, Cdc42WT; "activated" Cdc42, Cdc42V12; "dominant-negative" Cdc42, Cdc42N17) were observed in the cytoplasm and plasma membrane, but also in the Golgi complex 3–4 h after microinjection of cDNAs into the nucleus of NRK (Figure 1A) or HeLa cells (unpublished results). The Golgi localization was confirmed by double immunolabeling experiments with an-

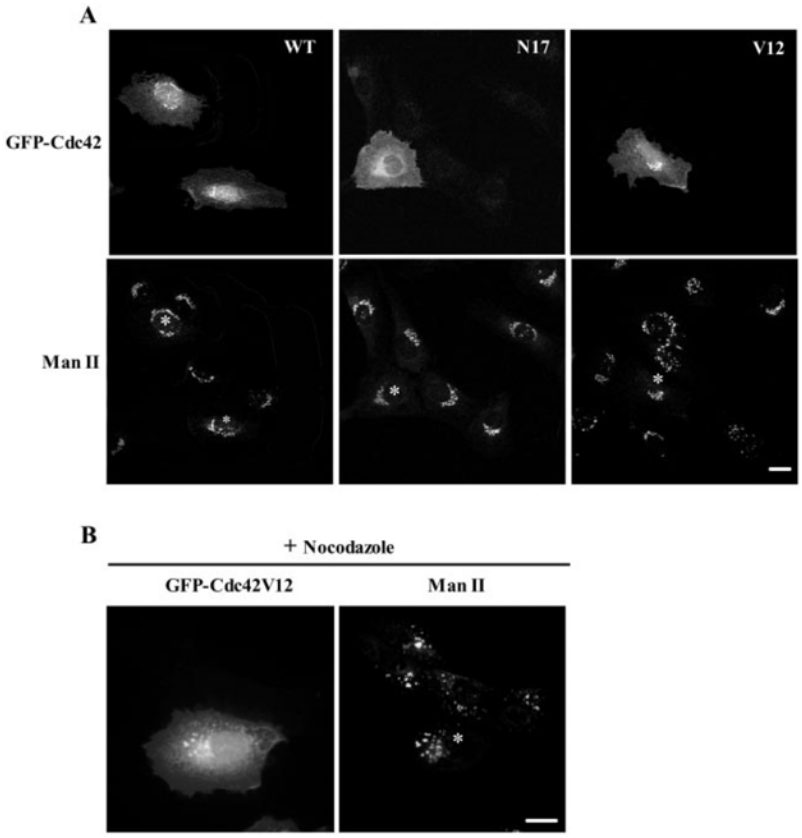

Figure 1. GFP-Cdc42 is localized in the Golgi complex. (A) NRK cells were microinjected into the nucleus with expressing vectors for wild-type (WT), dominant-negative (N17), and dominant-positive (V12) GFP-Cdc42 forms. After microinjection (3–4 h), the cells were processed for immunofluorescence microscopy and examined by confocal microscopy. The organization of the Golgi complex was visualized with anti–Mannosidase II (ManII) antibodies. Bar, 10 μm. (B) Microinjected NRK cells (asterisks) with GFP-Cdc42V12 construct were, after 3–4 h of expression, treated with nocodazole, which causes fragmentation and dispersal of Golgi fragments throughout the cell. The Golgi-associated Cdc42 colocalizes with ManII in small punctate Golgi structures. Bar, 10 μm.

ti–Mannosidase II antibodies and by Golgi-disruption experiments with nocodazole (Figure 1B) or BFA (unpublished results). The GFP-Cdc42 signal in the Golgi complex, at the light microscopy level, was more intense for the wild-type and activated Cdc42 than the dominant-negative Cdc42N17 (Figure 1A). The presence of Cdc42 in the Golgi complex has been recently reported in astrocytes also using the GFP-tagged form of wild-type Cdc42 and in fibroblasts using polyclonal antibodies raised against a peptide mapping near the carboxy terminus of the human Cdc42 protein (Erickson *et al.*, 1996; Etienne-Manneville and Hall, 2001).

We next examined the subcellular localization of the activated and dominant-negative forms of GFP-tagged Cdc42 in HeLa cells using cryoimmunoelectron microscopy (Figure 2, A–C, and Table 1). Only transfected cells were labeled with anti-GFP antibodies demonstrating the specificity of the labeling (Figure 2A). In cells transfected with Cdc42V12, gold particles were visualized in the plasma membrane (Figure 2A) and in the Golgi area (Figure 2, B and C) but also to a lesser extent throughout the cytoplasm (nonmembrane bound; Table 1). In the Golgi region, GFP-Cdc42V12 was present in the Golgi cisternae (Figure 2A) and associated vesicles (Figure 2C). Within the Golgi stack, Cdc42 was enriched in the lateral portions of the Golgi cisternae (indicated in Figure 2B as double-headed arrows). Some of the reactive peri-Golgi transport intermediates (TIs) showed the typical 10-nm-thick COPI coat (Figure 2C). Quantitative ultrastructural analysis for Cdc42N17 transfected cells showed that the inactive Cdc42 mutant was mostly nonmembrane bound, and when observed in the Golgi stack, it was uniformly distributed along cisternae (Table 1). These ultrastructural observations suggest a role of Cdc42 in Golgi-derived intracellular trafficking.

ER-to-Golgi Protein Transport Is Cdc42 Independent

Activated Cdc42 specifically interacts with the γ subunit of coatomer, suggesting a direct role in vesicular transport (Wu *et al.*, 2000). We examined whether the ER-to-Golgi transport of VSV-G was blocked in HeLa cells expressing Cdc42 mutants. In control cells, ts045 VSV-G mutant moves from the ER to the Golgi complex when cells are transferred from restrictive (40°C) to permissive temperature (32°C; Figure 3 C-F; GFP-Cdc42-expressing cells were detected by GFP fluorescence, marked by an asterisk). The kinetics of the ER-to-Golgi transport of VSV-G was monitored by immunofluorescence, which revealed that this transport remained unaltered in HeLa cells overexpressing the wild-type, activated or dominant negative Cdc42. The unaltered ER-to-Golgi transport was not due to the lack of signaling activity by the expressed GFP-Cdc42 proteins, because cells transfected with activated GFP-Cdc42 showed the expected filopodia formation (unpublished results).

We next tested whether the rebuilding of the Golgi complex after BFA removal is impaired by overexpression

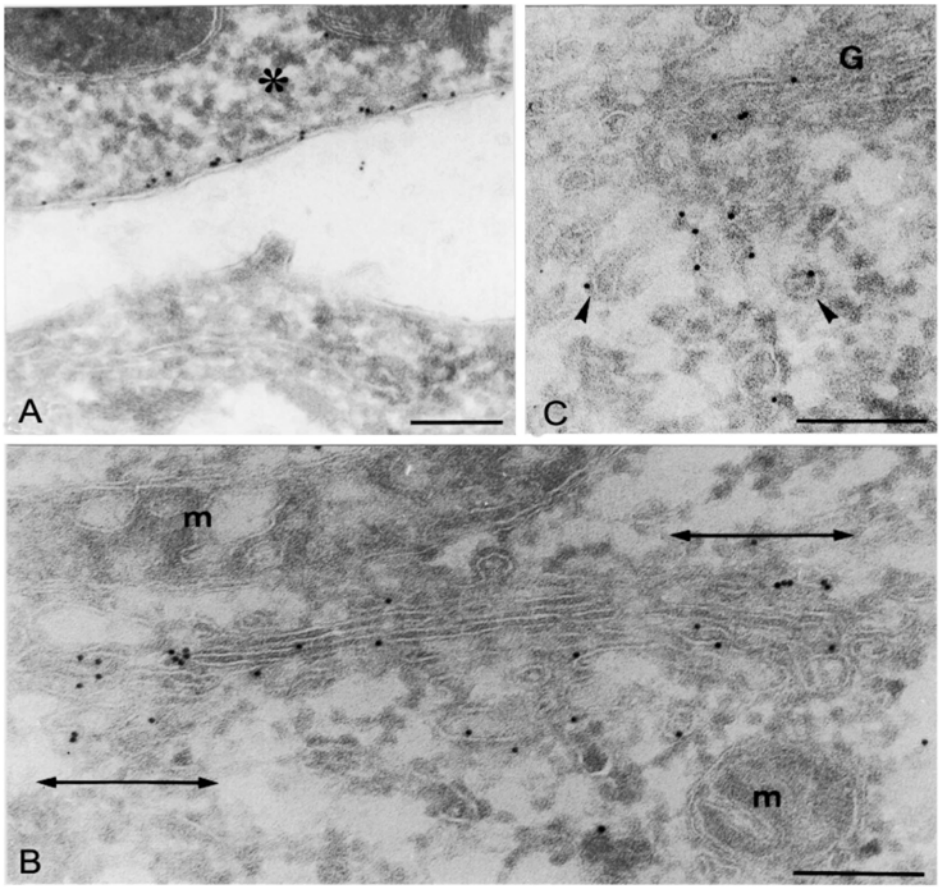

Figure 2. Activated GFP-Cdc42 is enriched in the lateral portions of Golgi cisternae and in peri-Golgi transport intermediates. Transfected HeLa cells with GFP-Cdc42V12 vector were fixed and processed for cryoimmunogold electron microscopy using polyclonal antibodies to GFP. Note the gold decoration for GFP-Cdc42V12 in the cytoplasmic part of the plasma membrane in a transfected cell (asterisk), whereas no gold particles are observed in the neighboring nontransfected cell (A). In the Golgi area (B and C), activated GFP-Cdc42 is predominantly located in the lateral portions of the Golgi cisternae (B) and in COPI-coated (arrowheads) and noncoated peri-Golgi transport intermediates (C). G, Golgi stack; m, mitochondria. Bars, 200 nm.

of Cdc42 mutants. Because ER-to-Golgi transport of VSV-G was unaltered, we reasoned that the rebuilding of the Golgi complex would not be affected. Transiently transfected HeLa cells expressing Cdc42 variants were first treated with BFA to induce fusion of Golgi membranes with the ER. Subsequently, BFA was withdrawn and the kinetics of the morphological appearance of the Golgi complex was examined by immunofluorescence. We found no significant differences in the reformation of perinuclear Golgi complex in these conditions (unpublished results). Thus, Cdc42 is not involved in ER-to-Golgi transport or the rebuilding of the Golgi complex.

Golgi-to-ER Membrane Flow and the Subcellular Distribution of the KDEL Receptor Are Altered in Cells Overexpressing Cdc42

We analyzed whether Cdc42 is involved in Golgi-to-ER membrane dynamics and transport. We first monitored whether the kinetics of the Golgi complex disassembly induced by BFA was Cdc42 dependent (Figure 4). Transfected Hela cells were treated with BFA and processed for immunolabeling at various times. The kinetics of the BFA-induced Golgi membranes merging into the ER remained unaltered in cells expressing wild-type or dominant-negative forms of

Table 1. Subcellular distribution of the gold labelling in the Golgi area of HeLa cells transfected with GFP-tagged activated and dominant-negative Cdc42 constructs (Cdc42V12 and Cdc42N17, respectively)

	Golgi cisternae		Peri-Golgi transport intermediates	Non–membrane bound
	Lateral portions	Central portions		
Cdc42V12	36.6 ± 3.2*	23.8 ± 3.5*	33.4 ± 3.2	6.3 ± 1.8
Cdc42N17	16.2 ± 4.0	13.5 ± 4.0	16.6 ± 4.1	53.7 ± 5.3

Numbers represent the percentages (mean ± SEM) of the total gold labeling over the different locations of the Golgi area. See MATERIALS AND METHODS for details. * Statistical Student's *t* test; $p \leq 0.01$.

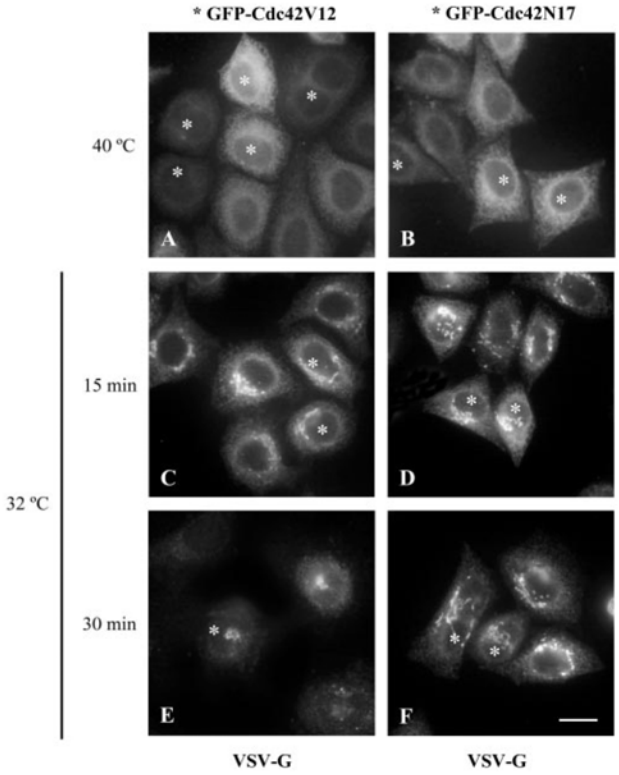

Figure 3. The ER-to-Golgi transport is unaltered in cells expressing Cdc42. HeLa cells were transfected with GFP-Cdc42V12 or GFP-Cdc42N17 vectors and incubated for 12 h. Subsequently, the cells were infected with the thermosensitive ts045 VSV-G mutant incubated at restrictive temperature (40°C). At this temperature VSV-G protein is retained in the ER (A and B). When cells were transferred to the permissive temperature of 32°C, the VSV-G glycoprotein exited the ER and was transported to the Golgi complex (C–F). At indicated transport times (C–F), cells were processed for indirect immunofluorescence for VSV-G glycoprotein. In this and the other panels, the GFP-Cdc42-expressing cells were detected by GFP fluorescence, as marked by asterisks (*). Note that both the dominant-positive (A, C, and E, asterisks) and dominant-negative Cdc42 mutants (B, D, and F, asterisks) show the same kinetics of transport of the VSV-G from the ER to the Golgi complex as the nontransfected neighboring cells. Bar, 10 μm.

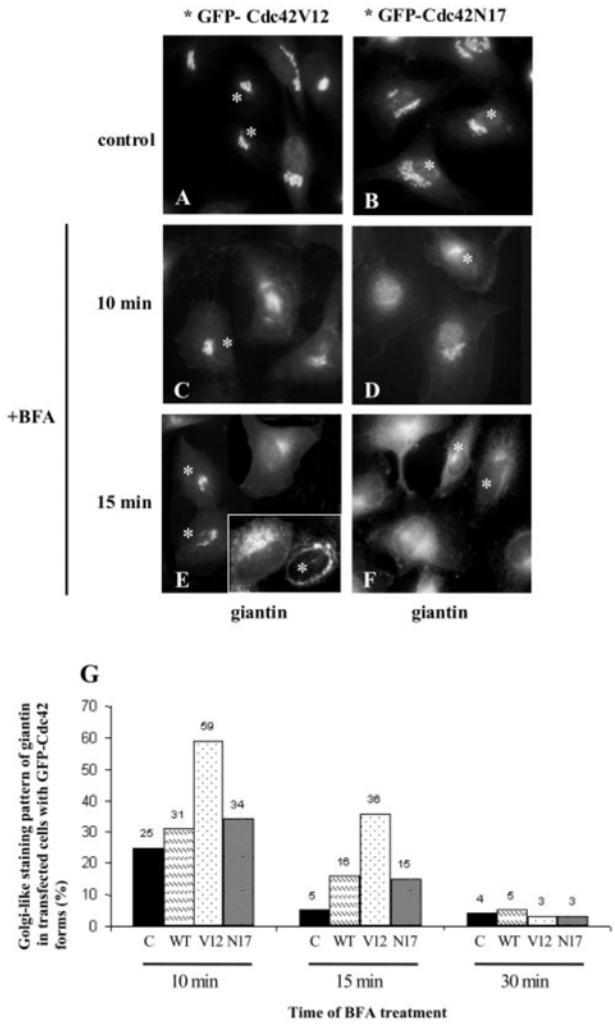

Figure 4. The kinetics of Golgi disassembly is slowed in cells expressing activated Cdc42. HeLa cells were incubated for 12 h after transfection with the GFP-Cdc42 vectors. The cells were treated with BFA (5 μg/ml) and the kinetics of the fusion of Golgi membranes with the ER was monitored by immunofluorescence using antibodies against the Golgi protein giantin. After BFA treatment, the pericentriolar Golgi complex can be visualized in transfected cells with GFP-Cdc42V12 (C and E, asterisks) but not in cells expressing the GFP-Cdc42N17 (D and F, asterisks) or in neighboring nontransfected cells (C–F). Inset in E shows the characteristic BFA-induced Golgi tubulation in a transfected cell with GFP-Cdc42V12 mutant (asterisk), whereas the neighbor nontransfected cells show a more ER-like staining pattern. (G) Quantitative analysis of the morphological observations. Data represent the average of two independent experiments, in which at least 200 cells were counted for each experiment. (C, nontransfected cells; WT, cells transfected with the wild-type form of Cdc42; V12, cells transfected with Cdc42V12 mutant; N17, cells transfected with Cdc42N17). Bar, 10 μm.

Cdc42 (Figure 4, D, F, and G). In contrast, in cells expressing activated Cdc42V12 the Golgi disassembly was significantly slower (Figure 4, C, E, and G).

To confirm that Cdc42 is involved in Golgi-to-ER membrane dynamics, we examined the subcellular redistribution of KDEL receptor (Figure 5). The steady state distribution of this protein changed from mainly Golgi-like (Figure 5, A and B) to a punctate cytoplasmic staining pattern when HeLa cells were transferred from 37–15°C (Figure 5, C–H). This is because the KDEL receptor is trapped in the intermediate compartment at this temperature. When cells expressing activated Cdc42 were incubated at 15°C, a larger percentage of KDELr molecules remained in a juxtanuclear Golgi-like compartment (Figure 5, C, E, and G, asterisks). In contrast, their nontransfected neighboring cells (Figure 5, C, E, and G) or cells transfected with the dominant-negative Cdc42 (Figure 5, D, F, and H, asterisks) showed no delay (Figure 5I for a quantitative analysis). These results show that Cdc42 is

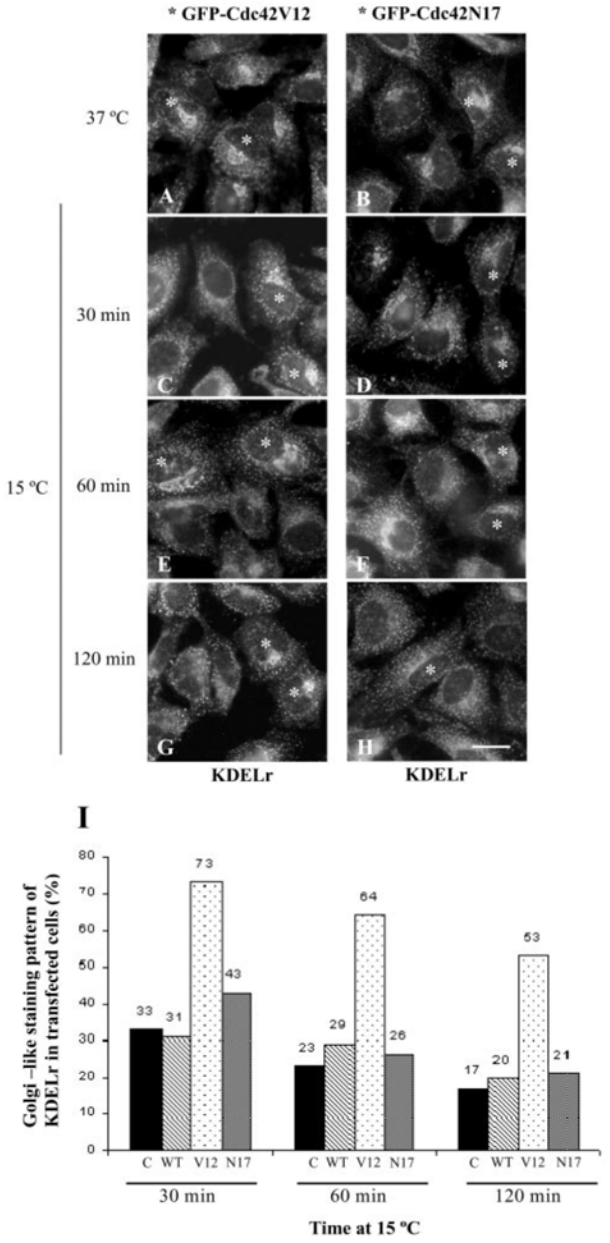

Figure 5. Cdc42 blocks the redistribution of the KDEL receptor when cells are incubated at 15°C. HeLa cells were transfected as described in the legends of Figures 3 and 4. At 37°C, the steady state distribution of the KDEL receptor (KDELr) shows both the Golgi-like and a diffuse punctate staining. When cells were incubated at 15°C for various times, the steady state distribution of KDELr changes to exclusive punctate staining in nontransfected (C–H) and GFP-Cdc42N17–transfected cells (asterisks in D, F, and H). In contrast, in GFP-Cdc42V12–transfected cells, the KDELr shows the Golgi-like staining pattern (C, E, and G; asterisks). (I) A quantitative visual analysis of the percentage of neighboring nontransfected (control, C) and GFP-Cdc42–transfected cells (WT, N17, and V12 forms) with a Golgi-like staining pattern. Results are the average of two independent experiments, in which at least 200 cells were counted for each experiment. Bar, 10 μm.

involved in the retrograde arm of bidirectional transport between the ER and the Golgi complex.

Golgi-to-ER Transport of Shiga Toxin and Golgi Enzymes Is Blocked by the Overexpression of Cdc42

From the previous experiments, we hypothesized that Cdc42 regulates protein recycling from Golgi to the ER. To confirm this, we studied the Golgi-to-ER trafficking of the Golgi-resident protein galactosyltransferase (Gal-T) and a cargo marker, Shiga toxin. Microinjection of the GTPase-deficient Sar1 mutant protein (Sar1[H79G], Sar1^{dn}) into living cells blocks the recycling of Golgi proteins and traps them in the ER (Aridor *et al.*, 1995; Storrie *et al.*, 1998; Seemann *et al.*, 2000). We expressed Sar1^{dn} either alone or together with GFP-Cdc42 constructs (Figure 6). After microinjection of the expression constructs, cells were incubated at 37°C for 6–7 h and processed for indirect immunofluorescence. Expression of Sar1^{dn} led to the expected ER accumulation of Gal-T (Figure 6A, asterisk) and the mixed ER-like and punctate staining patterns for the KDEL receptor (Figure 6D, asterisk). Similar results were obtained when cells were comicroinjected into the nucleus with vectors expressing Sar1^{dn} and the negative mutant GFP-Cdc42N17 (Figure 6, C and F, asterisks). In contrast, microinjected cells coexpressing Sar1^{dn} and the wild-type GFP-Cdc42WT or activated GFP-Cdc42V12 mutant showed the characteristic Golgi-like staining pattern for Gal-T (Figure 6B, asterisks) and the KDEL receptor (Figure 6E, asterisk). Both staining patterns were similar to that shown by neighboring noninjected cells. A quantitative analysis of these morphological results is shown in Figure 6G.

Similar experiments were performed using Shiga toxin (Figure 7), a well-established cargo marker of the Golgi-to-ER protein transport pathway (Sandvig *et al.*, 1992). Sar1^{dn} was microinjected into the nucleus of HeLa cells and incubated at 37°C for 90 min. Thereafter, cells were incubated with native cy3-Shiga toxin (ST-B) for 2 h at 20°C, the result of which was that the internalized toxin accumulated to early/recycling endosomes (Mallard *et al.*, 1998). Cells were subsequently transferred to 37°C to synchronize the ST-B transport to the ER. In Sar1^{dn} microinjected cells, the Golgi localization of ST-B was replaced by a diffuse cytoplasmic staining pattern, which is characteristic of the ER (Figure 7, C and E, asterisks). This is not the case of the neighboring nonmicroinjected cells, which showed a permanent steady state Golgi localization for ST-B (Figure 7, C and E). This is because native ST-B cycles continuously between the Golgi and the ER, but its passage through the ER is rapid (Johannes and Goud, 1998). Notice that, after 3 h of expression of Sar1^{dn}, the Golgi complex was virtually unaltered as assessed by Gal-T staining (Figure 7E, inset and asterisk). This illustrates that the appearance of ST-B in the ER is caused by its transport from the Golgi and not merely the merging of Golgi and ER membranes induced by Sar1^{dn}. Once in the ER, ST-B is retained by the blocking effect of Sar1^{dn} protein on the COPII-dependent ER export machinery (Barlowe, 1998, for review). When cells were comicroinjected with Sar1^{dn} and GFP-Cdc42V12 (asterisks in Figure 7, D and F), ST-B remained in the Golgi with a steady state distribution indistinguishable from that of the neighboring nonmicroinjected cells. This was not observed when cells were comicroinjected with Sar1^{dn} and the dominant-negative Cdc42N17 (see Figure 7G for the quantitative analysis of morphological observations). These data indicate that overexpression of Cdc42WT or Cdc42V12 impairs the Golgi-to-ER membrane transport.

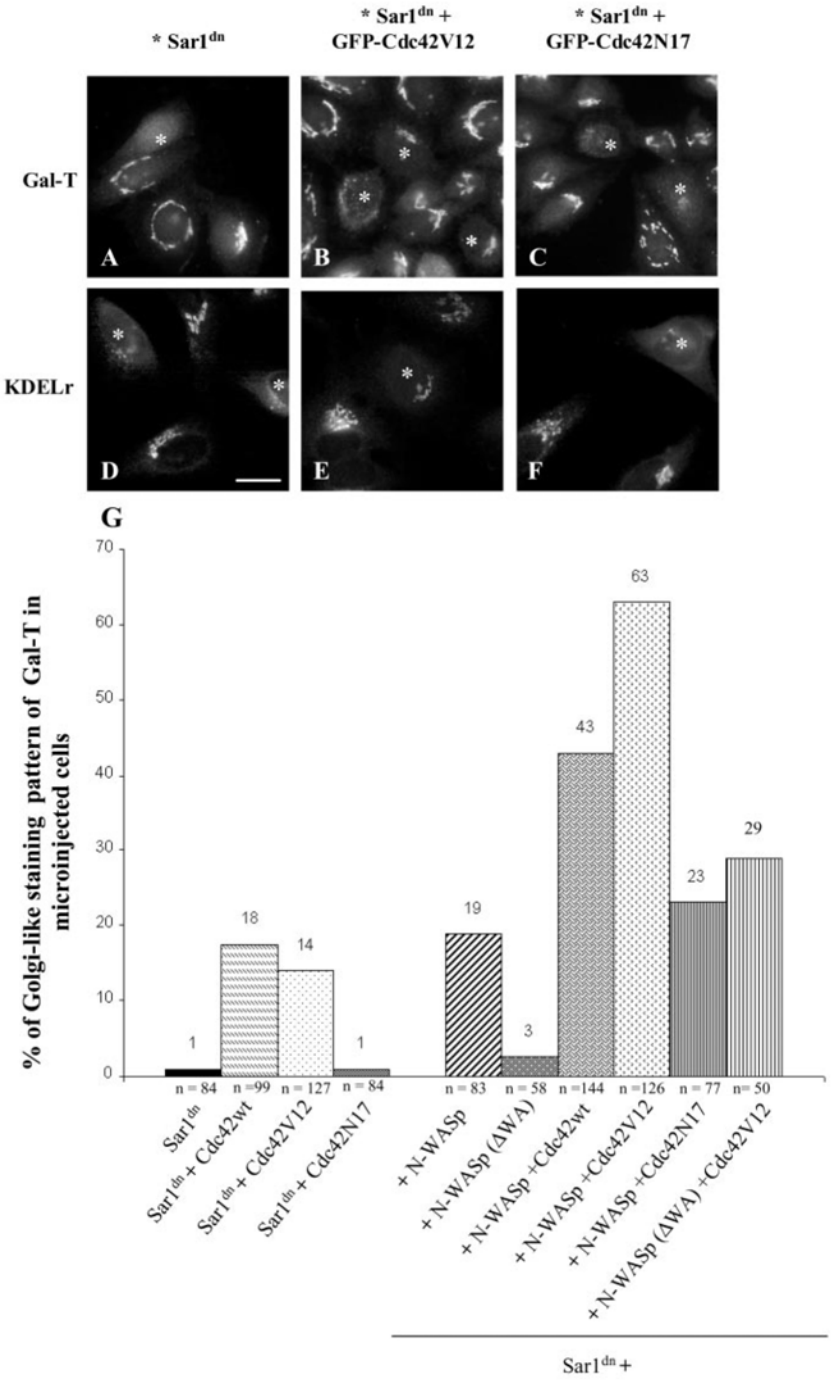

Figure 6. Cdc42 blocks the Sar1^{dn}-induced ER accumulation of Golgi enzymes and the KDEL receptor. Sar1^{dn} construct alone (A and D; asterisks) and Sar1^{dn} plus GFP-Cdc42V12 (B, E; asterisks) or GFP-Cdc42N17 mutant constructs (C and F; asterisks) were microinjected into the nucleus of HeLa cells. After 7 h of expression, the cells were fixed and processed for indirect immunofluorescence with antibodies against galactosyltransferase (Gal-T; A–C) or the KDEL receptor (KDELr; D–F). In comicroinjected cells with Sar1^{dn} plus activated GFP-Cdc42, Gal-T (B, asterisks), and KDELr (E, asterisk) reveal a Golgi-like staining pattern like the neighboring control (nonmicroinjected) cells. In contrast, cells microinjected with Sar1^{dn} alone (A and D; asterisks) or with Sar1^{dn} plus the dominant-negative GFP-Cdc42N17 (C and F; asterisks) show the characteristic ER accumulation of both Gal-T and KDELr induced by Sar1^{dn}. (G) Microinjected cells were visually quantified for the presence of Gal-T in the Golgi complex. Results are the mean of two independent experiments, and the number of counted microinjected cells is indicated (n). Bar, 10 μm.

Activated Cdc42 Recruits N-WASP to the Golgi Complex

WASP and its ubiquitous form N-WASP bind, among others, to Cdc42 and PIP_2, thus integrating and coordinating the signaling pathways that control actin nucleation/polymerization (Snapper and Rosen, 1999 for review; Rohatgi *et al.*, 2000). Hence, we examined whether the regulatory effect of Cdc42 on the retrograde Golgi-to-ER membrane transport requires N-WASP. Cells were coinjected with the GFP-tagged wild-type form of N-WASP and untagged forms of Cdc42. In cells microinjected with GFP-N-WASP alone or together with Cdc42N17, no colabeling of N-WASP (visualized by the GFP fluorescence signal) with Gal-T in the Golgi

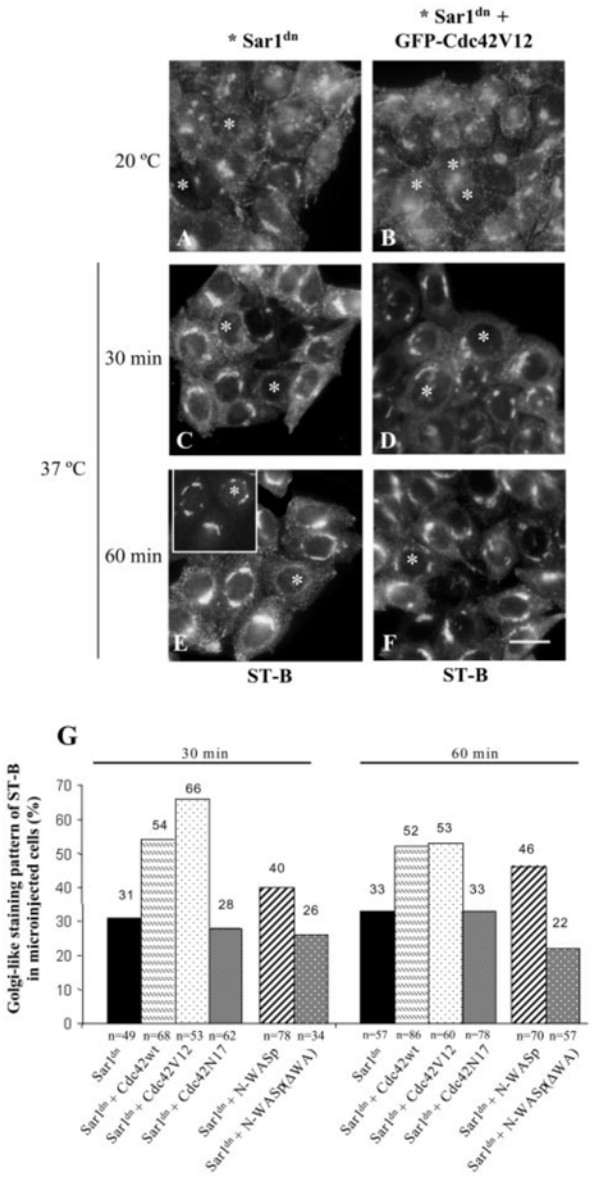

Figure 7. Cdc42 blocks transport of native Shiga toxin B-fragment from Golgi to the ER. HeLa cells were microinjected as described in Figure 6. After 1.5 h of expression, cells were incubated with cy3-tagged native Shiga toxin (ST-B) for 45 min at 37°C, rinsed, and transferred to 20°C for 2 h to allow internalized ST-B to be retained in the early/recycling endosomes (A and B). Cells were then shifted to 37°C to elicit retrograde transport to the ER via the Golgi complex (C–F). Unlike $Sar1^{dn}$-expressing cells (C and E; asterisks), ST-B remained accumulated in the Golgi complex in GFP-Cdc42V12 expressing cells (D and F; asterisks). After 60 min of transport, the Golgi complex (stained to giantin) remained virtually unaltered in $Sar1^{dn}$-expressing cells (E, insert; asterisk), but ST-B (asterisk in E) showed the expected ER-like staining pattern as a consequence of its transport from Golgi to the ER. Cells shown in E and its inset were double-stained with antibodies to giantin and ST-B. (G) Quantitative validation of the morphological data in cells microinjected with different vectors. Results are the mean of two independent experiments and the number of counted microinjected cells is indicated (n). Bar, 10 μm.

complex was observed (Figure 8A, A′, and A″). However, GFP-N-WASP was located in the Golgi complex in cells coinjected with Cdc42V12 (Figure 8, B, B′, and B″). These morphological observations indicate that activated Cdc42 induces the recruitment of N-WASP to the Golgi complex.

N-WASP Mediates Golgi-to-ER Transport Inhibition Induced by Cdc42

To examine the functional involvement of N-WASP in the Golgi complex and in retrograde protein transport, we coinjected GFP-N-WASP and $Sar1^{dn}$ and monitored the accumulation of Gal-T in the ER by immunofluorescence. In cells coexpressing $Sar1^{dn}$ and the GFP-N-WASP, Gal-T was retained in the Golgi complex (Figure 9A, left, asterisks). In contrast, cells expressing $Sar1^{dn}$ and GFP-N-WASP(ΔWA), a mutant that lacks the Arp2/3 binding domain and thus blocks endogenous N-WASP for membrane binding and target(s), showed no inhibitory effect on the $Sar1^{dn}$-induced ER accumulation of Gal-T (Figure 9A, right, asterisk). We found a much higher percentage of cells that showed inhibition in the retrograde transport of Golgi enzymes when the cells coexpressed N-WASP with wild-type Cdc42 or with dominant positive Cdc42V12 (Figure 6G). This was not the case for cells coexpressing N-WASP and the dominant negative Cdc42N17, which showed a similar inhibitory effect to N-WASP alone (Figure 6G). Nonetheless, this was expected because, unlike Cdc42V12, Cdc42N17 does not bind to WASP/N-WASP (Burbelo *et al.*, 1995). The coexpression of Cdc42V12 and N-WASP(ΔWA) also resulted in the accumulation of Gal-T in the ER (Figure 10). Therefore, N-WASP(ΔWA) alleviate the expected negative regulation of the retrograde transport by activated Cdc42 (Figure 6G).

Similar results were also obtained when the retrograde transport of ST-B was examined. The coexpression of $Sar1^{dn}$ plus GFP-N-WASP blocked the transport of ST-B from Golgi to the ER (Figure 9B, left, asterisks). This did not occur when cells coexpressed $Sar1^{dn}$ plus GFP-N-WASP(ΔWA) (Figure 9B, right, asterisks). A quantitative validation of these morphological observations is shown in Figure 7G. Together, results indicate that Cdc42 regulates the Golgi-to-ER protein transport via N-WASP.

DISCUSSION

Cdc42 participates in the maintenance and establishment of cell polarity (Adams *et al.*, 1990; Stowers *et al.*, 1995; Etienne-Manneville and Hall, 2001; Gotta *et al.*, 2001), and it is also involved in sorting at the TGN by regulating post-Golgi trafficking and generation of vesicles in polarized MDCK cells (Kroschewski *et al.*, 1999; Cohen *et al.*, 2001; Müsch *et al.*, 2001; Rojas *et al.*, 2001). However, the involvement of the Rho family of GTPases in the early steps of the secretory pathway is unknown. We have previously demonstrated that the Golgi-associated actin filaments are not regulated by Rho A (Valderrama *et al.*, 2000). However, recent results suggest that Cdc42 is a key regulator in secretory pathway trafficking because (1) both Cdc42 and its binding partner IQGAP are Golgi-associated in an ARF-dependent manner (Erickson *et al.*,

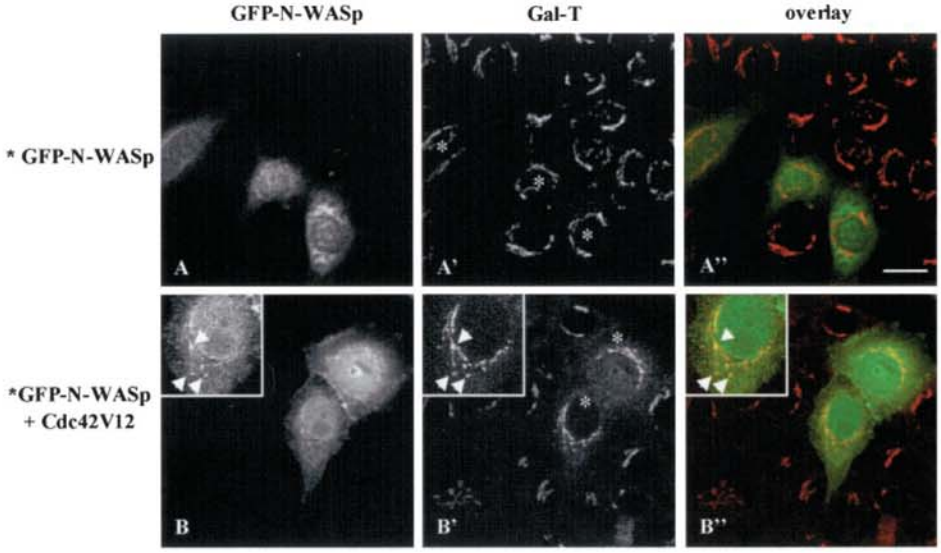

Figure 8. Activated Cdc42 recruits N-WASP to the Golgi complex. HeLa cells were coinjected with GFP-tagged wild-type N-WASP vector alone (A) or together with untagged Cdc42V12 construct (B). After 4 h of expression, cells were stained with antibodies to Gal-T and examined by confocal microscopy. Unlike cells expressing GFP-N-WASP alone (A, A', and A''), GFP fluorescence corresponding to N-WASP (B) in cells that coexpress Cdc42V12 partially colocalize with Gal-T in the Golgi complex (B') by the appearance of yellow color when images were superimposed (B'', overlay). Bars, 10 μm.

1996; McCallum *et al.*, 1998; Etienne-Manneville and Hall, 2001); and (2) Cdc42 governs Golgi complex polarization in wounded cells (Nobes and Hall, 1999), and it binds to the γ component of the COPI coatomer (Wu *et al.*, 2000). We have previously demonstrated that actin is involved in Golgi-to-ER transport but not in the ER-to-Golgi protein transport or in the Golgi rebuilding (which occurs after BFA withdrawal; Valderrama *et al.*, 2001). We here show that Cdc42 is not required for ER-to-Golgi transport either. These results are at variance with those of Wu *et al.* (2000), who reported that a rapid cycling mutant of Cdc42 that spends more time in the GTP-bound form (Cdc42F28L) interacts with γCOP. In addition, the expression of Cdc42F28L modestly stimulates secretory protein transport as measured by acquisition of carbohydrate modification after release of the VSV-G from the ER. In accordance with the biochemical observations of Wu *et al.*, (2000), our ultrastructural data show that Cdc42V12 is located to COPI-coated transport intermediates, which more likely are involved only in retrograde Golgi-to-ER transport (Letourneur *et al.*, 1994; Martínez-Menárguez *et al.*, 2001). Our results with Cdc42V12 are consistent with this idea. Unfortunately, Wu *et al.* (2000) only examined anterograde transport. Finally, the discrepancy of the results in the anterograde transport could simply be attributable to the use of different Cdc42 mutants in the two studies.

Molecular Mechanisms Regulating Actin-Golgi Membranes Interaction

The dissection of the actin-membrane interface is critical for understanding the events that occur at the Golgi membranes during transport and signaling. Endogenous vesicles in extracts occasionally nucleate actin polymerization (Taunton, 2001 for review). Small GTPases of the Rho family regulate actin dynamics through numerous downstream effectors (Hall, 1998) such as members of the Wiskott-Aldrich syndrome protein family (WASP/N-WASP; Aspenstrom *et al.*, 1996; Symons *et al.*, 1996), phospholipase D (PLD; Han *et al.*, 1998), phosphatidylinositide 3-kinase (PI3K; Zheng *et al.*, 1994), and IQGAPs (McCallum *et al.*, 1998) among others. Both PLD and PI3K are involved in the generation of transport carriers and post-Golgi trafficking, respectively (Corvera and Czech, 1998, Roth *et al.*, 1999 for reviews). In this respect, we have reported that PI3K seems to regulate the association of actin microfilaments with the Golgi complex (di Campli *et al.*, 1999), which could be complementary to the effects of N-WASP (see below). A much higher percentage of microinjected cells showed an inhibition in the retrograde $Sar1^{dn}$-induced ER accumulation of Golgi enzymes when N-WASP was coexpressed with Cdc42WT or Cdc42V12. These results indicate that N-WASP transduces signals from Cdc42 to the nucleation/polymerization of actin and it could give rise to the following situations: (1) The Golgi membranes nucleate and polymerize actin as do phagosomes (Defacque *et al.*, 2000), endosomes, and lysosomes (Taunton *et al.*, 2000). Preliminary data indicate that Golgi membranes promote actin nucleation (T. Babià and G. Egea, unpublished observations); (2) retrograde transport intermediates form actin comet tails for their propulsion through Arp2/3 complex, similar to those induced by *Listeria*, *Shigella*, and Vaccinia and in raft-enriched secretory and endocytic vesicles (Frischknecht *et al.*, 1999a, 1999b; Taunton *et al.*, 2000; Rozelle *et al.*, 2000). However, the coexistence of an actin-independent mechanism for Cdc42 in the Golgi complex cannot be ruled out, as suggested by the findings that the release of TGN-derived apical transport vesicles is inhibited by latrunculin B and stimulated by activated Cdc42 (Müsch *et al.*, 2001). In fact, PLD and PI3K, both targets of Cdc42, are involved in the generation of transport carriers and in post-Golgi trafficking in vitro, respectively, as previously mentioned.

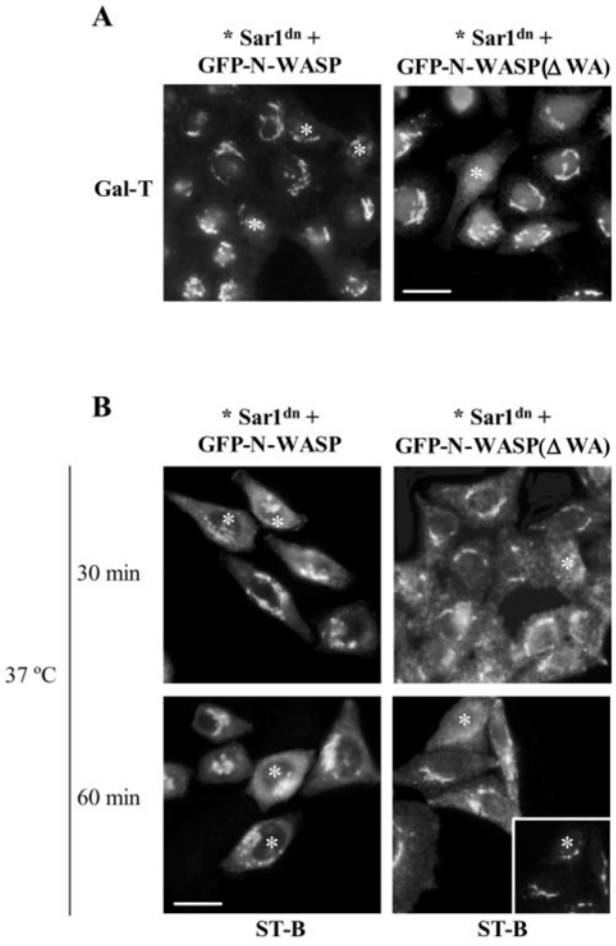

Figure 9. N-WASP blocks the Sar1^{dn}-induced ER accumulation of Golgi enzymes and the retrograde transport of Shiga toxin from Golgi to the ER. (A) Sar1^{dn} plus wild-type GFP-N-WASP (left; asterisks) or Sar1^{dn} plus GFP-N-WASP(ΔWA) (right; asterisks) constructs were coinjected into the nucleus of HeLa cells. After 3–4 h, cells were processed for indirect immunofluorescence with anti–Gal-T antibodies. Unlike N-WASP(ΔWA), N-WASP prevents the Sar1^{dn}-induced ER accumulation of Gal-T. These morphological observations were quantified and results are shown in Figure 7G. Bar, 10 μm. (B) The experimental procedure is described in the legend to Figure 7. N-WASP blocks transport of ST-B from Golgi to the ER (left; asterisks). However, in cells expressing GFP-N-WASP(ΔWA), ST-B accumulates in the ER (right; asterisks). Non-microinjected cells show the characteristic steady state Golgi localization of ST-B. The inset shows the double immunostaining to giantin, which reveals of the presence of a virtually intact Golgi complex at this time of Sar1^{dn} expression. Bar, 10 μm.

Formation of Transport Intermediates in the Golgi Complex via Cdc42→N-WASP→(?)Arp2/3

WASP/N-WASP is activated by the lipid second-messenger phosphatidylinositol 4,5-biphosphate (PIP_2) and the GTP-bound, prenylated Cdc42 (Zigmond *et al.*, 1997; Ma *et al.*, 1998a, 1998b; Rohatgi *et al.*, 1999; Higgs and Pollard, 2000; see Zigmond, 2000 and Higgs and Pollard, 2001 for reviews). Cdc42 and PIP_2 can synergize to activate N-WASP, which in turns triggers actin polymerization via the Arp3/3 complex depending on the localization of both activators on the membrane surface (Prehoda *et al.*, 2000; Rohatgi *et al.*, 2001). Unlike dominant-negative Cdc42N17, activated Cdc42V12 is particularly enriched in the lateral portions of the Golgi cisternae, where most of peri-Golgi transport intermediates are formed. Furthermore, the enzymes responsible for the synthesis of PIP_2 phosphatidylinositol-4-OH kinase-β and type I phosphatidylinositol 4-phosphate 5-kinase are both recruited in an ARF-dependent manner to isolated Golgi membranes (Godi *et al.*, 1999; Jones *et al.*, 2000). Thus, there could be a molecular interaction in the lateral portions of Golgi cisternae among activated Cdc42, the aforementioned PI and PIP kinases and PIP_2, whose primary role in the signaling pathway may be to activate GTP/GDP nucleotide exchange on Cdc42 (Rohatgi *et al.*, 2000). This interaction could locally and simultaneously regulate protein transport through the formation of transport intermediates or through the peri-Golgi actin assembly. It could be argued that this process does not occur in the Golgi complex because at steady state WASP/N-WASP and Arp2/3 are not located at the Golgi membranes. In fact, most of WASP/N-WASP is not associated with the actin cytoskeleton or membranes, indicating that WASP molecules are not bound to GTP-Cdc42 or PIP_2 because both are associated with membranes (Regazzi *et al.*, 1992; Nomanbhoy and Cerione, 1999). However, we found that N-WASP is localized to the Golgi complex in cells overexpressing active Cdc42. This suggests that at physiological conditions such a process, albeit transient and local, also occurs in the Golgi complex. Unlike N-WASP, N-WASP(ΔWA), a mutant that lacks Arp2/3 binding domain, did not alter retrograde transport. Furthermore, when it was coexpressed with Cdc42V12, the expected negative regulation of the retrograde transport by GTP-Cdc42 was also inhibited (Figure 10). This result strongly suggests that N-WASP mediates the Cdc42 response via an interaction with Arp2/3. This complex has in addition to its actin nucleation activity the ability to cross-link actin filaments into a characteristic dendritic network (Mullins *et al.*, 1998). Thus, both the disassembly of microfilaments (Valderrama *et al.*, 2001) and the possible formation of a dense peri-Golgi dendritic actin network by N-WASP-Arp2/3 impair Golgi-to-ER protein transport. We speculate that when microfilaments are disassembled transport intermediates do not interact with them. As a result, they are not transported to the ER either directly or after translocation to microtubules. In contrast, the dense peri-Golgi dendritic actin network induced by N-WASp-Arp2/3 could form a physical barrier that acts as a cage that obstructs the formation or the transport of transport intermediates. This is analogous to the effect of F-actin located underneath the plasma membrane in secretory cells (Morales *et al.*, 2000; Trifaro *et al.*, 2000 for review). Therefore, from our previous findings (Valderrama *et al.*, 2001) and from those reported here, we propose that changes in the peri-Golgi actin dynamics (for example, imbalance in the peri-Golgi G-/F-actin ratio) have direct consequence on the efficacy of retrograde protein transport.

Finally, the regulation of retrograde pathway by Cdc42 and N-WASP equally affects the COPI-dependent and -independent pathways in the Golgi-to-ER protein transport, because overexpression of both Cdc42 and N-WASP hin-

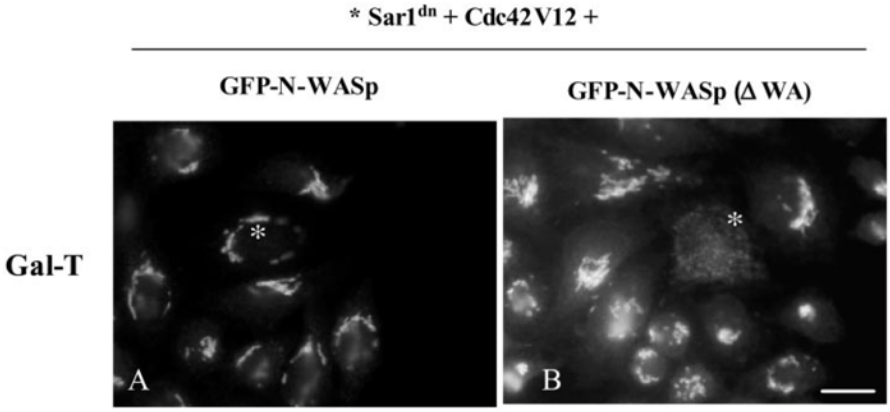

Figure 10. N-WASP(ΔWA) alleviates the blocking effect of activated Cdc42 on the Sar1^{dn}-induced ER accumulation of Gal-T. Sar1^{dn} plus untagged Cdc42V12 plus wild-type GFP-N-WASP or Sar1^{dn} plus untagged Cdc42V12 plus GFP-N-WASP(ΔWA) constructs were coinjected into the nucleus of HeLa cells (A and B, respectively; asterisks). After 7 h, cells were processed for indirect immunofluorescence with anti–Gal-T antibodies. Unlike cells that overexpress wild-type N-WASP (A), those expressing the mutant form of N-WASP lacking the Arp2/3 binding domain (N-WASP(ΔWA)) showed that Gal-T was redistributed to the ER by Sar1^{dn} despite Cdc42V12 (B). These morphological observations were quantified and results are shown in Figure 6G. Bar, 10 μm.

dered the Sar1^{dn}-induced ER accumulation of Gal-T and the Golgi-to-ER transport of native ST-B (COPI-independent), and the subcellular distribution of the KDEL receptor (COPI-dependent; Storrie *et al.*, 2000, for review). These results are consistent with those obtained when actin microfilaments were disassembled by latrunculin B and botulinum C2 toxin (Valderrama *et al.*, 2001).

Why Does the Cdc42 Dominant Negative Form Have No Effect in the Golgi-to-ER Pathway?

We show that, unlike Cdc42V12, Cdc42N17 is mostly located in the cytoplasm (6% vs. 54%, respectively; Table 1), and therefore it cannot interfere in the signaling route(s) activated by Cdc42V12. Hence, the physiological function of Cdc42 in ER/Golgi trafficking can only be revealed when this GTPase is attached to membranes that only happens when, like other GTPases, it is in GTP state. What likely happens in physiological conditions? Most of the endogenous pool of Cdc42 is in GDP-bound form and more probably bound to Rho-GDP dissociation inhibitor protein (Rho-GDI; Nomanbhoy *et al.*, 1999; Faure and Dagher, 2001 for review). Consequently, Cdc42 is located in the cytoplasm and therefore inactive. At steady state, only a small pool of this Cdc42 is activated, which is recruited to plasma membrane and Golgi membranes, where as expected it will activate its cognate downstream targets. We assume that the activation of Cdc42 is rapidly and locally produced (for example, in the lateral portions of the Golgi cisternae). Immediately after, GTP-Cdc42 is also rapidly inactivated by local GAPs. We rationalize that when we overexpress a mutant that encodes GTP-Cdc42, the effects of this GTP-Cdc42 will be much more apparent. In contrast, we cannot reveal a role when we overexpress a mutant that encodes a constitutively GDP-bound Cdc42, which is inactive and located in the cytoplasm. In other words, the overexpression of Cdc42N17 has no physiological effect in these processes, which involves a prior membrane attachment and activation of the GTPase for carrying out its biological function. On the other hand, there is now evidence demonstrating that the effects of a constitutively active GTPase may not necessarily be antagonistic to the effects of a dominant inhibitory form of the same GTPase (Arozarena *et al.*, 2001; Müsch *et al.*, 2001).

In conclusion, the regulatory mechanism involved in the role of actin filaments in the Golgi-to-ER membrane transport is mediated via Cdc42 and N-WASP. Our findings also raise the possibility that motile transport intermediates propelled by actin comets could mediate Golgi-to-ER protein transport.

ACKNOWLEDGMENTS

The authors thank Piero Crespo (IIB, UAM-CSIC, Madrid), Ferran Valderrama (ICRF, London) and Teresa Babià (Kelly Científico, Barcelona) for helpful discussions; Maite Muñoz for technical support; Anna Bosch (Serveis Científico-Tècnics, University of Barcelona) for advice with the confocal microscope; Robin Rycroft for editorial assistance; and Francisco Sánchez-Madrid, Hans-Peter Hauri, Hans-Dieter Soling, David Shima, Rainer Pepperkok, Eric Berger, Kelly Moremen, Ludger Johannes, and Bruno Goud for reagents and antibodies. This study was funded by CICYT grants SAF00–0042 and CIRIT AGP2000 to G.E.; CICYT grants PM99–0137 and BFI2000–0156 to J.B.; and IDIBAPS, CIRIT, and FIS predoctoral fellowships to A.L., O.M., and J.M.D., respectively.

REFERENCES

Adams, A.E., Johnson, D.I., Longnecker, R.M., Sloat, B.F., and Pringle, J.R. (1990). CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. J. Cell Biol. *111*, 131–142.

Apodaca, G. (2001). Endocytic traffic in polarized epithelial cells: role of the actin and microtubule cytoskeleton. Traffic *2*, 149–159.

Aridor, M., Bannykh, S.I., Rowe, T., and Balch, W.E. (1995). Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. J. Cell Biol. *131*, 875–893.

Arozarena, I., Matallanas, D., and Crespo, P. (2001). Maintenance of CDC42 GDP-bound state by Rho-GDI inhibits MAP kinase activation by the exchange factor Ras-GRF. Evidence for Ras-GRF function being inhibited by Cdc42-GDP but unaffected by CDC42-GTP. J. Biol. Chem. *276*, 21878–21884.

Aspenstrom, P., Lindberg, U., and Hall, A. (1996). Two GTPases, Cdc42 and Rac, bind directly to a protein implicated in the immu-

nodeficiency disorder Wiskott-Aldrich syndrome. Curr. Biol. *6*, 70–75.

Barlowe, C. (1998). COPII and selective export from the endoplasmic reticulum. Biochim. Biophys. Acta *1404*, 67–76.

Beck, K.A., Buchanan, J.A., Malhotra, V., and Nelson, W.J. (1994). Golgi spectrin: identification of an erythroid beta-spectrin homolog associated with the Golgi complex. J. Cell Biol. *127*, 707–723.

Bonatti, S., Migliaccio, G., and Simons, K. (1989). Palmitylation of viral membrane glycoproteins takes place after exit from the endoplasmic reticulum. J. Biol. Chem. *264*, 12590–12595.

Burbelo, P.D., Drechsel, D., and Hall, A. (1995). A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J. Biol. Chem. *270*, 29071–29074.

Buss, F., Kendrick-Jones, J., Lionne, C., Knight, A.E., Cote, G.P., and Paul, L.J. (1998). The localization of myosin VI at the Golgi complex and leading edge of fibroblasts and its phosphorylation and recruitment into membrane ruffles of A431 cells after growth factor stimulation. J. Cell Biol. *143*, 1535–1545.

Chimini, G., and Chavrier, P. (2000). Function of Rho family proteins in actin dynamics during phagocytosis and engulfment. Nat. Cell Biol. *2*, E191–E196.

Cohen, D., Müsch, A., and Rodriguez-Boulan, E. (2001). Selective control of basolateral membrane protein polarity by cdc42. Traffic *2*, 556–564.

Corvera, S., and Czech, M.P. (1998). Direct targets of phosphoinositide 3-kinase products in membrane traffic and signal transduction. Trends Cell Biol. *8*, 442–446.

Defacque, H., Egeberg, M., Habermann, A., Diakonova, M., Roy, C., Mangeat, P., Voelter, W., Marriott, G., Pfannstiel, J., Faulstich, H., and Griffiths, G. (2000). Involvement of ezrin/moesin in de novo actin assembly on phagosomal membranes. EMBO J. *19*, 199–212.

del Pozo, M.A., Vicente-Manzanares, M., Tejedor, R., Serrador, J.M., and Sanchez-Madrid, F. (1999). Rho GTPases control migration and polarization of adhesion molecules and cytoskeletal ERM components in T lymphocytes. Eur. J. Immunol. *29*, 3609–3620.

DePina, A.S., and Langford, G.M. (1999). Vesicle transport: the role of actin filaments and myosin motors. Microsc. Res. Tech. *47*, 93–106.

di Campli, A., Valderrama, F., Babia, T., De Matteis, M.A., Luini, A., and Egea, G. (1999). Morphological changes in the Golgi complex correlate with actin cytoskeleton rearrangements. Cell Motil. Cytoskeleton *43*, 334–348.

Ellis, S., and Mellor, H. (2000). Regulation of endocytic traffic by rho family GTPases. Trends Cell Biol. *10*, 85–88.

Erickson, J.W., Zhang, C., Kahn, R.A., Evans, T., and Cerione, R.A. (1996). Mammalian Cdc42 is a brefeldin A-sensitive component of the Golgi apparatus. J. Biol. Chem. *271*, 26850–26854.

Etienne-Manneville, S., and Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCδ. Cell *106*, 489–498.

Faure, J., and Dagher, M.C. (2001). Interactions between Rho GTPases and Rho GDP dissociation inhibitor (Rho-GDI). Biochimie *83*, 409–414.

Frischknecht, F., Moreau, V., Rottger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G., and Way, M. (1999a). Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signaling. Nature *401*, 926–929.

Frischknecht, F., Cudmore, S., Moreau, V., Reckmann, I., Rottger, S., and Way, M. (1999b). Tyrosine phosphorylation is required for actin-based motility of vaccinia but not *Listeria* or *Shigella*. Curr. Biol. *9*, 89–92.

Garrett, W.S., Chen, L.M., Kroschewski, R., Ebersold, M., Turley, S., Trombetta, S., Galan, J.E., and Mellman, I. (2000). Developmental control of endocytosis in dendritic cells by Cdc42. Cell *102*, 325–334.

Godi, A., Santone, I., Pertile, P., Devarajan, P., Stabach, P.R., Morrow, J.S., Di Tullio, G., Polishchuk, R., Petrucci, T.C., Luini, A., and De Matteis, M.A. (1998). ADP ribosylation factor regulates spectrin binding to the Golgi complex. Proc. Natl. Acad. Sci. USA *95*, 8607–8612.

Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Iurisci, C., Luini, A., Corda, D., and De Matteis, M.A. (1999). ARF mediates recruitment of PtdIns-4-OH kinase-β and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex. Nat. Cell Biol. *1*, 280–287.

Gotta, M., Abraham, M.C., and Ahringer, J. (2001). CDC-42 controls early cell polarity and spindle orientation in *C. elegans*. Curr. Biol. *11*, 482–488.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509–514.

Han, J.S., Kim, H.C., Chung, J.K., Kang, H.S., Donaldson, J., and Koh, J.K. (1998). The potential role for CDC42 protein from rat brain cytosol in phospholipase D activation. Biochem. Mol. Biol. Int. *45*, 1089–1103.

Heimann, K., Percival, J.M., Weinberger, R., Gunning, P., and Stow, J.L. (1999). Specific isoforms of actin-binding proteins on distinct populations of Golgi-derived vesicles. J. Biol. Chem. *274*, 10743–10750.

Higgs, H.N., and Pollard, T.D. (2000). Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex. J. Cell Biol. *150*, 1311–1320.

Higgs, H.N., and Pollard, T.D. (2001). Regulation of actin filament network formation through Arp2/3 complex: Activation by a diverse array of proteins. Annu. Rev. Biochem. *70*, 649–676.

Johannes, L., and Goud, B. (1998). Surfing on a retrograde wave: how does Shiga toxin reach the endoplasmic reticulum? Trends Cell Biol. *8*, 158–162.

Jones, D.H., Morris, J.B., Morgan, C.P., Kondo, H., Irvine, R.F., and Cockcroft, S. (2000). Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the Golgi compartment. J. Biol. Chem. *275*, 13962–13966.

Jou, T.S., Leung, S.M., Fung, L.M., Ruiz, W.G., Nelson, W.J., and Apodaca, G. (2000). Selective alterations in biosynthetic and endocytic protein traffic in Madin-Darby canine kidney epithelial cells expressing mutants of the small GTPase Rac1. Mol. Biol. Cell *11*, 287–304.

Kroschewski, R., Hall, A., and Mellman, I. (1999). Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells. Nat. Cell Biol. *1*, 8–13.

Kuge, O., Dascher, C., Orci, L., Rowe, T., Amherdt, M., Plutner, H., Ravazzola, M., Tanigawa, G., Rothman, J.E., and Balch, W.E. (1994). Sar1 promotes vesicle budding from the endoplasmic reticulum but not Golgi compartments. J. Cell Biol. *125*, 51–65.

Lamaze, C., Chuang, T.H., Terlecky, L.J., Bokoch, G.M., and Schmid, S.L. (1996). Regulation of receptor-mediated endocytosis by Rho and Rac. Nature *382*, 177–179.

Letourneur, F., Gaynor, E.C., Hennecke, S., Demolliere, C., Duden, R., Emr, S.D., Riezman, H., and Cosson, P. (1994). Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. Cell *79*, 1199–1207.

Ma, L., Rohatgi, R., and Kirschner, M.W. (1998a). The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. Proc. Natl. Acad. Sci. USA *95*, 15362–15367.

Ma, L., Cantley, L.C., Janmey, P.A., and Kirschner, M.W. (1998b). Corequirement of specific phosphoinositides and small GTP-binding protein Cdc42 in inducing actin assembly in *Xenopus* egg extracts. J. Cell Biol. *140*, 1125–1136.

Mallard, F., Antony, C., Tenza, D., Salamero, J., Goud, B., and Johannes, L. (1998). Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. J. Cell Biol. *143*, 973–990.

Martínez-Menárguez, J.A., Geuze, H.J., Slot, J.W., and Klumperman, J. (1999). Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. Cell *98*, 81–90.

Martínez-Menárguez, J.A., Prekeris, R., Oorschot, V.M., Scheller, R., Slot, J.W., Geuze, H.J., and Klumperman, J. (2001). Peri-Golgi vesicles contain retrograde but not anterograde proteins consistent with the cisternal progession model of intra-Golgi transport. J Cell Biol. *155*, 1213–1224.

McCallum, S.J., Erickson, J.W., and Cerione, R.A. (1998). Characterization of the association of the actin-binding protein, IQGAP, and activated Cdc42 with Golgi membranes. J. Biol. Chem. *273*, 22537–22544.

Morales, M., Colicos, M.A., and Goda, Y. (2000). Actin-dependent regulation of neurotransmitter release at central synapses. Neuron *27*, 539–550.

Moreau, V., Frischknecht, F., Reckmann, I., Vincentelli, R., Rabut, G., Stewart, D., and Way, M. (2000). A complex of N-WASP and WIP integrates signaling cascades that lead to actin polymerization. Nat. Cell Biol. *2*, 441–448.

Mullins, R.D., Heuser, J.A., and Pollard, T.D. (1998). The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proc. Natl. Acad. Sci. USA *95*, 6181–6186.

Müsch, A., Cohen, D., and Rodriguez-Boulan, E. (1997). Myosin II is involved in the production of constitutive transport vesicles from the TGN. J. Cell Biol. *138*, 291–306.

Müsch, A., Cohen, D., Kreitzer, G., and Rodriguez-Boulan, E. (2001). cdc42 regulates the exit of apical, and basolateral proteins from the trans-Golgi network. EMBO J. *20*, 2171–2179.

Nobes, C.D., and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. *144*, 1235–1244.

Nomanbhoy, T., and Cerione, R.A. (1999). Fluorescence assays of Cdc42 interactions with target/effector proteins. Biochemistry *38*, 15878–15884.

Nomanbhoy, T.K., Erickson, J.W., and Cerione, R.A. (1999). Kinetics of Cdc42 membrane extraction by Rho-GDI monitored by real-time fluorescence resonance energy transfer. Biochemistry *38*, 1744–1750.

Prehoda, K.E., Scott, J.A., Mullins, R.D., and Lim, W.A. (2000). Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. Science *290*, 801–806.

Qualmann, B., Kessels, M.M., and Kelly, R.B. (2000). Molecular links between endocytosis and the actin cytoskeleton. J. Cell Biol. *150*, F111–F116.

Regazzi, R., Kikuchi, A., Takai, Y., and Wollheim, C.B. (1992). The small GTP-binding proteins in the cytosol of insulin-secreting cells are complexed to GDP dissociation inhibitor proteins. J. Biol. Chem. *267*, 17512–17519.

Ridley, A.J. (2001). Rho proteins: linking signaling with membrane trafficking. Traffic *2*, 303–310.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M.W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. Cell *97*, 221–231.

Rohatgi, R., Ho, H.Y., and Kirschner, M.W. (2000). Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. J. Cell Biol. *150*, 1299–1310.

Rohatgi, R., Nollau, P., Ho, H.Y., Kirschner, M.W., and Mayer, B.J. (2001). Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. J. Biol. Chem. *276*, 26448–26452.

Rojas, R., Ruiz, W.G., Leung, S.M., Jou, T.S., and Apodaca, G. (2001). Cdc42-dependent modulation of tight junctions and membrane protein traffic in polarized Madin-Darby canine kidney cells. Mol. Biol. Cell *12*, 2257–2274.

Roth, M.G., Bi, K., Ktistakis, N.T., and Yu, S. (1999). Phospholipase D as an effector for ADP-ribosylation factor in the regulation of vesicular traffic. Chem. Phys. Lipids *98*, 141–152.

Rozelle, A.L., Machesky, L.M., Yamamoto, M., Driessens, M.H., Insall, R.H., Roth, M.G., Luby-Phelps, K., Marriott, G., Hall, A., and Yin, H.L. (2000). Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. Curr. Biol. *10*, 311–320.

Sandvig, K., Garred, O., Prydz, K., Kozlov, J.V., Hansen, S.H., and van Deurs, B. (1992). Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature *358*, 510–512.

Seemann, J., Jokitalo, E., Pypaert, M., and Warren, G. (2000). Matrix proteins can generate the higher order architecture of the Golgi apparatus. Nature *407*, 1022–1026.

Snapper, S.B., and Rosen, F.S. (1999). The Wiskott-Aldrich syndrome protein (WASP): roles in signaling and cytoskeletal organization. Annu. Rev. Immunol. *17*, 905–929.

Storrie, B., White, J., Rottger, S., Stelzer, E.H., Suganuma, T., and Nilsson, T. (1998). Recycling of Golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering. J. Cell Biol. *143*, 1505–1521.

Storrie, B., Pepperkok, R., and Nilsson, T. (2000). Breaking the COPI monopoly on Golgi recycling. Trends Cell Biol. *10*, 385–391.

Stow, J.L., Fath, K.R., and Burgess, D.R. (1998). Budding roles for myosin II on the Golgi. Trends Cell Biol. *8*, 138–141.

Stowers, L., Yelon, D., Berg, L.J., and Chant, J. (1995). Regulation of the polarization of T cells toward antigen-presenting cells by Ras-related GTPase CDC42. Proc. Natl. Acad. Sci. USA *92*, 5027–5031.

Symons, M., Derry, J.M., Karlak, B., Jiang, S., Lemahieu, V., Mccormick, F., Francke, U., and Abo, A. (1996). Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. Cell *84*, 723–734.

Taunton, J., Rowning, B.A., Coughlin, M.L., Wu, M., Moon, R.T., Mitchison, T.J., and Larabell, C.A. (2000). Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. J. Cell Biol. *148*, 519–530.

Taunton, J. (2001). Actin filament nucleation by endosomes, lysosomes and secretory vesicles. Curr. Opin. Cell Biol. *13*, 85–91.

Thyberg, J., and Moskalewski, S. (1999). Role of microtubules in the organization of the Golgi complex. Exp. Cell Res. *246*, 263–279.

Trifaro, J., Rose, S.D., Lejen, T., and Elzagallaai, A. (2000). Two pathways control chromaffin cell cortical F-actin dynamics during exocytosis. Biochimie *82*, 339–352.

Valderrama, F., Babia, T., Ayala, I., Kok, J.W., Renau-Piqueras, J., and Egea, G. (1998). Actin microfilaments are essential for the cytological positioning and morphology of the Golgi complex. Eur J. Cell Biol. *76*, 9–17.

Valderrama, F., Luna, A., Babia, T., Martinez-Menarguez, J.A., Ballesta, J., Barth, H., Chaponnier, C., Renau-Piqueras, J., and Egea, G. (2000). The Golgi-associated COPI-coated buds and vesicles contain β/γ-actin. Proc. Natl. Acad. Sci. USA *97*, 1560–1565.

Valderrama, F., Durán, M., Babia, T., Barth, H., Renau-Piqueras, J., and Egea, G. (2001). Actin microfilaments facilitate the retrograde transport from the Golgi complex to the endoplasmic reticulum in mammalian cells. Traffic *2*, 717–726.

West, M.A., Prescott, A.R., Eskelinen, E.L., Ridley, A.J., and Watts, C. (2000). Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. Curr. Biol. *10*, 839–848.

Wu, W.J., Erickson, J.W., Lin, R., and Cerione, R.A. (2000). The gamma-subunit of the coatomer complex binds Cdc42 to mediate transformation. Nature *405*, 800–804.

Zheng, Y., Bagrodia, S., and Cerione, R.A. (1994). Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. J. Biol. Chem. *269*, 18727–18730.

Zigmond, S.H., Joyce, M., Borleis, J., Bokoch, G.M., and Devreotes, P.N. (1997). Regulation of actin polymerization in cell-free systems by GTPγS and Cdc42. J. Cell Biol. *138*, 363–374.

Zigmond, S.H. (2000). How WASP regulates actin polymerization. J. Cell Biol. *150*, F117–F120.

Traffic 2004; 5: 838–846
Blackwell Munksgaard

doi: 10.1111/j.1600-0854.2004.00225.x

Association of Cdc42/N-WASP/Arp2/3 Signaling Pathway with Golgi Membranes

Olga B. Matas[1], Josè Ángel Martínez-Menárguez[2] and Gustavo Egea[1,*]

[1] Departament de Biologia Cel·lular i Anatomia Patològica, Facultat de Medicina, Universitat de Barcelona-IDIBAPS, 08036 Barcelona, Spain
[2] Departamento de Biología Celular, Facultad de Medicina, Universidad de Murcia, E-30071 Murcia, Spain
** Corresponding author: Gustavo Egea, gegea@ub.edu*

Recent findings indicate that Cdc42 regulates Golgi-to-ER (endoplasmic reticulum) protein transport through N-WASP and Arp2/3 (Luna et al. 2002, Mol. Biol. Cell, 13:866–879). To analyse the components of the Cdc42-governed signaling pathway in the secretory pathway, we localized Cdc42, N-WASP and Arp2/3 in the Golgi complex by cryoimmunoelectron microscopy. Cdc42 is found throughout the Golgi stack, particularly in *cis*/middle cisternae, whereas N-WASP and Arp3 (a component of the Arp2/3 complex) are restricted to *cis* cisternae. Arp3 also colocalized in peri-Golgi tubulovesicular structures with either KDEL receptor or GM130. Even though Arp3 is not found in TGN46-positive cisternal elements, a small fraction of Arp3-labeled tubulo-vesicular elements showed TGN46 labeling. Active Cdc42 (GTP-bound form) induced relocation of N-WASP and Arp3 to the lateral rims of Golgi cisternae. These results show that the actin nucleation and polymerization signaling pathway governed by Cdc42/N-WASP/Arp operates in the Golgi complex of mammalian cells, further implicating actin dynamics in Golgi-associated membrane trafficking.

Key words: actin, cytoskeleton, Golgi complex, membrane traffic, Rho GTPases, secretory pathway

Received 24 May 2004, revised and accepted for publication 4 August 2004

The involvement of dynamic actin cytoskeleton in the formation and locomotion of carrier proteins is becoming clearer, particularly in the endocytic pathway (1,2). Recently, progress has been made in revealing the role of actin filaments and identifying actin-binding proteins in the Golgi complex (3). However, the regulation of this actin–Golgi interaction is poorly understood. Cdc42 is seen in the Golgi complex (4–6) and regulates post-Golgi (7–10) and endoplasmic reticulum (ER)/Golgi protein transport (6,11,12). Some Cdc42 downstream effectors such as PAK4 (13) and cdck5-p35 kinase (14) or exchange factors like the Cdc42 GEF protein Fgd1 (15) have been seen in the Golgi complex but their function there is unknown. We and others have recently reported that N-WASP and Arp2/3 are the downstream effectors of Cdc42, mediating regulation of retrograde (Golgi-to-ER) (6) and anterograde (ER-to-Golgi) membrane trafficking at the ER/Golgi interface (12). To understand the significance of the Cdc42-governed actin dynamics in Golgi-derived transport events, it is essential to locate the molecular components of this signaling pathway in the Golgi complex. Here we examine the localization of Cdc42, N-WASP and Arp2/3 in the Golgi complex by cryoimmunoelectron microscopy. Our data indicate that the actin dynamics signaling pathway mediated by Cdc42/N-WASP/Arp is associated not only with plasma membrane trafficking events like clathrin-mediated endocytosis (16) but also with the endomembrane system of the secretory pathway and, in particular, the Golgi complex.

Results

Cdc42, N-WASP and Arp2/3 are polarized in the Golgi stack

Using high-resolution cryoimmunogold electron microscopy and anti-GFP (green fluorescent protein) antibodies (since anti-N-WASP antibodies are unsuitable for immunoelectron microscopy), GFP-N-WASP was seen in Golgi cisternae (Figure 1) and in peri-Golgi tubulovesicular structures, some of which showed the characteristic electron-dense coat of COPI-coated vesicles (Figure 1, arrows). No gold labeling in the Golgi complex was observed either in HeLa cells transfected with the expressing vector for GFP alone or in neighboring nontranfected cells (data not shown). We next examined the localization of endogenous Arp3, which was shown by epifluorescence in punctate cytoplasmic structures and partially colocalized with the *cis*-Golgi matrix protein GM130 both in NIH3T3 and HeLa (Figure 2A and B, respectively) cells. As a control of Arp3 immunolocalization, HeLa cells were treated with platelet-derived gorwth factor (PDGF) (100 ng/mL, 10 min), which induced the expected enrichment of Arp3 in the plasma membrane (17; data not shown). At the ultrastructural level, Arp3 was seen in Golgi cisternae (Figure 2C) and peri-Golgi tubulovesicular structures (Figure 2C, arrows). Subsequently, we examined whether Cdc42, N-WASP and Arp3 showed a *cis–trans* polarized distribution through the Golgi stack. For this purpose, double immunogold labeling experiments were performed with the *cis*-Golgi marker GM130 (18) and the *trans*-Golgi network (TGN) marker TGN46 (19). In the Golgi stacks of GFP-Cdc42-transfected HeLa cells, Cdc42 showed a wider distribution than GM130 (Figure 3A). Quantitative immunocytochemical data indicated that Cdc42 was present throughout the

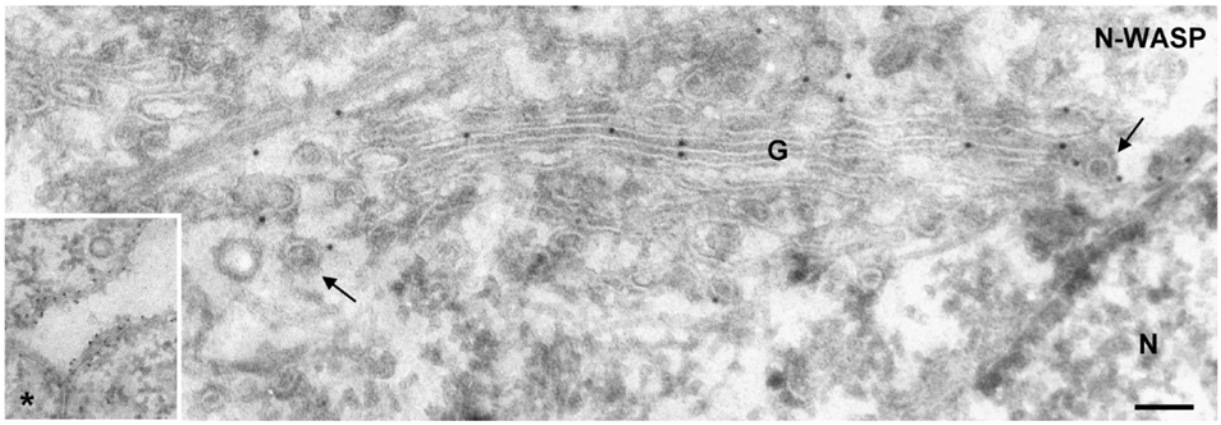

Figure 1: N-WASP localization in Golgi membranes. GFP-N-WASP-transfected HeLa cells were fixed and processed for cryoimmunogold electron microscopy using polyclonal antibodies to GFP followed by protein A-gold (10 nm). N-WASP is seen in Golgi cisternae and in COP-coated transport intermediates (arrows). Insert shows gold labeling for GFP-N-WASP in the plasma membrane of two GFP-N-WASP-transfected HeLa cells but not in a neighboring nontransfected cell (asterisk). G, Golgi; N, nucleus. Bar, 100 nm.

Golgi stack but particularly enriched in *cis*/middle cisternae (Table 1). N-WASP was mostly located in the first two *cis*-Golgi cisternae (Table 1) and Arp3 showed a more restricted distribution, colocalizing with GM130 in the first *cis*-Golgi cisterna (Figure 3B and Table 1). Double labeling experiments with TGN46 showed that neither N-WASP (not shown) nor Arp3 (Figure 3C) was significantly present in *trans*/TGN cisternae, although, strikingly, some peri-Golgi tubulovesicular structures showed colocalization of Arp3 and TGN46 (see next paragraph).

Arp3-positive peri-Golgi tubulovesicular structures contain either KDELr or TGN46

Arp3 was seen in the *cis* cisternae of the Golgi stack but gold labeling was also observed in peri-Golgi tubulovesicular structures (Figure 2C). As a consequence of the continuous membrane flow to and from the Golgi complex, the molecular content of these tubulovesicular structures depends on the subcompartment in which they are generated. We therefore performed double labeling experiments to characterize these Arp3-positive peri-Golgi tubulovesicular structures. We analyzed them for the presence of GM130 (Figure 4A), KDEL receptor (KDELr) (Figure 4B) and TGN46 (Figure 4c). For quantification, tubulovesicular structures within 200 nm of the lateral side of a Golgi stack were examined. Almost 30% of all Arp3-positive tubulovesicular structures contained GM130 or KDELr and 15% contained TGN46 (Table 2). Therefore, besides *cis*-Golgi cisterna, Arp3 is present in dynamic tubulovesicular structures that continuously cycle in the ER/Golgi interface. Notably, Arp3 is also located in TGN-derived tubulovesicular profiles, although it is not seen in *trans*/TGN cisternae.

Activated Cdc42 induces the relocation of N-WASP and Arp3 in the lateral rims of Golgi cisternae

In HeLa cells expressing Cdc42V12, the dominant-positive mutant form of Cdc42, we observed that guanosine-5′-triphosphate (GTP)-bound Cdc42 is enriched in the lateral portions of Golgi cisternae (6). We therefore examined whether N-WASP and Arp3 are also relocated in Golgi cisternae (lateral vs. central portions) accompanying GTP-Cdc42. HeLa cells cotransfected with vectors expressing GFP-N-WASP and the myc-tagged dominant positive (myc-Cdc42V12) or dominant negative (myc-Cdc42N17) mutant

Table 1: Relative *cis*-to-*trans* distribution (C1-to-C5, respectively) of Cdc42, N-WASP and Arp3 in the Golgi stack

	C1	C2	C3	C4	C5
Cdc42V12	23.4 ± 4.0	33.6 ± 5.4	23.8 ± 3.6	11.1 ± 3.1	8.4 ± 3.9
N-WASP	50.4 ± 6.2	33.8 ± 4.9	13.8 ± 5.0	0.7 ± 0.7	1.3 ± 2.2
Arp3	68.1 ± 7.2	18.0 ± 5.8	8.3 ± 4.3	4.7 ± 2.4	1.3 ± 2.2

Golgi labeling was scored in nontransfected Hela cells (Arp3) or in HeLa cells transfected with GFP-Cdc42V12 (Cdc42) or GFP-N-WASP (N-WASP) expression vectors. The first *cis*-Golgi cisterna was identified using anti-GM130 antibodies (18). Numbers represent the percentage (mean ± SEM) of the total gold labeling of the Golgi stack.

Table 2: Relative codistribution of Arp3 with marker proteins in peri-Golgi tubulovesicular structures

	Peri-Golgi tubulovesicular protein marker		
	GM130	KDELr	TGN46
Arp3	29.8 ± 5.9	27.4 ± 7.0	15.7 ± 5.7

Over 100 positive tubulovesicular structures were counted for each double labeling experiment. Numbers indicate the percentage (mean ± SEM) of positive structures for Arp3 that also labeled for the protein indicated.

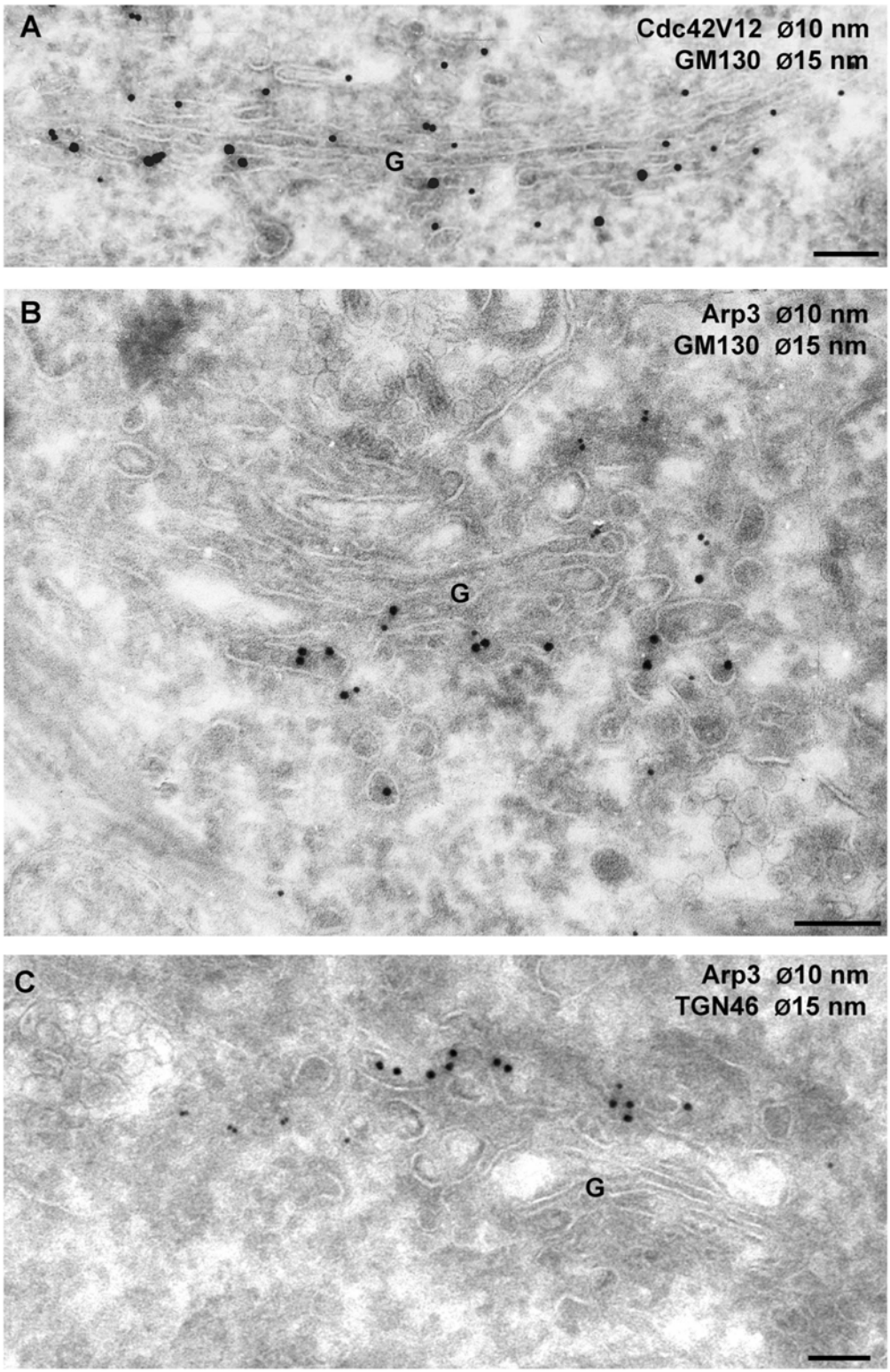

Figure 3: Polarized localization of Cdc42 and Arp3. A) GFP-Cdc42V12-transfected and (B and C) nontransfected HeLa cells were fixed and processed for double immunogold labeling with polyclonal anti-GFP (A) or anti-Arp3 (B and C) with monoclonal anti-GM130 (A and B) or anti-TGN46 (C) antibodies followed by protein A-gold. Cdc42 shows a broader cisternal distribution than GM130, whereas Arp3 narrowly colocalizes with GM130 at the *cis* cisterna (B). Conversely, Arp3 and TGN46 gold labeling are segregated (C). Bars, 100 nm.

forms of Cdc42 were fixed and subsequently processed for quantitative double immunogold labeling experiments in ultrathin cryosections (Table 3). N-WASP in GFP-N-WASP-transfected cells was slightly enriched in the central portions of cisternae but the differences with the lateral portions were not significant (Table 3, control condition). In cells expressing both GFP-N-WASP and myc-Cdc42V12, N-WASP was relocated in the lateral portions of Golgi cisternae, the differences with the central portions being significant (Table 3, Cdc42V12 condition). The coexpression with myc-Cdc42N17 did not alter the relative distribution of N-WASP along the Golgi cisternae (Table 3, Cdc42N17 condition). We next analyzed the relative distribution in the Golgi of Arp3 in nontransfected (control) and in GFP-Cdc42V12- and GFP-Cdc42N17-transfected cells. Both in control and in Cdc42N17-transfected cells (Figure 5B; Table 4), Arp3 was also uniformly distributed along Golgi cisternae. However, Arp3 was significantly enriched in the lateral portions in Cdc42V12-expressing cells (Figure 5A; Table 4).

Discussion

Our results indicate that the actin assembly and polymerization signaling pathway governed by Cdc42/N-WASP/Arp is not restricted to endocytic events (16,20–22) but also occurs in the Golgi complex. Using cryoimmunogold electron microcopy techniques, we observed that Cdc42 is present throughout the Golgi stack, albeit more enriched in *cis*/middle cisternae, whereas N-WASP and Arp3 are restricted to *cis* cisternae. N-WASP and Arp3 are both significantly enriched in the lateral portions of Golgi cisternae in Cdc42V12- but not in Cdc42N17-expressing cells. This indicates that activated Cdc42 triggers the recruitment of N-WASP, and in turn Arp2/3, to the lateral rims of *cis*-Golgi cisternae where, according to the cisternal-maturation model, retrograde transport intermediates are formed (23). We hypothesize that the Cdc42-governed actin polymerization signaling pathway could be activated

Table 3: Relative distribution of N-WASP in the Golgi complex

	Lateral portions	Central portions	Peri-Golgi tubulovesicular structures
Control	14.1 ± 5.4	23.5 ± 5.0	62.4 ± 6.7
Cdc42V12	35.2 ± 3.2*	15.3 ± 4.1	49.4 ± 3.8
Cdc42N17	21.7 ± 3.8	21.2 ± 3.3	57.1 ± 4.9

Quantitative immunogold labeling was carried out in HeLa cells transfected with GFP-N-WASP (control) or cotransfected with GFP-N-WASP/myc-Cdc42V12 (Cdc42V12) or GFP-N-WASP/myc-Cdc42N17 (Cdc42N17) vectors. The expressed proteins were visualized by using polyclonal anti-GFP and monoclonal anti-myc antibodies. Numbers represent the percentages (mean SEM) of the total gold labeling.

*Significant differences with respect to central portions; Student's *t*-test ($p \leq 0.001$).

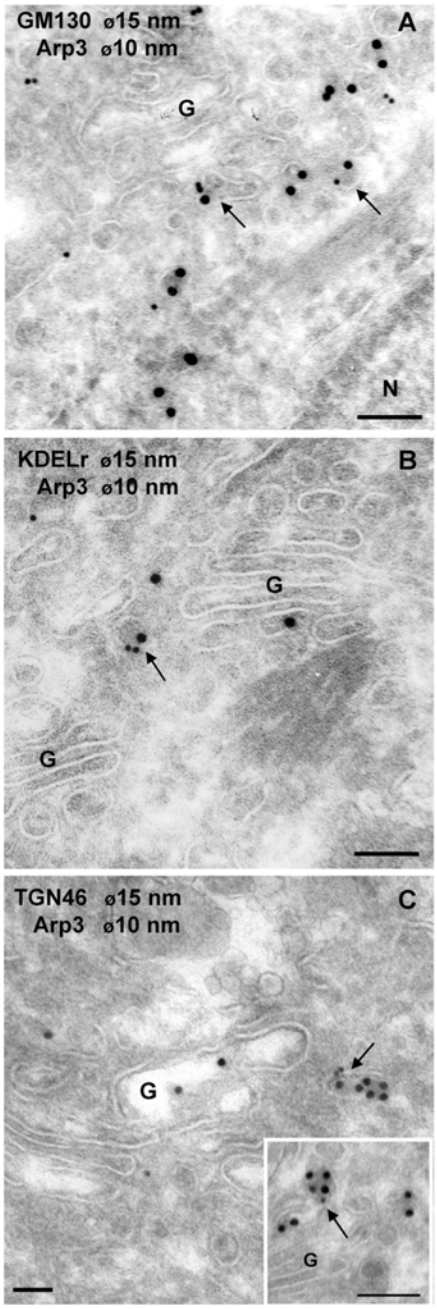

Figure 4: Arp3 colocalization with different cycling proteins in peri-Golgi tubulovesicular structures. Double immunogold labeling experiments in nontransfected HeLa cells with anti-Arp3 and anti-GM130 (A) or anti-KDELr (B) or anti-TGN46 (C) antibodies. Some peri-Golgi tubulovesicular structures are colabeled for Arp3 and the ER/Golgi interface cycling proteins GM130 or KDELr (arrows in A and B, respectively). GM130 and KDELr, Arp3 also colocalized to a lesser extent with TGN-46 in some of these peri-Golgi structures despite being virtually absent from *trans*/TGN cisternae. G, Golgi; N, nucleus. Bars, 100 nm.

Table 4: Relative distribution of Arp3 in the Golgi complex

	Golgi cisternae		
	Lateral portions	Central portions	Peri-Golgi tubulovesicular structures
Control	13.9 ± 5.8	16.9 ± 5.6	69.2 ± 7.1
Cdc42V12	23.5 ± 5.7*	11.5 ± 4.9	65.0 ± 7.7
Cdc42N17	14.4 ± 3.5	18.8 ± 3.9	67.2 ± 5.1

Quantitative immunogold labeling was performed in nontransfected HeLa cells (control) and in cells transfected with GFP-Cdc42V12 (Cdc42V12) or GFP-Cdc42N17 (Cdc42N17) expression vectors. The respective expressed proteins were visualized by using polyclonal anti-GFP and monoclonal anti-Arp3 antibodies. Numbers represent the percentages (mean ± SEM) of the total gold labeling.

*Significant differences with respect to central portions; Student's *t*-test ($p \leq 0.05$).

concomitantly to the formation of retrograde transport intermediates. In accordance with this hypothesis, Cdc42 associates with the γ component of coatomer (γCOP (11)), and a mutant form of N-WASP that lacks the Arp2/3 binding domain (N-WASPΔWA) alleviates the blocking effect of activated Cdc42 on the $Sar1^{dn}$-induced ER accumulation of Golgi-resident enzymes (6). These results strongly suggest that N-WASP and Arp2/3 are the main mediators in the Cdc42-induced regulatory effect on the ER/Golgi interface protein transport. The localization of N-WASP and Arp2/3 in *cis*-Golgi cisternae suggests that: (a) actin nucleation/polymerization contributes to budding of retrograde transport carriers; and (b) actin filaments in conjunction with myosins cause membrane elongation, which precedes the fission of transport carriers, or serve as tracks of myosin-mediated short-term locomotion of retrograde transport carriers.

In this regard, we have recently reported that nonmuscle myosin II mediates in the Golgi-to-ER membrane pathway (24).

Unlike Cdc42, neither N-WASP nor Arp2/3 are seen in *trans*/TGN cisternae but, strikingly, a significant pool of endogenous Arp3 colocalised with TGN46 in peri-Golgi tubulovesicular structures. This colocalization is consistent with the reported role of Arp2/3 complex in propelling some post-Golgi-derived transport intermediates away from TGN (25,26), and the recent observation of a (partial) association of Arc21 (other subunit of Arp2/3 complex) with TGN46-immunostained Golgi membranes (27). Most likely, this partial association of Arp2/3 with TGN membranes is the equivalent at electron microscopy level of the Arp3/TGN46-positive peri-Golgi tubulovesicular structures reported here. Although N-WASP is absent in *trans*-Golgi/TGN, Arp2/3 complex could be recruited and activated in these membranes by other effectors such as cortactin, which in turn interacts with dynamin2 (21,28,29), a GTPase involved in post-Golgi transport (30,31). The localization of Cdc42 in the *trans*-Golgi/TGN is in accordance with the involvement of Cdc42 in polarized post-Golgi trafficking (7–10) but our results suggest that it could not be mediated by N-WASP.

In conclusion, our results provide morphologic evidence for the localization and involvement of the Cdc42/N-WASP/Arp signaling pathway in Golgi-associated membrane trafficking, consistent with recent biochemical evidence that N-WASP and Arp2/3 binding to Golgi membranes requires their activation via signaling through the coatomer/Cdc42 complex (32). Moreover, the different subcellular localization of the molecular components of this signaling pathway in the Golgi stack and in peri-Golgi tubulovesicular structures strongly supports a role of actin dynamics both at the ER/Golgi interface and in post-Golgi trafficking, Cdc42, N-WASP and Arp2/3 mediating in the former and Cdc42 and/or Arp2/3 in the latter.

Materials and Methods

Constructs and antibodies

The plasmid encoding GFP-N-WASP was provided by M. Way (Imperial Cancer Research Fund, London). The plasmids encoding GFP-Cdc42 wild-type, dominant-positive and dominant-negative forms were provided by M. Way and F. Sánchez-Madrid (Hospital La Princesa, Madrid). The plasmids encoding myc-Cdc42V12 and myc-Cdc42N17 were provided by A. Hall (University College, London). Rabbit polyclonal antibodies anti-GFP, anti-KDELr and anti-Arp3 were provided by D. Shima (ICRF, London), H.-D. Söling (Max Planck, Gottingen, Germany) and M. D. Welch (University of California, San Francisco, CA), respectively. Mouse monoclonal antibodies anti-GM130 and anti-myc were from BD Transduction Laboratories (Lexington, KY) and BABCO-Covance Inc. (Princeton, NJ), respectively. Sheep polyclonal antibody to TGN46 was from SeroTec (Oxford, UK) and anti-giantin was from H.-P. Hauri (Biozentrum, Basel, Switzerland). Secondary Alexa-488 and Alexa-546 $F(ab')_2$ fragments were from Molecular Probes (Eugene, OR).

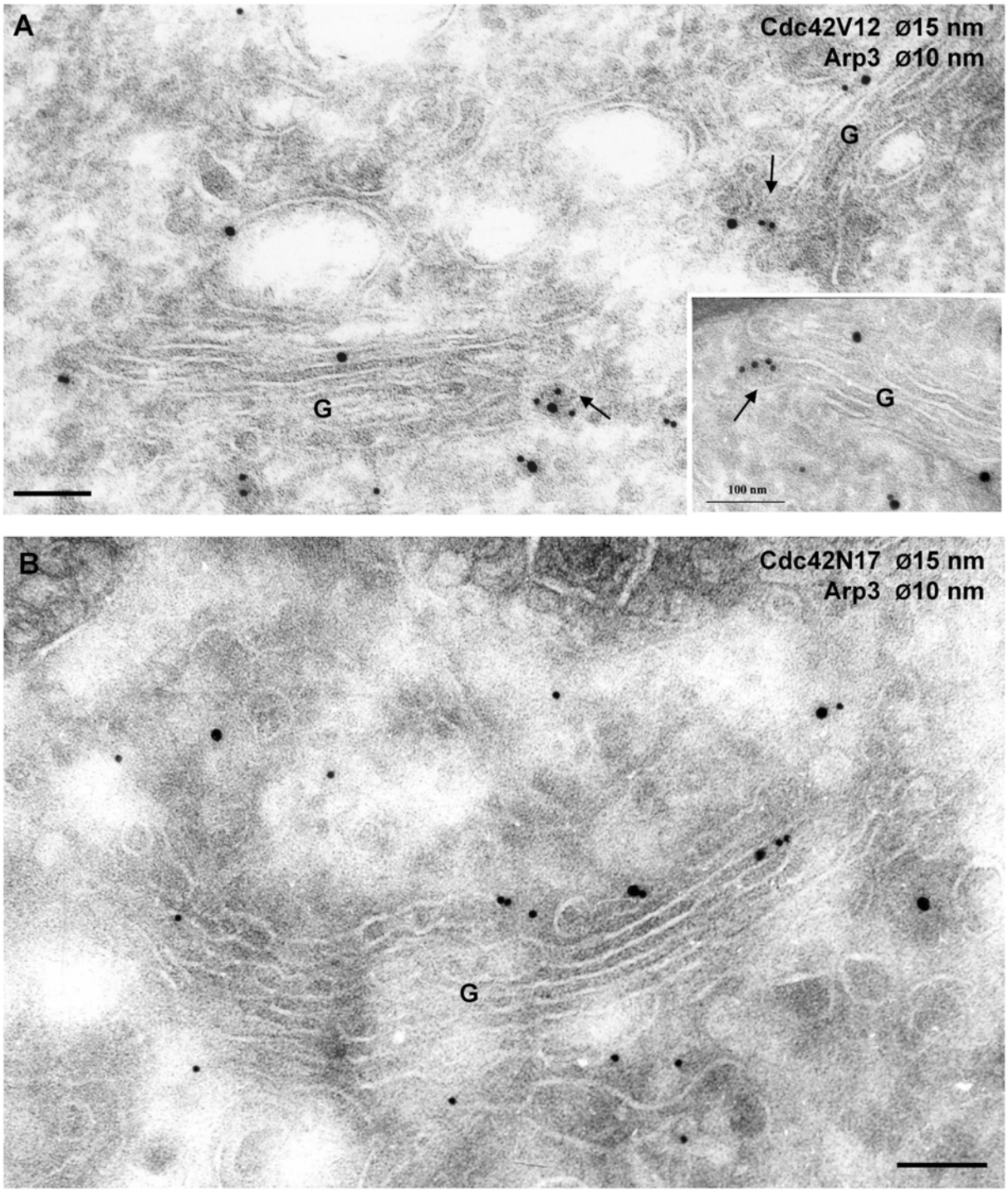

Figure 5: Arp3 localization in Golgi membranes of HeLa cells transfected with myc-Cdc42V12 (A) or myc-Cdc42N17 (B). Cells were fixed and processed for double immunogold labeling with monoclonal anti-myc and polyclonal anti-Arp3 antibodies followed by protein A-gold. In activated Cdc42-expressing cells (A), Arp3 is mostly located in the lateral portions of cisternae (insert in A, arrow) where it also colocalizes with activated Cdc42 (A, arrows). In contrast, in those cells expressing nonactivated Cdc42 (B), Arp3 and Cdc42 were uniformly distributed along the cisternae (B). G, Golgi stacks. Bars, 100 nm.

Cell culture

NIH3T3 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM from Invitrogen; Paisley, UK) containing 10% fetal calf serum (FCS). All culture media were supplemented with L-glutamine (10 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were grown in a humidified incubator at 37 °C and 5% CO_2.

Transient transfection

The transfection method Effectene was used (Qiagen, Valencia, CA). HeLa cells were grown in culture medium as described above and subconfluent cells (40–70%) were transfected according to the manufacturer's instructions. Cells were then expressed for 12–18 h.

Indirect immunofluorescence

Indirect immunofluorescence was carried out as previously described (33,34) with the following antibody dilutions: anti-Arp3, 1:100; anti-GM130, 1:500, antimyc 1:100 and Alexa-488- and Alexa-546-conjugated secondary antibodies, 1:1000. The coverslips were mounted on microscope slides using Mowiol (Calbiochem, Nottingham, UK). Microscopy and imaging were performed both in an Olympus BX60 epifluorescence microscope coupled with a cooled Olympus CCD camera and in a Leica TCS-NT confocal microscope.

Quantitative immunogold electron microscopy

Control and transfected HeLa cells were fixed and processed for gold staining (35). To establish the relative distribution of GFP-Cdc42, GFP-N-WASP and Arp2/3 in the Golgi area, cryosections were single or double immunolabeled with rabbit polyclonal antibodies against GFP, anti-KDELr or anti-Arp3, sheep polyclonal antibodies against TGN46 or monoclonal anti-myc or anti-GM130 antibodies followed by protein A-gold or rabbit anti-mouse/protein A-gold, respectively (36). In the Golgi area, gold particles were counted and ascribed to one of the following categories (6): (a) lateral portion (defined as the lateral zones of cisternae that show the characteristic terminal dilatation); (b) central portions (defined as the flattened zones of stacked cisternae); (c) peri-Golgi transport intermediates (defined as tubulovesicular membrane profiles located in the vicinity of the Golgi stacks).

The *cis*-to-*trans* Golgi distributions of Cdc42, N-WASP and Arp3 (Table 1) were calculated on 25 cross-sectioned Golgi stacks. The *cis*-side of the Golgi complex was identified by double immunogold labeling with anti-GM130 antibodies (18). Cisternae were numbered from *cis* (C1) to *trans* (C5). For all immunogold data, the number of gold particles assigned was expressed as a percentage (mean ± SEM) of the total gold labeling in the Golgi stack. For each experimental condition examined, at least 25 randomly selected Golgi areas were analyzed. To analyze the molecular composition of Arp3-positive peri-Golgi tubulovesicular structures, a series of double labeling experiments were performed in which Arp3 was combined with GM130, KDELr and TGN46, respectively. For each combination, 100 Arp3-positive tubulovesicular structures were examined. Statistical analysis was performed using Student's *t*-test.

Acknowledgments

This article is dedicated to the victims of the terrorist attack in Madrid on March 11. We would like to thank all those who provided us with antibodies and cDNAs; Stefan Fritz and Juan M. Duran for helpful discussions, Maite Muñoz for her continuous technical support, J. Ballesta (University of Murcia) for his interest and support, and Robin Rycroft for editorial assistance. This study was funded by grants to G.E. (BCM2003-01064 and in part by European Community's Human Potential Programme under contract HPRN-CT-2002–00259; Secretory Pathway Dynamics) and to J.A.M.-M. (BCM2003-03738 and Fundación Sèneca PB/49/FS/02).

References

1. Qualmann B, Kessels MM. Endocytosis and the cytoskeleton. Int Rev Cytol 2002;220:93–144.
2. Engqvist-Goldstein AEY, Drubin DG. Actin assembly and endocytosis: from yeast to mammals. Annu Rev Cell Dev Biol 2003;19:287–332.
3. Stamnes M. Regulating the actin cytoskeleton during vesicular transport. Curr Opin Cell Biol 2002;14:428–433.
4. Erickson JW, Zhang C, Kahn RA, Evans T, Cerione RA. Mammalian Cdc42 is a brefeldin A-sensitive component of the Golgi apparatus. J Biol Chem 1996;271:26850–26854.
5. Etienne-Manneville S, Hall A. Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCζ. Cell 2001;106:489–498.
6. Luna A, Matas OB, Martinez-Menarguez JA, Mato E, Duran JM, Ballesta J, Way M, Egea G. Regulation of protein transport from the golgi complex to the endoplasmic reticulum by CDC42 and N-WASP. Mol Biol Cell 2002;13:866–879.
7. Kroschewski R, Hall A, Mellman I. Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells. Nat Cell Biol 1999;1:8–13.
8. Cohen D, Musch A, Rodriguez-Boulan E. Selective control of basolateral membrane protein polarity by cdc42. Traffic 2001;2:556–564.
9. Rojas R, Ruiz WG, Leung SM, Jou TS, Apodaca G. Cdc42-dependent modulation of tight junctions and membrane protein traffic in polarized madin-darby canine kidney cells. Mol Biol Cell 2001;12:2257–2274.
10. Musch A, Cohen D, Kreitzer G, Rodriguez-Boulan E. Cdc42 regulates the exit of apical and basolateral proteins from the trans-Golgi network. EMBO J 2001;20:2171–2179.
11. Wu WJ, Erickson JW, Lin R, Cerione RA. The gamma-subunit of the coatomer complex binds Cdc42 to mediate transformation. Nature 2000;405:800–804.
12. Fucini RV, Chen JL, Sharma C, Kessels MM, Stamnes M. Golgi vesicle proteins are linked to the assembly of an actin complex defined by mAbp1. Mol Biol Cell 2002;13:621–631.
13. Abo A, Qu J, Cammarano MS, Dan C, Fritsch A, Baud V, Belisle B, Minden A. PAK4, a novel effector for Cdc42Hs, is implicated in the reorganization of the actin cytoskeleton and in the formation of filopodia. EMBO J 1998;17:6527–6540.
14. Paglini G, Peris L, Diez-Guerra J, Quiroga S, Caceres A. The Cdk5-p35 kinase associates with the Golgi apparatus and regulates membrane traffic. EMBO Rep 2001;2:1139–1144.

15. Estrada L, Caron E, Gorski J. Fgd1, the Cdc42 guanine nucleotide exchange factor responsible for faciogenital dysplasia, is localized to the subcortical actin cytoskeleton and Golgi membrane. Hum Mol Genet 2001;10:485–495.
16. Merrifield CJ, Qualmann B, Kessels MM, Almers W. Neural Wiskott Aldrich Syndrome Protein (N-WASP) and the Arp2/3 complex are recruited to sites of clathrin-mediated endocytosis in cultured fibroblasts. Eur J Cell Biol 2004;83:13–18.
17. Krueger EW, Orth JD, Cao H, McNiven MA. A dynamin-cortactin-Arp2/3 complex mediates actin reorganization in growth factor-stimulated cells. Mol Biol Cell 2003;14:1085–1096.
18. Nakamura N, Rabouille C, Watson R, Nilsson T, Hui N, Slusarewicz P, Kreis TE, Warren G. Characterization of a *cis*-Golgi matrix protein, GM130. J Cell Biol 1995;131:1715–1726.
19. Prescott AR, Lucocq JM, James J, Lister JM, Ponnambalam S. Distinct compartmentalization of TGN46 and β1,4-galactosyltransferase in HeLa cells. Eur J Cell Biol 1997;72:238–246.
20. Kessels MM, Qualmann B. Syndapins integrate N-WASP in receptor-mediated endocytosis. EMBO J 2002;21:6083–6094.
21. Schafer DA, Weed SA, Binns D, Karginov AV, Parsons JT, Cooper JA. Dynamin2 and cortactin regulate actin assembly and filament organization. Curr Biol 2002;12:1852–1857.
22. Cao H, Orth JD, Chen J, Weller SG, Heuser JE, McNiven MA. Cortactin is a component of clathrin-coated pits and participates in receptor-mediated endocytosis. Mol Cell Biol 2003;23:2162–2170.
23. Polishchuk RS, Mironov AA. Structural aspects of Golgi function. Cell Mol Life Sci 2004;61:146–158.
24. Duran JM, Valderrama F, Castel S, Magdalena J, Tomas M, Hosoya H, Renau-Piqueras J, Malhotra V, Egea G. Myosin motors and not actin comets are mediators of the actin-based Golgi-to-endoplasmic reticulum protein transport. Mol Biol Cell 2003;14: 445–459.
25. Rozelle AL, Machesky LM, Yamamoto M, Driessens MH, Insall RH, Roth MG, Luby-Phelps K, Marriott G, Hall A, Yin HL. Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. Curr Biol 2000;10:311–320.
26. Frischknecht F, Moreau V, Rottger S, Gonfloni S, Reckmann I, Superti-Furga G, Way M. Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. Nature 1999;401:926–929.
27. Carreno S, Engqvist-Goldstein AE, Zhang CX, McDonald KL, Drubin DG. Actin dynamics coupled to clathrin-coated vesicle formation at the trans-Golgi network. J Cell Biol 2004;21:781–788.
28. Weaver AM, Young ME, Lee WL, Cooper JA. Integration of signals to the Arp2/3 complex. Curr Opin Cell Biol 2003;15:23–30.
29. Kim K, Hou P, Gorski JL, Cooper JA. Effect of Fgd1 on cortactin in Arp2/3 complex-mediated actin assembly.Biochemistry 2004; 43: 2422–2427.
30. Jones SM, Howell KE, Henley JR, Cao H, McNiven MA. Role of dynamin in the formation of transport vesicles from the trans-Golgi network. Science 1998;279:573–577.
31. Kasai K, Shin HW, Shinotsuka C, Murakami K, Nakayama K. Dynamin II is involved in endocytosis but not in the formation of transport vesicles from the trans-Golgi network. J Biochem 1999; 125:780–789.
32. Chen JL, Lacomis L, Erdjument-Bromage H, Tempst P, Stamnes M. Cytosol-derived proteins are sufficient for Arp2/3 recruitment and ARF/coatomer-dependent actin polymerization on Golgi membranes. FEBS Lett 2004;21:281–286.
33. Valderrama F, Babia T, Ayala I, Kok JW, Renau-Piqueras J, Egea G. Actin microfilaments are essential for the cytological positioning and morphology of the Golgi complex. Eur J Cell Biol 1998;76:9–17.
34. Valderrama F, Luna A, Babia T, Martinez-Menarguez JA, Ballesta J, Barth H, Chaponnier C, Renau-Piqueras J, Egea G. The golgi-associated COPI-coated buds and vesicles contain β/γ-actin. Proc Natl Acad Sci U S A 2000;97:1560–1565.
35. Slot JW, Geuze HJ, Gigengack S, Lienhard GE, James DE. Immunolocalization of the insulin regulatable glucose transporter in brown adipose-tissue of the rat. J Cell Biol 1991;113:123–135.
36. Martinez-Menarguez JA, Prekeris R, Oorschot VMJ, Scheller R, Slot JW, Geuze HJ, Klumperman J. Peri-Golgi vesicles contain retrograde but not anterograde proteins consistent with the cisternal progression model of intra-Golgi. J Cell Biol 2001;155:1213–1224.

Membrane trafficking at the ER/Golgi interface: functional implications of RhoA and Rac1

Olga B. Matas, Stefan Fritz, Ana Luna[1], and Gustavo Egea[*]

Departament de Biologia Cel·lular i Anatomia Patològica, Facultat de Medicina, Universitat de Barcelona-IDIBAPS, 08036 Barcelona, Spain

[1]Present address: DPC Dipesa, Dept. Técnico, 08029 Barcelona (Spain)

Running title: RhoA and Rac1 at the ER/Golgi interface

*Address correspondence to:
Gustavo Egea, Dept. Biologia Cel·lular i Anatomia Patològica, Facultat de Medicina, Universitat de Barcelona, C/ Casanova 143, E-08036 Barcelona (Spain)

Phone: 34-93-402-19-09; Fax: 34-93-403-52-60

E-mail: gegea@ub.edu

ABSTRACT

N-WASP and Arp 2/3, the components of the actin nucleation/polymerization signaling pathway governed by Cdc42, are located in Golgi membranes and regulate ER/Golgi interface protein transport. In the present study, we examined whether RhoA and Rac1, like Cdc42, are also involved in this early secretory pathway. Unlike Cdc42, RhoA and Rac1 were not observed in the Golgi complex of different clonal cell lines nor were they present in isolated Golgi membranes. Expression of dominant-positive or dominant-negative mutants of RhoA or Rac1 proteins in HeLa cells did not alter either the assembly or the disassembly of the Golgi complex following the addition or withdrawal of BFA, respectively, the ER-to-Golgi VSV-G transport or the $Sar1^{dn}$-induced ER accumulation of Golgi proteins. Moreover, unlike Cdc42-expressing cells, the 15º C-induced subcellular redistribution of the KDEL receptor remained unaltered. Only cells that constitutively express the activated Cdc42 mutant (Cdc42Q61L), or that were microinjected with activated Cdc42Q61L protein, exhibited a significant change in Golgi complex morphology. Collectively, our results demonstrate that RhoA and Rac1 are not located in the Golgi complex, nor do they directly or indirectly regulate membrane trafficking at the ER/Golgi interface. This finding, in turn, confirms that Cdc42 is the only Rho GTPase to have a specific function on the Golgi complex.

Key words: actin, Rho GTPases, secretory pathway, cytoskeleton, Golgi apparatus, ER/Golgi interface

INTRODUCTION

While the Rho family of proteins were known to be key regulators of actin dynamics (Etienne-Manneville and Hall, 2001, Hall, 1998), over the past few years evidence has accumulated indicating that these proteins are also essential regulators in endocytic and secretory membrane trafficking (Ridley, 2001; Symons and Rusk, 2003). Rho proteins mediate these as well as other functions depending on their GTPase cycle: either GDP-bound (active) or GTP-bound (inactive). The three types of regulatory proteins controlling this GTPase cycle, guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (Symons and Settleman, 2000), not only govern nucleotide exchange, but also act as rate-limiting factors in Rho GTPase functions. Moreover, recent evidence indicates that Rho GTPases, and their nucleotide exchange regulators, are highly localized within the endomembrane system, controlling relevant trafficking events (Higgins and McMahon, 2002; Symons and Settleman, 2000; Symons and Risk, 2003).

Significant progress has been made in the dissection of the downstream signaling events that mediate Rho, Rac, Cdc42 and their respective regulators in endocytic membrane trafficking. In fact, signaling molecules have been observed acting in close concert with actin dynamics regulation (Engvist and Dubrin, 2003; Qualmann and Mellor, 2003; Symons and Rusk, 2003). Conversely, the most compelling evidence of a role for Rho GTPases in the secretory pathway in mammalian cells derives from studies on Cdc42. Together with N-WASP and Arp2/3, Cdc42 is located in the Golgi complex (Chen et al., 2004; Erickson et al., 1996; Etienne-Manneville and Hall, 2001; Luna et al., 2002; Matas et al., 2004) wherein regulate ER/Golgi interface and post-Golgi trafficking (Cohen et al., 2001; Fuccini et al., 2002; Kroschewski et al., 1999; Luna et al., 2002; Müsch et al., 2001; Rojas et al., 2001; Wu et al., 2000). Cytoskeletal dynamics at the Golgi complex is regulated by the interaction between the coatomer (□-COP) and Cdc42 (Fucini et al., 2002; Wu et al., 2000), a complex that, interestingly, correlates with Cdc42-induced cell transformation (Wu et al., 2000). These findings on Cdc42 illustrate the concept of coordinated vesicular trafficking and actin dynamics for Rho GTPases cell functions.

Here we examined whether RhoA and Rac1, like Cdc42, could coordinate vesicular

trafficking and actin dynamics at the ER/Golgi interface. Our results definitively indicate that neither of these two GTPases is located in the Golgi complex, and that neither regulates membrane trafficking at the ER/Golgi interface. These findings leave Cdc42 as the unique Rho GTPase protein capable of playing a functional role in the Golgi complex.

MATERIALS AND METHODS

Constructs and antibodies. The plasmids encoding GFP-RhoA, GFP-Rac1 and GFP-Cdc42 wild-type, dominant-positive (RhoAV14; Rac1V12 or RacQ61; Cdc42V12) and dominant-negative (RhoAN17; Rac1N17; Cdc42N17) forms were provided by M. Way (Imperial Cancer Research Fund, London) and F. Sánchez-Madrid (Hospital La Princesa, Madrid); those encoding GFP-VSV-G and the dominant-negative Sar1 mutant (Sar1 [H79G], $Sar1^{dn}$) were kindly provided by Jennifer Lippincott-Schwartz (NIH, Bethesda, MA, USA) and Rainer Pepperkok (EMBL, Heidelberg), respectively. Rabbit polyclonal antibodies anti-Mannosidase II (ManII) and anti-KDEL receptor (KDELr) were from K. Moremen (University of Georgia, Georgia) and Stressgen (San Diego, CA), respectively. Mouse monoclonal antibodies to myc and to GM130 were obtained from BABCO-Covance Inc (Princeton, New Jersey) and Transduction Laboratories (San Diego, CA), respectively; those to Cdc42, Rho and Rac1 were purchased from BD Biosciences Pharmingen (San Diego, CA); and monoclonal anti-giantin antibodies were a gift from H.-P. Hauri (Biozentrum, Basel), respectively. Sheep polyclonal antiserum to TGN46 was obtained from SeroTec (Oxford, UK). Mouse monoclonal antibodies to Secondary Alexa-488 or Alexa-546 $F(ab')_2$ fragment and cascade blue dextran were acquired from Molecular Probes (Eugene, OR). For Western blotting experiments, we used the secondary antibody ImmunoPure goat anti-mouse IgG from Pierce (Rockford, IL). TRITC-phalloidin was purchased from Sigma (St. Louis, MI, USA).

Cell culture. Human melanoma A375P cell line, HeLa cells and NRK cells were cultured in Dulbecco's modified Eagle's medium (DMEM from Gibco/Brl Life Technologies; Paisley, UK) containing 10% fetal calf serum (FCS). NIH3T3 stable cell lines were cultured in DMEM medium containing 10% normal calf serum (NCS). All culture media were supplemented with L-glutamine (10 mM), penicillin (100 U/ml) and streptomycin (100 □g/ml). Cells were grown in a humidified incubator at 37 ºC and 5% CO_2.

Cell extract, Golgi purification and Western blot analysis. NRK cell extracts were obtained from culturing cells until confluence in ten p100 Petri dishes. Cells were treated with PBS containing TX-100 (1%) and PMSF (1 mM) for 2 h at 4º C.

Thereafter, the lysate was centrifuged in an Eppendorf 5415R centrifuge at 16.000 rpm for 30 min at 4ºC. Protein content of the supernatant was determined by the Bradford method and subsequently precipitated by adding TCA (10%).
Fractions enriched in Golgi membranes were isolated from rat liver and HeLa cells by flotation in a sucrose gradient as previously described (Chen et al., 2004). Samples were prepared for SDS-PAGE by boiling in sample buffer. Proteins were electrophoretically transferred onto nitrocellulose membranes. Blots were blocked in 5% nonfat dry milk in TBS with 0.1% Tween-20 (vol/vol) (TBST) for 2 h at room temperature. Primary antibody incubations were performed in TBS containing 1.5% BSA for 2 h at room temperature and used as recommended by the distributor. Secondary antibody incubations were performed in TBST containing 5% nonfat dry milk at 1:20,000 for 1 h at room temperature. Protein bands were visualized using a Supersignal West Pico Chemiluminiscence Substrate Kit from Pierce (Rockford, IL).

Microinjection. For protein microinjection, A375P cells were grown for 1-2 days on normal glass coverslips and cultured in DMEM plus 10 % FCS medium containing 25 mM HEPES, and supplemented with penicillin, streptomycin and glutamine. Constitutively activated Rac and Cdc42 mutant proteins (RacQ61L-GST and Cdc42Q61L-GST, respectively; Cytoskeleton, Denver, CO, USA) were first diluted to 200 μg/ml and then microinjected into cytoplasm using an automated microinjection system (Carl Zeiss, Jena, Germany). For microinjection of single or double cDNA constructs, NRK or HeLa cells, respectively, were cultured as described above. The cDNA constructs were first diluted to 50-100 ng/ml and then injected into the cell nucleus. Thereafter, the coverslips were transferred to a Petri dish containing fresh culture medium and returned to the incubator for 30 min (protein microinjection) or 3-6 h (construct microinjection).

Transient transfection. The transfection method used was Effectene (Qiagen, Valencia, CA). HeLa cells were grown in culture medium as described above and sub-confluent cells (40-70 %) were transfected according to the manufacture's instructions. Cells were then expressed for 12-18 h.

ER-to-Golgi VSV-G transport assay. HeLa cells were transiently co-transfected with GFP-VSV-G temperature sensitive mutant cDNA and the dominant-negative or dominant-positive mutant forms of myc-tagged RhoA or Rac1 expression vectors. After 24 hours at 40 ºC, the VSV-G protein accumulated in the ER without any observed interference in RhoA or Rac1 protein expression. Cells were maintained for 1 additional hour at a non-permissive temperature in the presence of cycloheximide (100 µg/ml). Cells were then transferred to a permissive temperature (32 ºC) for different time periods. Subsequently, cells were fixed and processed for immunofluorescenc

Indirect Immunofluorescence. Indirect immunofluorescence was carried out as previously described (Valderrama et al., 1998; 2000) with the following antibody dilutions: anti-ManII, 1:2000; anti-giantin, 1:500; anti-GM130, 1:10000; anti-myc, 1:1000; anti-KDELr, 1:200; Alexa-488- and Alexa-546-conjugated secondary antibodies, 1:1000. Coverslips were mounted on the microscope slides using Mowiol (Calbiochem, Nottingham, UK). Microscopy and imaging were performed both with an Olympus BX60 epifluorescence microscope coupled to a cooled Olympus CCD camera, and with a Leica TCS-NT confocal microscope.

Results and Discussion

We and other laboratories have previously reported the localization of Cdc42 in the Golgi complex (Erickson et al., 1996; Etienne-Manneville and Hall, 2001; Luna et al., 2002). Here we examined whether RhoA and Rac1, like Cdc42, are also located in this organelle and whether they also regulate ER/Golgi interface membrane trafficking. HeLa (not shown) and NRK cells were microinjected with plasmids expressing GFP-tagged positive- or negative-dominant mutant forms of Cdc42, Rac1, or RhoA (Fig. 1). Unlike Cdc42V12 (dominant-positive; Fig. 1 A, B) and Cdc42N17 (dominant-negative; Fig. 1C, D) (Luna et al., 2002), neither RacV12/RacN17 (Fig. 1 E-H) nor RhoV14/RhoN19 (Fig. 1 I-L) mutants co-localized with mannosidase II (ManII) in the Golgi complex. Rac1 mutant proteins accumulated mainly in the nucleus and in membrane ruffles (Fig. 1E and G; Kraynov et al., 2000); RhoA mutants accumulated in the cytoplasm (Fig. 1 I and K). Isolated Golgi membranes from rat liver (Fig. 1M) were also examined by western blotting for the presence of these three Rho GTPases. As shown in Fig. 1M, while only Cdc42 was present in isolated Golgi membranes, all three GTPases were detected, as expected, in cell extracts. Thus, immunohistochemical and biochemical experimental approaches indicate that RhoA and Rac1 do not localize to the Golgi complex. Nevertheless, the possibility that both GTPases could indirectly regulate ER/Golgi interface membrane dynamics by the activation of downstream effectors cannot be ruled out. To test this possibility, HeLa cells were transfected with GFP-tagged forms of activated RhoA (RhoV14) or Rac1 (RacQ61L), and treated initially with brefeldin A (BFA) to thereby examine the retrograde membrane flow leading to the disassembly of the Golgi complex and its subsequent fusion into the ER (Fig. 2A-F). Afterwards, BFA was washed out to analyze the anterograde membrane flow from the ER leading to the reassembly of the Golgi complex (Fig. 2G-L). In non-transfected and RhoV14-transfected cells, the kinetics of Golgi complex disassembly and reassembly were the same (Fig. 2A-L). Similar results were obtained both in cells transfected with GFP-RacV12, and in stable NHI3T3 cell lines that constitutively expressed activated Rac1 or RhoA mutants (data not shown). We also examined the ER-to-Golgi transport of GFP-VSV-G glycoprotein in transfected HeLa cells expressing the myc-tagged dominant-positive or the dominant-negative mutant forms of RhoA or Rac1 (Fig. 3). VSV-G transport was initiated by incubating cells from a non-permissive

(40º C; Fig. 3A, D) to a permissive (32º C; Fig. 3 B, C, F, H) temperature. After 15 (Fig. 3 B, F) and 30 min (Fig. 3C, H) at 32 ºC, the viral protein had already reached the Golgi complex, exhibiting the same kinetics in transfected (Fig. 3 F-I) as in non-transfected cells (Fig. 3 B, C). The retrograde Golgi-to-ER relocation of the Golgi matrix protein giantin, induced by the expression of the GTPase-deficient Sar1 mutant protein (Sar1H79G; $Sar1^{dn}$; Aridor et al., 1995), was also examined in microinjected cells co-expressing GFP-RacQ61L or GFP-Cdc42V12 (Fig. 4B, asterisks in inset). Unlike GFP-Cdc42V12 (Fig. 4D-F, asterisks; see also Luna et al., 2002), in cells co-expressing $Sar1^{dn}$ and dominant-positive Rac1, disassembly of the giantin-stained Golgi complex and subsequent ER accumulation was observed (Fig. 4A-C, asterisk). Finally, the redistribution of the ER/Golgi interface cycling protein KDEL receptor in HeLa cells cultured at 15º C was identical in neighboring non-transfected (Fig. 5 B, D, F, H) to RacV12-transfected (Fig. 5, B, D, F, H, asterisks) or RhoV12-transfected (not shown) HeLa cells.

We have demonstrated here that RhoA and Rac1, unlike Cdc42, are neither located in the Golgi complex nor do they affect Golgi morphology or transport along the early secretory pathway. In regards to RhoA, these observations are consistent with previous findings in which RhoA was discarded as a regulating component of Golgi-associated actin dynamics (Fuccini et al., 2002; Valderrama et al., 2000). In hippocampal neurons, however, the Rho-binding protein Citron-N and RhoA were both observed in the Golgi complex of Citron-N-overexpressing cells, even though their function remains unknown (Camera et al., 2003). The localization of RhoA and RhoA-binding proteins in the Golgi complex could be characteristic of neuronal cells since none of several clonal cell lines examined in this study (HeLa, NRK, Vero and A375 cells) exhibited any co-localization with well-established Golgi protein markers. On the other hand, Rac1 has been widely implicated both in endocytic trafficking events and in the exocytic processes of secretory cells (Qualmann and Mellor, 2003). Therefore Rac1, like RhoD (Murphy et al., 1996), might regulate secretory vesicular trafficking and actin dynamics in coordinated processes. However, the observations that (1) Rac1 is not required in the ER/Golgi interface membrane transport; (2) the steady-state distribution of the cycling TGN marker TGN46 is also unaltered in cells overexpressing the dominant-positive or dominant-negative form of Rac1 (O.B.M. and G.E., unpublished results); and (3) Rac1-

mediated formation of lamellipodia and PKD-dependent constitutive membrane trafficking are independent processes in migrating cells (Prigozhina and Waterman-Storer, 2004) collectively indicate that Rac1, like RhoA, does not regulate secretory membrane trafficking. However, recent evidence implicates the Lowe syndrome protein OCRL1 as the Rac1-interacting protein observed at the TGN level (Faucherre et al., 2003). The physiological relevance to membrane trafficking of such interaction remains as yet unknown.

Finally, the stable NIH3T3 cell lines cultured at low-fetal calf serum to better visualize actin cytoskeleton responses (Fig. 6 A, C, D, F; Jiménez et al., 1995; Perona et al., 1997) were also used to examine any putative correlation between the different activated Rho GTpases-induced actin cytoskeleton organizations and Golgi complex architecture. Interestingly, cells that constitutively expressed activated Cdc42 exhibited Golgi complex with extended perinuclear morphology (Fig. 6D). Conversely, control, RhoV12- and RacQ61L-expressing cells showed a compact Golgi morphology (Figs. 1B, F and H, respectively). Similar results were also obtained in human A375P cells microinjected with the activated Cdc42 (Cdc42Q61L-GST) or Rac1 (RacQ61L-GST) mutant proteins (data not shown). Collectively, these results suggest that Cdc42, unlike RhoA and Rac1, is not only present in the Golgi complex, regulating ER/Golgi interface protein transport (Fucini et al., 2002; Luna et al., 2002; Wu et al., 2000), but also influences Golgi complex morphology. Most likely, this is carried out through the regulation of the actin assembly on Golgi membranes.

In conclusion, the findings reported here are of relevant interest because they not only definitively discard the involvement of RhoA and Rac1 membrane dynamics in the ER/Golgi interface, but also confirm that Cdc42 has a specific effect on Golgi structure and function in comparison to other studied Rho GTPases.

Acknowledgements. The authors thank Maite Muñoz for technical support, Juan C. Lacal (IIB, CSIC, Madrid) for kindly providing the stable NIH3T3 cell lines, to numerous colleagues for supplying antibodies and plasmids (see Material and Methods), and to Robin Rycroft for editorial assistance. This study was funded by grants to G.E. (BCM2003-01064 and, in part, by the European Community's Human Potential Programme, contract HPRN-CT-2002-00259; Secretory Pathway Dynamics).

REFERENCES

Aridor M., Bannykh S. I., Rowe T., Balch W. E., 1995. Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. J Cell Biol 131, 875-893.

Camera P., Da Silva J. S., Griffiths G., Giuffrida M. G., Ferrara L., Schubert V., Imarisio S., Silengo L., Dotti, C. G., Di Cunto, F., 2003. Citron-N is a neuronal Rho-associated protein involved in Golgi organization through actin cytoskeleton regulation. Nature Cell Biology 5, 1071-1078.

Chen J. L., Lacomis L., Erdjument-Bromage H., Tempst P., Stamnes M., 2004. Cytosol-derived proteins are sufficient for Arp2/3 recruitment and ARF/coatomer-dependent actin polymerization on Golgi membranes. Febs Letters 566, 281-286.

Cohen D., Musch A., Rodriguez-Boulan E., 2001. Selective control of basolateral membrane protein polarity by cdc42. Traffic 2, 556-564.

Engqvist-Goldstein A. E. Y., Drubin D. G., 2003. Actin assembly and endocytosis: From yeast to mammals. Annual Review of Cell and Developmental Biology 19, 287-332.

Erickson J. W., Zhang C., Kahn R. A., Evans T., Cerione R. A., 1996. Mammalian Cdc42 is a brefeldin A-sensitive component of the Golgi apparatus. J Biol Chem 271, 26850-26854.

Etienne-Manneville S., Hall A., 2001. Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell 106, 489-498.

Faucherre A., Desbois P., Satre V., Lunardi J., Dorseuil O., Gacon G., 2003. Lowe syndrome protein OCRL1 interacts with Rac GTPase in the trans-Golgi network. Human Molecular Genetics 12, 2449-2456.

Fucini R. V., Chen, J. L., Sharma C., Kessels M. M., Stamnes M., 2002. Golgi Vesicle Proteins Are Linked to the Assembly of an Actin Complex Defined by mAbp1. Mol Biol Cell 13, 621-631.

Hall A., 1998. Rho GTPases and the actin cytoskeleton. Science 279, 509-514.

Higgins M. K., McMahon H. T., 2002. Snap-shots of clathrin-mediated endocytosis. Trends in Biochemical Sciences 27, 257-263.

Jiménez B., Arends M., Esteve P., Perona R., Sánchez R., Cajal S. R., Wyllie A., Lacal J. C., 1995. Induction of Apoptosis in Nih3T3 Cells After Serum Deprivation by Overexpression of Rho-P21, A Gtpase Protein of the Ras Superfamily. Oncogene 10, 811-816

Kraynov V.S., Chamberlain C., Bokoch G.M., Schwartz M.A., Slabaugh S., Hahn K.M., 2000. Localized Rac activation dynamics visualized in living cells. Science 290, 333-337.

Kroschewski R., Hall A., Mellman I., 1999. Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells. Nat Cell Biol 1, 8-13.

Luna A., Matas O. B., Martínez-Menárguez J. A., Mato E., Duran J.M., Ballesta J., Way M., Egea G., 2002. Regulation of protein transport from the golgi complex to the endoplasmic reticulum by CDC42 and N-WASP. Molecular Biology of the Cell 13, 866-879.

Matas O. B., Martinez-Menarguez J. A., Egea G., 2004. Association of Cdc42/N-WASP/Arp2/3 signaling pathway with golgi membranes. Traffic 5, 838-846.

Murphy C., Saffrich R., Grummt W., Gournier H., Rybin V., Rubino M., Auvinen P., Lutcke A., Parton R. G., Zerial M., 1996. Endosome dynamics regulated by a Rho protein. Nature 384, 427-432.

Musch A., Cohen D., Kreitzer G., Rodriguez-Boulan E., 2001. cdc42 regulates the exit of apical and basolateral proteins from the trans-Golgi network. EMBO J 20, 2171-2179.

Perona R., Montaner S., Saniger L., Sanchez-Perez I., Bravo R., Lacal J. C., 1997. Activation of the nuclear factor-kappa B by Rho, CDC42, and Rac-1 proteins. Genes and Development 11, 463-475

Prigozhina N. L., Waterman-Storer C. M., 2004. Protein kinase D-mediated anterograde membrane trafficking is required for fibroblast motility. Current Biology 14, 88-98.

Qualmann B., Mellor H., 2003. Regulation of endocytic traffic by rho GTPases. Biochemical Journal 371, 233-241.

Ridley A. J., 2001. Rho family proteins: coordinating cell responses. Trends in Cell Biology 11, 471-477.

Rojas R., Ruiz W. G., Leung S. M., Jou T. S., Apodaca G., 2001. Cdc42-dependent modulation of tight junctions and membrane protein traffic in polarized madin-darby canine kidney cells. Mol Biol Cell 12, 2257-2274.

Symons M., Settleman J., 2000. Rho family GTPases: more than simple switches. Trends in Cell Biology 10, 415-419.

Symons M., Rusk N., 2003. Control of vesicular trafficking by Rho GTPases. Current Biology 13, R409-R418.

Valderrama F., Babia T., Ayala I., Kok J. W., Renau-Piqueras J., Egea G., 1998. Actin microfilaments are essential for the cytological positioning and morphology of the Golgi complex. Eur J Cell Biol 76, 9-17.

Valderrama F., Luna A., Babia T., Martinez-Menarguez J. A., Ballesta J., Barth H., Chaponnier C., Renau-Piqueras J., Egea G., 2000. The golgi-associated COPI-coated buds and vesicles contain beta/gamma -actin. Proc Natl Acad Sci U S A 97, 1560-1565.

Wu W.J., Erickson J. W., Lin R., Cerione R. A., 2000. The gamma-subunit of the coatomer complex binds Cdc42 to mediate transformation. Nature 405, 800-804.

Figure 1

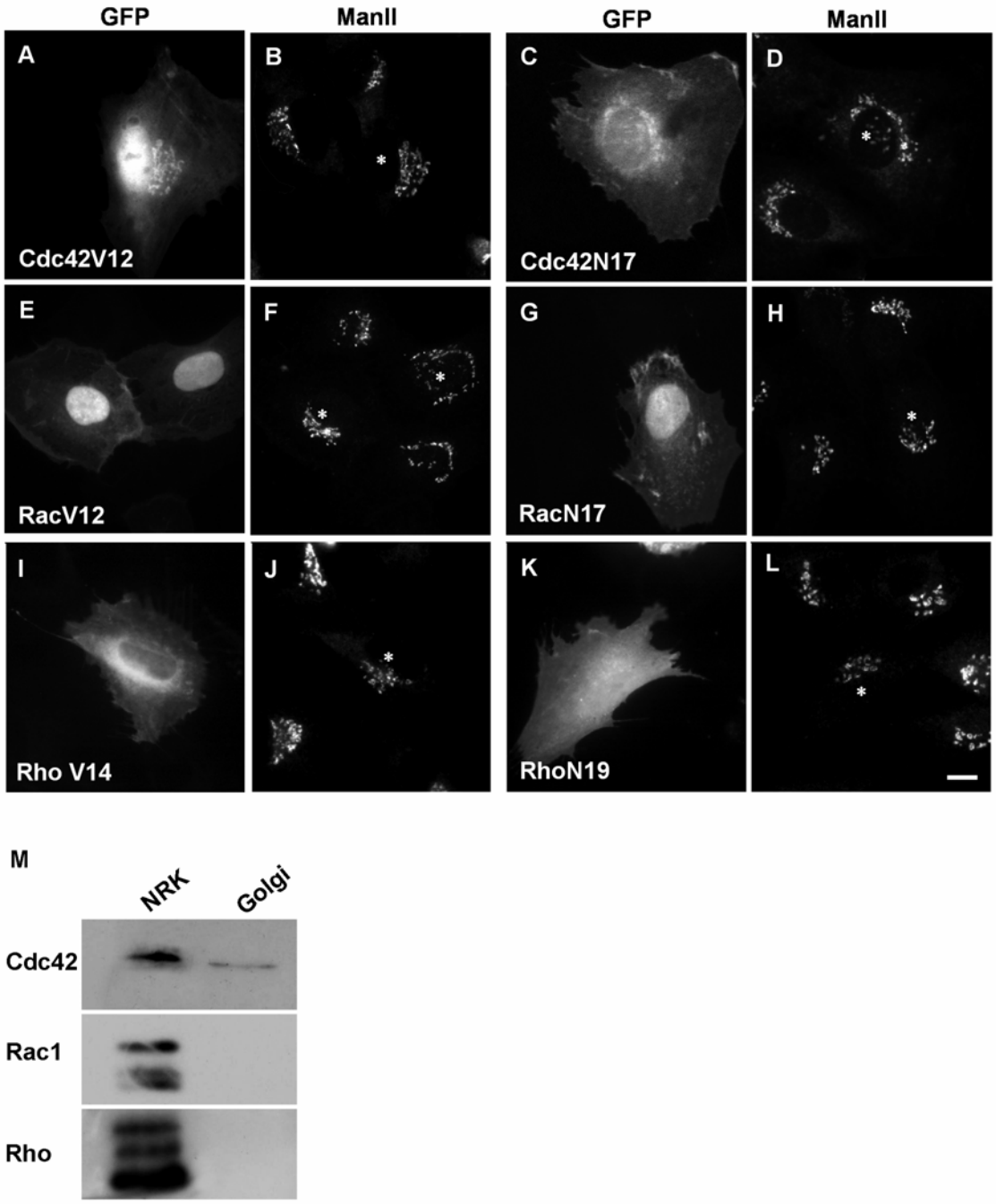
GFP
ManII
GFP
ManII
A
B
C
D
Cdc42V12
Cdc42N17
E
F
G
H
RacV12
RacN17
I
J
K
L
Rho V14
RhoN19
M
NRK
Golgi
Cdc42
Rac1
Rho

Figure 1. **Unlike Cdc42, neither RhoA nor Rac1 are located in the Golgi complex**. NRK cells were microinjected into the nucleus with expressing vectors for the dominant-positive or dominant-negative mutant forms of GFP-Cdc42 (Cdc42 V12 and Cdc42N17, respectively), GFP-Rac1 (RacV12 and RacN17) and GFP-Rho (RhoV14 and RhoN19). After microinjection (3-4 h), cells were processed for immunofluorescence confocal microscopy. The Golgi complex was visualized with anti-ManII antibodies. Unlike Cdc42 (Cdc42V12/N17, A-D), neither Rac1 (RacV12/N17, E-H) nor RhoA (RhoAV14/N19; I-L) co-localized with Man II in the Golgi complex. NRK cell extracts (NRK) and isolated rat liver Golgi membranes (Golgi) were submitted to SDS-PAGE and western blotting to determine the presence of Cdc42, Rac1 and Rho using their respective monoclonal antibodies. Unlike Rac1 and Rho, only Cdc42 was detected in Golgi membranes. Asterisks indicate microinjected cells. Bar, 10 μm.

Figure 2

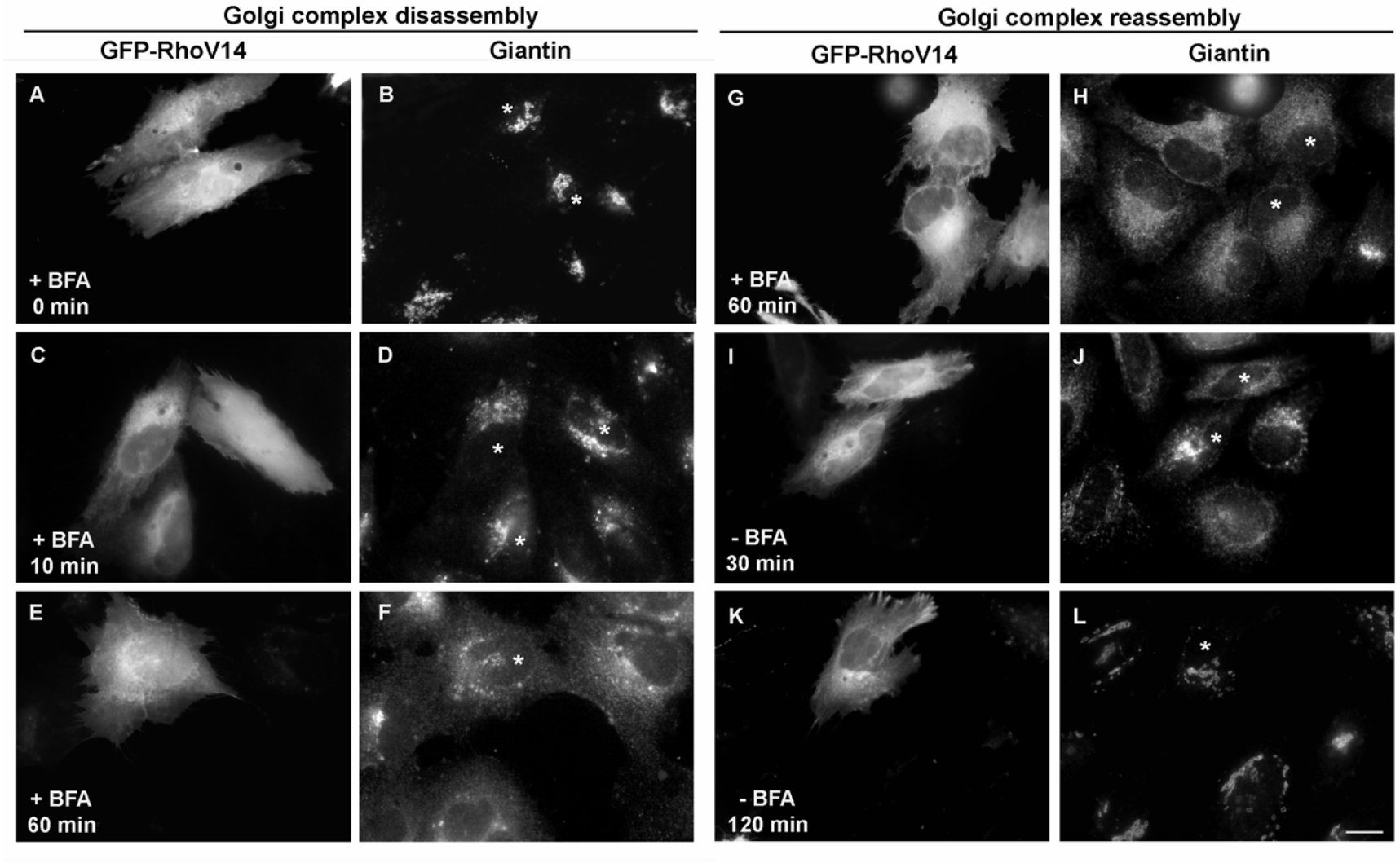
Golgi complex disassembly
GFP-RhoV14
Giantin
Golgi complex reassembly
GFP-RhoV14
Giantin
A
+ BFA
0 min
B
G
+ BFA
60 min
H
C
+ BFA
10 min
D
I
- BFA
30 min
J
E
+ BFA
60 min
F
K
- BFA
120 min
L

Figure 2. **The dominant-positive mutant form of RhoA does not perturb Golgi complex disassembly and reassembly**. GFP-RhoAV12-transfected HeLa cells were treated with BFA to monitor the Golgi disassembly (A-F). Subsequently, BFA was withdrawn and the Golgi complex reassembly was monitored (G-L). Following their respective treatments, cells were processed for immunofluorescence microscopy. Transfected cells were identified by the green fluorescence of GFP (A, C, E, G, I, and K) and the Golgi complex was visualized with anti-giantin antibodies (B, D, F, H, J, and L). Note that the kinetics of the Golgi complex disassembly and reassembly are the same in transfected (asterisks) as in neighboring non-transfected cells. Bar, 10 μm

Figure 3

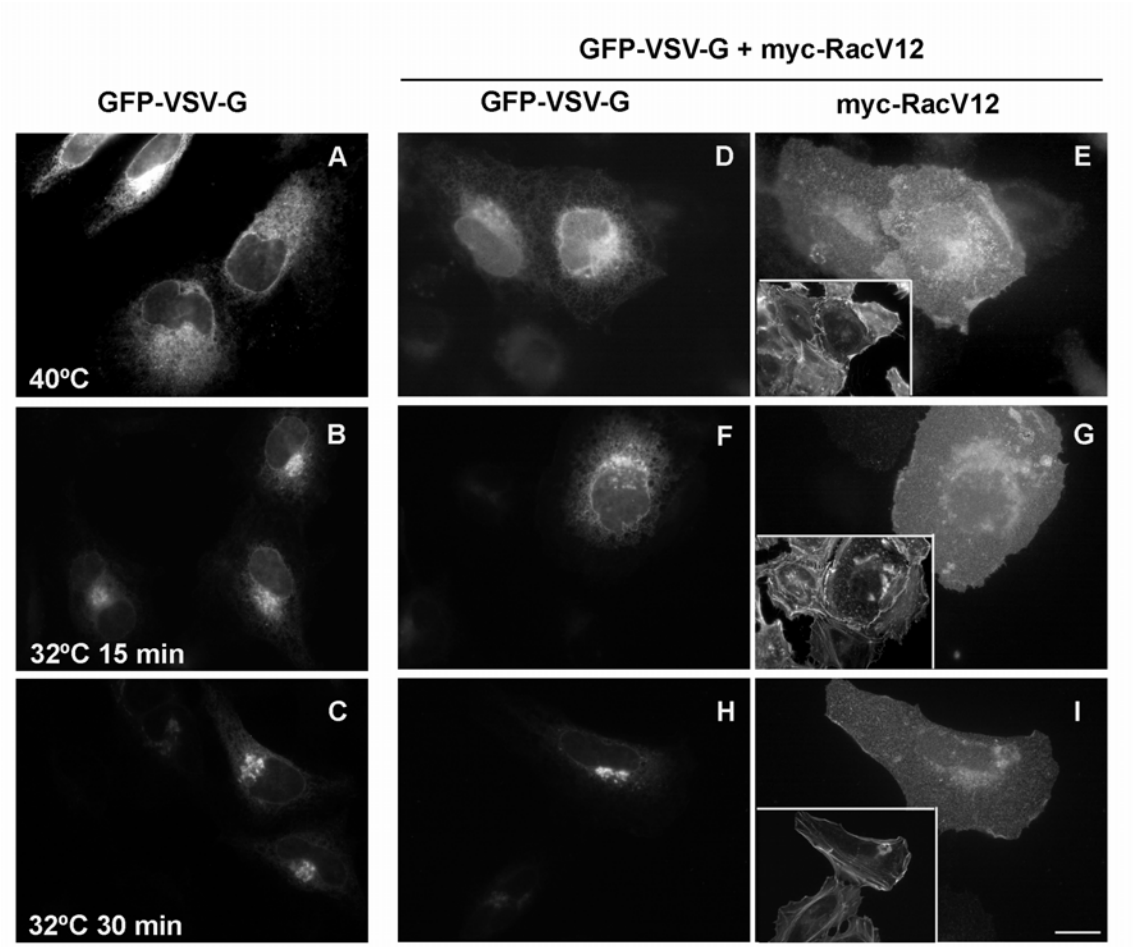
GFP-VSV-G + myc-RacV12
GFP-VSV-G
GFP-VSV-G
myc-RacV12
A
D
E
40ºC
B
F
G
32ºC 15 min
C
H
I
32ºC 30 min

Figure 3. ER-to-Golgi VSV-G transport remains unaltered in HeLa cells expressing the dominant-positive mutant form of Rac1. HeLa cells were transfected with GFP-VSV-G alone (A-C) or co-transfected with GFP-VSV-G and myc-RacV12 (D-I) expressing vectors. Cells incubated at non-permissive temperature (40ºC) show VSV-G accumulation in the ER (A and D). At permissive-temperature (32ºC), VSV-G is transported to the Golgi complex (15 min in B and F; 30 min in C and H). Note that ER-to-Golgi VSV-G transport occurs with the same kinetics both in non-cotransfected (B and C) and in Rac1V12 (F-I)-cotransfected cells. Insets in E, G and I show the actin cytoskeleton rearrangement induced by activated Rac1 mutant. Bar, 10 μm

Figure 4

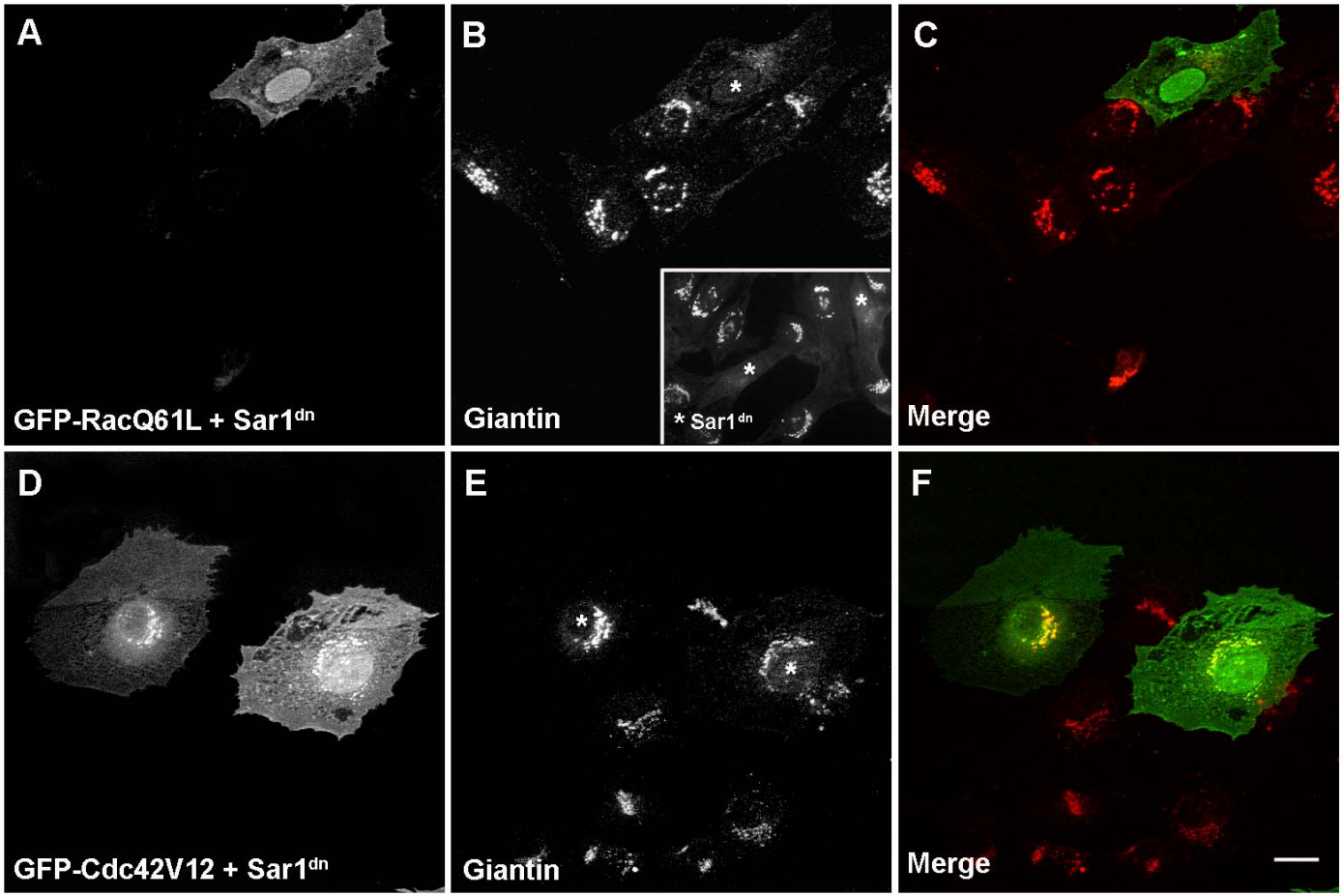
A
GFP-RacQ61L + Sar1dn
B
Giantin
* Sar1dn
C
Merge
D
GFP-Cdc42V12 + Sar1dn
E
Giantin
F
Merge

Figure 4. **Activated Rac1 does not perturb the Sar1^{dn}-induced disassembly of the Golgi complex**. Sar1^{dn} plus GFP-RacQ61L (A) or GFP-Cdc42V12 (D) mutant constructs were microinjected into the nucleus of HeLa cells. Following 6-8 h of expression, cells were fixed and processed for immunofluorescence confocal microscopy using antibodies to giantin (B, E). In cells microinjected with Sar1^{dn} plus GFP-Cdc42V12, giantin revealed a characteristic Golgi-like staining pattern (E, asterisks), whereas in those cells microinjected with RacV12 plus Sar1^{dn}, giantin exhibited a more ER-like staining pattern (B, asterisk) as observed in cells microinjected with Sar1^{dn} alone (insert in B, asterisks). Merge images are shown in C and F. Bar, 10 μm.

Figure 5

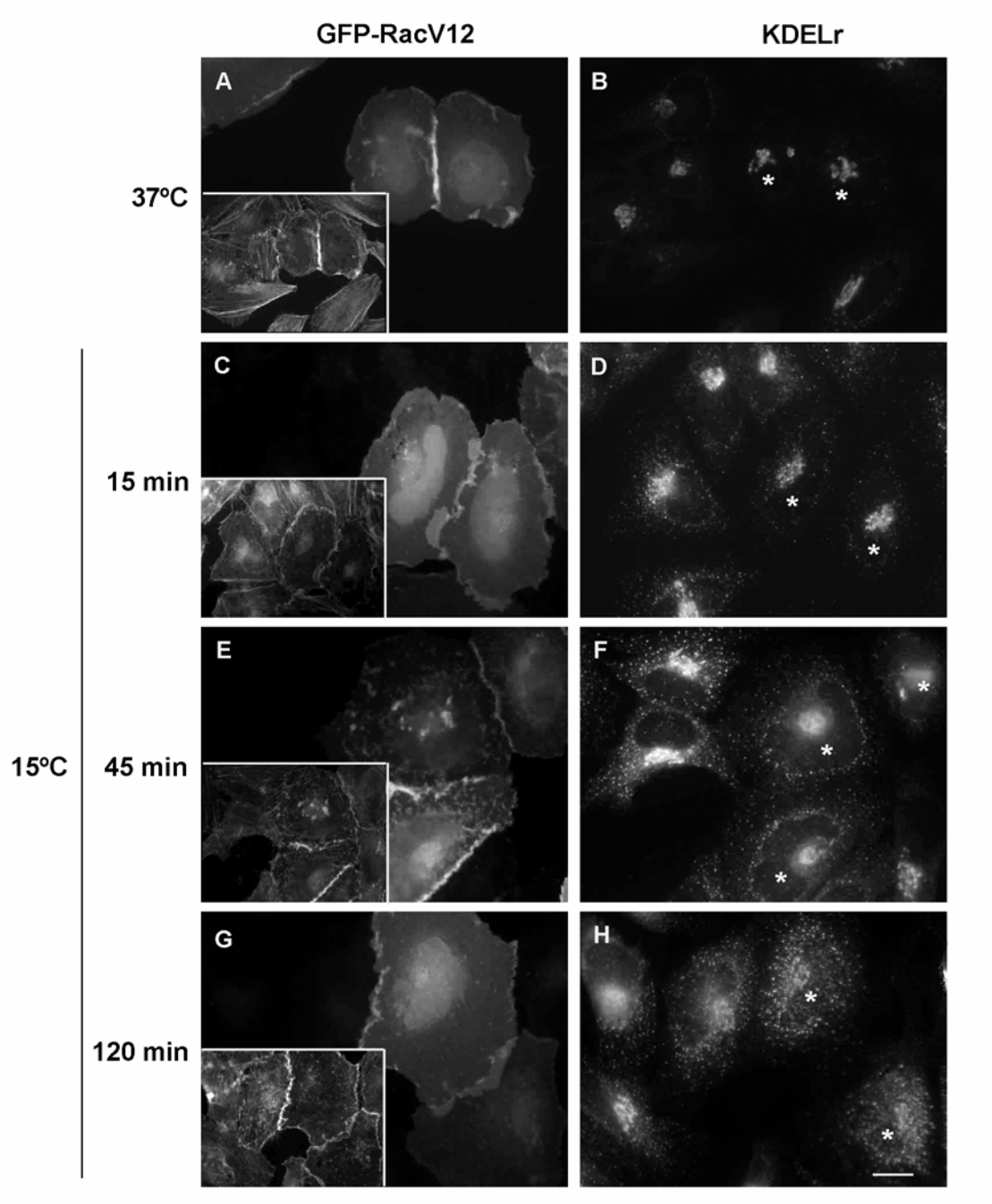

Fig. 5. KDEL receptor redistribution induced by low-temperature (15ºC) is not perturbed by expressing activated Rac1. HeLa cells were transfected with GFP-Rac1V12 for 18 h, fixed and immunostained to KDELr. At 37º C, the steady-state distribution of the KDEL receptor exhibited a Golgi-like and a diffuse punctate staining (B). When cells were incubated at 15º C for various time periods, the steady-state staining pattern of the KDEL receptor progressively changed to exclusively punctate staining, both in neighboring non-transfected (D, F, H) and Rac1V12-transfected cells (asterisks in D, F, H). Insets in A-G show the expected actin cytoskeleton rearrangement (plasma membrane ruffling) induced by expressing activated Rac1 mutant form. Bar, 10 μm.

Figure 6

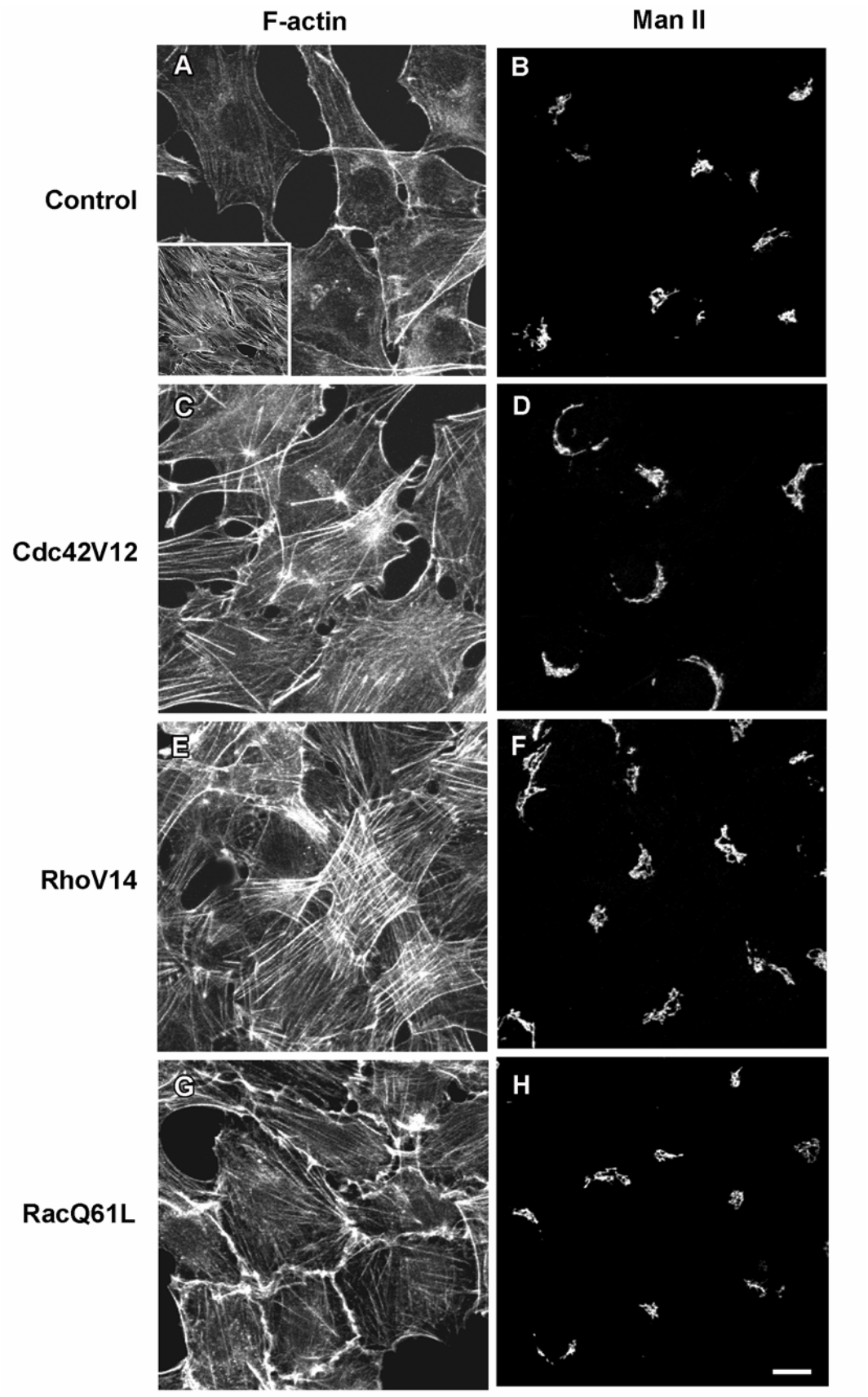

Figure 6. Cells that constitutively express activated Cdc42 exhibit a different Golgi complex morphology. Confocal fluorescence microscopy of control low FBS-cultured stable NIH3T3 cells (A and B) as well as those that constitutively express activated Cdc42 (Cdc42V12; C and D), RhoA (RhoV14; E and F), or Rac1 (RacQ61L; G and H), stained to F-actin (TRITC-phalloidin; A, C, E, and G) and to the Golgi-resident enzyme mannosidase II (ManII; B, D, F, and H). Insert in (A) shows TRITC-phalloidin staining in normal FBS-cultured stable NIH3T3 cells. Note that the perinuclear Golgi complex morphology in most Cdc42V12-expressing cells is much more extended in comparison to those observed in other cell lines. Bar, 10 μm.