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

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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(W9004WA), 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).

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 y 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(AWA) 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-Madriz (Hospital La Princesa, Madrid). Cy3-tagged native Shiga toxin fragment B was a gift from 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-GFP–Shiga toxin fragment B was a gift from Francisco Sañez-Madriz (Hospital La Princesa, Madrid). Cy3-tagged native Shiga toxin fragment B was a gift from 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–GFP–Shiga toxin fragment B was a gift from Sigma Chemical Co. (St. Louis, MO). DMEM and fetal calf 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-KDEL, 1:1000; anti–Gal-T, 1:100; anti–antigiant, 1:500; anti–VSVG, 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 (Martinez-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 (characteristic dilatation), 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 Regulates Golgi-to-ER Transport

Cdc42 correlates with Golgi-to-ER transport, which is thought to be mediated by the N-WASPDictyostelium discoideum)/2 fragments were

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% CO2.

Microinjection Experiments

For microinjection, HeLa and NRK cells were grown for 1–2 d on Eppendorf Celllocate 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 Sar1dn 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 Sar1dn 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, FluCGENE 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.

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RESULTS

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

GFP-tagged Cdc42 proteins (wild-type, Cdc42WT; “activating” 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-
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
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.

### 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)

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<th>Golgi cisternae</th>
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<th>Peri-Golgi transport intermediates</th>
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<td></td>
<td>Lateral portions</td>
<td>Central portions</td>
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<tr>
<td>Cdc42V12</td>
<td>36.6 ± 3.2*</td>
<td>23.8 ± 3.5*</td>
<td>33.4 ± 3.2</td>
<td>6.3 ± 1.8</td>
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<tr>
<td>Cdc42N17</td>
<td>16.2 ± 4.0</td>
<td>13.5 ± 4.0</td>
<td>16.6 ± 4.1</td>
<td>53.7 ± 5.3</td>
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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 ≤ 0.01.
Cdc42 (Figure 4, D, F, and G). In contrast, in cells expressing activated Cdc42 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
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], Sar1dn) 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 Sar1dn 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 Sar1dn 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 Sar1dn and the negative mutant GFP-Cdc42N17 (Figure 6, C and F, asterisks). In contrast, microinjected cells coexpressing Sar1dn 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). Sar1dn 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 Sar1dn 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). 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 Sar1dn, 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 Sar1dn. Once in the ER, ST-B is retained by the blocking effect of Sar1dn protein on the COPII-dependent ER export machinery (Bowell, 1998, for review). When cells were comicroinjected with Sar1dn 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 Sar1dn 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 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.
Activated Cdc42 Recruits N-WASP to the Golgi Complex

WASP and its ubiquitous form N-WASP bind, among others, to Cdc42 and PIP2, 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 6. Cdc42 blocks the Sar1dn-induced ER accumulation of Golgi enzymes and the KDEL receptor. Sar1dn construct alone (A and D; asterisks) and Sar1dn 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 Sar1dn 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 Sar1dn alone (A and D; asterisks), or with Sar1dn plus the dominant-negative GFP-Cdc42N17 (C and F; asterisks) show the characteristic ER accumulation of both Gal-T and KDELr induced by Sar1dn. (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.

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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 Sar1dn and monitored the accumulation of Gal-T in the ER by immunofluorescence. In cells coexpressing Sar1dn and the GFP-N-WASP, Gal-T was retained in the Golgi complex (Figure 9A, left, asterisks). In contrast, cells expressing Sar1dn and GFP-N-WASP(H9004WA), 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 Sar1dn-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(H9004WA) also resulted in the accumulation of Gal-T in the ER (Figure 10). Therefore, N-WASP(H9004WA) alleviated 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 Sar1dn 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 Sar1dn plus GFP-N-WASP(H9004WA) (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., 1999).
either. These results are at variance with those of Wu et al. (2000), who reported that a rapid cycling mutant of Cdc42 (Cdc42F28L) interacts with γCOP and spends more time in the GTP-bound form (2000), who reported that a rapid cycling mutant of Cdc42

We have previously demonstrated that actin is involved 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 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 (Letourneau 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 Sar1α-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. Babić 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.
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et al. bound, prenylated Cdc42 (Zigmond et al., 2000 and Higgs and Pollard, 2001 for reviews).


Formation of Transport Intermediates in the Golgi Complex via Cdc42→N-WASP→(7)Arp2/3

WASP/N-WASP is activated by the lipid second-messenger phosphatidylinositol 4,5-biphosphate (PIP2) 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 PIP2 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 PIP2 phosphatidylinositol-4-OH kinase-β and type 1 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 PIP2, 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 PIP2, 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(AWA), 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 (Moraes 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-
duced the Sar1<sup>dn</sup>-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 Cdc42<sup>V12</sup>, Cdc42<sup>N17</sup> is mostly localized in the cytoplasm (6% vs. 54%, respectively; Table 1), and therefore it cannot interfere in the signaling route(s) activated in the cytoplasm (6% vs. 54%, respectively; Table 1), We show that, unlike Cdc42<sup>V12</sup>, Cdc42<sup>N17</sup> is mostly located in the cytoplasm. In other words, the overexpression of Cdc42<sup>N17</sup> 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.

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