Actin Microfilaments Facilitate the Retrograde Transport from the Golgi Complex to the Endoplasmic Reticulum in Mammalian Cells

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Actin cytoskeleton consists of actin and associated proteins and requires constant reorganization and regulation for its functions in such diverse cellular events as cell-shape changes, cell motility, chemotaxis, development, signal transduction, RNA localization, cell polarity and endocytosis (1). The high dynamics and the structural and molecular complexity of the actin cytoskeleton make it difficult to study its involvement in these processes. One of the most widely used approaches is to disturb its structure and organization. Several naturally derived substances interfere with actin cytoskeleton, including cytochalasins and phalloidins derived from fungi; latrunculin, swinholide and jasplakinolide from sponges (2); and some clostridial ADP-ribosyltransferases (2,3). Although they all alter the organization of actin cytoskeleton, their utilization often gives rise to disparate observations. One of the clearest examples are cytochalasins. The disappearance of filamentous actin (F-actin) in cells treated with these agents has been attributed to the capping activity on actin filaments, which prevents their assembly (4,5). However, the treatment with cytochalasins does not lead to net depolymerization of actin microfilaments, since in cytochalasin D (cyD)-treated cells, the binding of heavy meromyosin chain remains unaltered, and the expected complementary change in the levels of G- and F-actin in cell extracts and in the ratio between Triton-soluble and insoluble actin do not occur (6 and references therein; 7). This is not the case for Clostridium botulinum C2 toxin and latrunculins. The former is a binary toxin that ADP-ribosylates G-actin; the latter is a Red Sea sponge product that binds G-actin noncovalently. Both agents prevent actin assembly (8,9) and disrupt actin filaments (7,9,10).

Although the involvement of actin microfilaments in membrane trafficking is more established in the endocytic pathway (11 and references therein), actin and several actin-associated/binding proteins have also been implicated in the morphology and in the membrane transport to and from the Golgi complex (12–14,15 and references therein, 16–21). However, in both intracellular membrane routes there are disparate observations depending on the cell type, the technical approach and the anti-actin agents used (22–25). We recently reported that α- and γ-actin isoforms are localized to COPI-coated and noncoated transport intermediates (18). This result suggests the involvement of actin in mem-
brane trafficking in the early steps of the secretory pathway. Using cytochalasin D, we did not observe alterations in the endoplasmic reticulum (ER)-Golgi membrane dynamics, except for the morphology and subcellular positioning of the Golgi complex (17). We therefore re-examined the role of actin microfilaments in the ER/Golgi membrane dynamics using botulinum C2 toxin and latrunculin B as actin-disrupting agents.

Results and Discussion

The Golgi complex is compacted in cells treated with cytochalasin D (cyD), latrunculin B or C2 toxin, but only the last two produce net depolymerization of actin filaments

CyD, C2 toxin and latrunculin B (LT-B) decreased the number of stress fibers (Figure 1D,F,H, respectively) when compared to control NRK cells (Figure 1B). Concomitantly, the Golgi complex showed similar compact morphology (Figure 1C,E,G, respectively). When the filamentous actin (F-actin) content was measured in cell extracts from cyD-, C2 toxin- or LT-B-treated cells, only the latter two showed a significant decrease (Figure 1I). This result is in accordance with previous data showing that cyD does not induce net depolymerization of actin filaments (6 and references therein) or changes in the distribution of actin in Triton X-100-soluble or insoluble cell fractions (7). Taken together, these results show that C2 toxin and LT-B produce much higher net depolymerization of actin filaments than cyD. We next re-examined the functional role of actin microfilaments in the ER-Golgi membrane dynamics using C2 toxin and LT-B.

The reassembly of the Golgi complex upon BFA withdrawal and the ER-to-Golgi transport of the vesicular stomatitis virus (VSV)-G are unaltered in latrunculin B- and C2 toxin-treated cells

We reported that microfilaments are not required for the anterograde ER-to-Golgi membrane transport using cyD (17). We thus repeated the same experiments using LT-B and C2 toxin. NRK cells were first treated with brefeldin A (BFA, a fungal metabolite that induces relocation of Golgi enzymes to the ER), subsequently incubated with LT-B or C2 toxin, and BFA was then withdrawn from the culture medium. The rebuilding of the Golgi complex was visualized by fluorescence microscopy. In control, C2 toxin- and LT-B-treated cells, no differences were observed in either the kinetics of the reassembly of the Golgi complex (Figure 2) or the ER-to-Golgi transport of the VSV-G glycoprotein (Figure 3). Taken together, these findings demonstrate that actin microfilaments are not required for the rebuilding of the Golgi complex or for the anterograde ER-to-Golgi transport.

The Golgi complex disassembly induced by BFA is delayed in latrunculin B- and C2 toxin-treated cells

We next assessed whether the disassembly of the Golgi complex induced by BFA was altered when actin cytoskeleton was disrupted by LT-B or C2 toxin (Figure 4). NRK cells

![Figure 1: Golgi complex is compacted in NRK cells treated with cytochalasin D (cyD), botulinum C2 toxin (C2 toxin) or latrunculin B (LT-B) but only C2 toxin and latrunculin B produce net depolymerization of filamentous actin. Double confocal immunofluorescence microscopy experiments in untreated (A and B), and cyD- (C and D), C2 toxin- (E and F) or LT-B-treated cells (G and H) stained with anti-Man II antibodies (A, C, E and G) and TRITC-phalloidin (B, D, F and H). The F-actin content was quantified using the fluorescent phalloidin-binding assay (I). The results are the mean ± SEM of three independent experiments. Bar, 10 μm.
Actin in the ER/Golgi Interface

Figure 2: The Golgi complex reassembly upon BFA withdrawal remains unaltered in NRK cells when actin microfilaments are disrupted with latrunculin-B (LT-B) or botulinum C2 toxin (C2 toxin). Cells were treated first with BFA and then with LT-B (B, E, H) or C2 toxin (C, F, I). Notice the characteristic BFA-induced ER-like staining pattern of the Golgi-resident protein Man II (A, B, C). Next, BFA was withdrawn from the culture medium and the kinetics of the Golgi reassembly was followed at fluorescence confocal microscopy in control (D, G), LT-B- (E, H) and in C2 toxin-treated cells (F, I). As expected, in LT-B and C2 toxin-treated cells, the reassembled Golgi complex showed a compacted morphology. Bar, 10 μm.

were first incubated with cyD, LT-B or C2 toxin and then treated with BFA for different times. In control (Figure 4A,E,I) and cyD-treated cells (Figure 4B,F,J) the kinetics of the appearance of the characteristic ER-like staining pattern, consistent with the translocation to the ER of the Man II-stained Golgi complex, was the same. However, in LT-B- (Figure 4C,G,K) and C2 toxin-treated cells (Figure 4D,H,L), the BFA-induced disassembly of the Golgi complex was significantly delayed. In particular, the time for which the morphology of the Golgi complex remained unaltered in 50% of the cells in LT-B or C2 toxin-treated cells was practically double that in cyD-treated or control cells (20 min vs 10 min, respectively; Figure 4J). Hence, the results indicate that microfilaments are involved in the Golgi-to-ER membrane flux.

The subcellular distribution of the Shiga toxin fragment B and the KDEL receptor is altered in cells with depolymerized actin

We used Shiga toxin as a marker of the retrograde Golgi-to-ER pathway. Shiga toxin binds to glycolipid GB3, which is expressed in HeLa but not in NRK cells (26), and transported to the ER via the early/recycling endosomes and the Golgi complex (27). HeLa cells are also sensitive to botulinum C2 toxin (28) and LT-B (7). Cells were incubated with cy3-labeled fragment B of the Shiga toxin bearing the ER-retention KDEL motif (ST-B-KDEL) (29); and its transport to the ER was monitored by fluorescence microscopy. Briefly, cells were incubated with the toxin at 4 °C, and after 45 min, heated to 19.5 °C to accumulate the internalized toxin in the early/recycling endosomes (Figure 5A,C). Heating the cells to 37 °C synchronized the transport of Shiga toxin to the ER. After 2 h at 37 °C, in control cells, ST-B-KDEL colocalized with the Golgi complex stained with anti-giantin antibodies (Figure 5E,F). After 4 h at 37 °C, the ST-B-KDEL was seen in the ER, but still colocalized with giantin (Figure 5L,J), and after 6 h, the ST-B-KDEL was mostly located in the ER (Figure 5M,N). In contrast, after 4 h and 6 h of transport, in LT-B treated cells (not shown) and C2 toxin-treated cells (Figure 5K,L,O,P), the ST-B-KDEL labeling was mostly observed in the Golgi complex. We validated the morphological observations by quantifying
Figure 3: ER-to-Golgi transport of the VSV-G protein is not altered in NRK cells treated with either botulinum C2 toxin (C2 toxin) or cytochalasin D (cyD). Cells were infected with the VSV ts045 temperature-sensitive mutant virus and after accumulation at 40°C VSV-G glycoprotein was monitored at immunofluorescence with anti-VSV-G antibodies. The kinetics of transport of VSV-G from the ER to the Golgi complex (stained to ManII) is the same in control (A, E, I, M) and in C2 toxin-treated cells (C, G, K, O). Biochemical studies were also performed. In that case, after virus infection cells were labeled with [35S]-methionine at 40°C and chased at 32°C. ER-to-Golgi transport was then analyzed by the acquisition of the Endo H-resistant form of the G glycoprotein (Q). The results shown are the mean of two experiments. Bar, 10μm.

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Figure 4: Golgi complex disassembly induced by BFA is delayed in NRK cells treated with botulinum C2 toxin (C2 toxin) or latrunculin-B (LT-B) but not with cytochalasin D (cyD). Confocal immunofluorescence microscopy experiments of the kinetics of the disassembly of the Man II-stained Golgi complex in control (A, E, I), in cyD (B, F, J), in LT-B (C, G, K) and in C2 toxin-treated cells (D, H, L). A quantitative analysis of these results is shown in M. The results are the mean ± SEM of three independent experiments; at least 200 cells, randomly chosen, were counted per experimental condition. Bar, 10 μm.
Figure 5: Golgi-to-ER transport of Shiga toxin B subunit is impaired in botulinum C2 toxin (C2 toxin)-treated HeLa cells. Double confocal immunofluorescence microscopy experiments of the retrograde transport kinetics of the cy3-labeled Shiga toxin fragment B containing the ER-retention KDEL signal (ST-B-Glyc-KDEL/ST-B-KDEL) in untreated (A, B, E, F, I, J, M, N) and C2 toxin-treated cells (C, D, G, H, K, L, O, P). Plasma membrane internalization of the ST-B-Glyc-KDEL was carried out at 19.5°C to accumulate it in early/recycling endosomes in the absence (A, B) or presence of C2 toxin (C, D). Thereafter, cells were heated to 37°C (E-P) to induce the synchronous retrograde transport of the ST-B-Glyc-KDEL to the ER through the Golgi complex, which was visualized with anti-giantin antibodies (B, D, F, H, J, L, N, P). (Q) Quantification of cells in which ST-B-Glyc-KDEL was still seen in the Golgi complex. The results are the mean ± SEM of three independent experiments; at least 200 cells, randomly chosen, were counted per experimental condition. Bar, 10 μm.

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the percentage of cells in which ST-B-KDEL still showed a Golgi-like staining pattern (Figure 5Q). This gives information on the emptying of the fluorescent ST-B-KDEL from the Golgi as a result of its transport to the ER, where it is retained. Unlike cyD-treated cells, C2 toxin- and LT-B-treated cells showed a significant retention of ST-B-KDEL in the Golgi complex (Figure 5Q). It is worth remarking that such retention was more efficient when actin microfilaments were disrupted by C2 toxin than by LT-B, possibly attributable to differences in their induction of net actin depolymerization (Figure 11).

Morphological experiments using native Shiga toxin (ST-B) gave similar results (data not shown). To provide more evidence for the role of actin in Golgi-to-ER transport, we next measured N-glycosylation in mutant Shiga toxin B-subunit containing an N-glycosylation site and a nonfunctional version of the KDEL motif (ST-B-Glyc-KDELGL) (29). It is important to take into account that at steady state ST-B-Glyc-KDEL and native ST-B are located in the Golgi complex, and that the percentage and kinetics of glycosylation of ST-B-Glyc-KDEL and ST-B-Glyc-KDELGL are practically the same (29). When ST-B-Glyc-KDELGL reaches the ER, it is core glycosylated. Hence, the measurement of N-glycosylation of ST-B-Glyc-KDELGL is an indicator of the arrival of Shiga toxin at the ER. In C2 toxin-treated cells, glycosylation of ST-B-Glyc-KDELGL underwent a partial inhibition (Figure 6). Hence, the morphological and biochemical results demonstrate that the arrival of Shiga toxin B subunit at the ER is impaired when actin microfilaments are disrupted. Consequently, Shiga toxin B-subunit would remain for longer either in the Golgi complex or in transit between the Golgi complex and the ER. The former suggests a role of actin in the generation of transport intermediates, the latter on the mechanism(s) of transport.

To further examine the possibility that actin depolymerization impairs the proximal Golgi-to-ER recycling route, we studied the subcellular distribution of the KDEL receptor (Figure 7). This protein is retained in the intermediate compartment when HeLa cells are cooled from 37°C to 15°C. This redistribution is observed by the disappearance of its Golgi-like staining pattern (Figure 7A,B), which is mostly replaced by the characteristic peripheral punctate pattern of the intermediate compartment (Figure 7C,D). In contrast, in C2 toxin-treated cells kept at 15°C for 2h the KDEL receptor still showed the juxtanuclear Golgi complex staining pattern, colocalizing with gaint tin (Figure 7G,H). After longer incubation times at 15°C, the KDEL receptor redistributed to acquire the punctate pattern, which was indistinguishable from that observed in control cells (not shown).

**The disruption of microtubules and microfilaments causes an additive inhibitory effect on the retrograde transport of Shiga toxin**

Next, we examined whether the codisruption of both microtubules and microfilaments would inhibit in an additive or synergic manner the retrograde transport from the Golgi complex to the ER. With this aim, we measured in a time-dependent manner the glycosylation of ST-B-Glyc-KDELGL in HeLa cells treated with nocodazole and C2 toxin, agents that disrupt microtubules and microfilaments, respectively. After 4h at 37°C, a higher inhibitory effect on glycosylation of Shiga toxin in comparison with cells treated with nocodazole or C2 toxin alone was observed (Figure 6). After 8h, this higher inhibitory effect in the glycosylation of Shiga toxin was also observed but it was not so evident. Together, results show that the inhibition in the retrograde transport caused by the disruption of both microtubules and microfilaments is additive rather than synergic. This partial inhibition in the arrival of Shiga toxin at the ER could be explained by the fact that nocodazole does not cause the disassembly of stable microtubules, which have been reported to be involved in the ER/Golgi interface (30,31), and that C2 toxin does not induce a total net depolymerization of microfilaments (Figure 11).

**Conclusions**

Results obtained using BFA, Shiga toxin and KDEL receptor indicate a role of actin in the membrane dynamics at the ER/Golgi interface and, in particular, in the Golgi-to-ER membrane pathway. The retrograde Golgi-to-ER membrane transport uses both COPI-dependent and COPI-independent mechanisms (32,33). The results obtained with the KDEL receptor and ST-B-KDEL on one hand, and those with the ST-B-Glyc-KDELGL on the other, suggest that actin microfilaments are involved in either the COPI-dependent or the COPI-independent pathways. Both possibilities are also consistent with the presence of β- and γ-actin in COPI-coated and noncoated transport intermediates (18). Hence, this comparative study in the early steps of the secretory pathway using cyD, LT-B and C2 toxin as actin disrupting agents indicates that actin microfilaments are involved in the retrograde Golgi-to-ER but not in the anterograde ER-to-Golgi pathway.
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Figure 7: C2 toxin-treated HeLa cells show an altered subcellular distribution of the KDEL receptor upon cooling the cells from 37°C to 15°C. Untreated (A, B, C, D) and C2 toxin-treated cells (E, F, G, H) were kept at 15°C for 2h. Cells were subsequently processed for immunofluorescence and double stained to KDEL receptor (A, C, E, G) and to giantin (B, D, F, H). Bar, 10 μm.

Furthermore, the biochemical results on the arrival of Shiga toxin to the ER suggest that the retrograde Golgi-to-ER membrane transport is an orchestrated movement of transport carriers along microtubule and microfilament tracks. The challenge is now to identify the molecular machinery involved and determine how actin microfilaments contribute to the retrograde Golgi-to-ER transport. Finally, the utilization of LT-B and, in particular, C2 toxin could help to clarify disparate

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results about the role of actin in the endocytic and biosynthetic/secretory processes.

Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM) and Fetal Calf Serum (FCS) were from Gibco/Berkeley Life Technologies (Paisley, UK); Endo-1-β-N-acetylgalactosamine 1-phosphate transferase (E1) and Endo-1-β-N-acetylglucosamine 1-phosphate transferase (E2) were from Boehringer Mannheim (Mannheim, Germany); rabbit anti-mouse IgG was from DAKO (Dako, AS, Denmark); Protein A/G agarose was from Sta. Cruz Biotechnology Inc. (Sta. Cruz, CA, USA); ProMix L-[35S]-labeling mix from Amersham (Buckinghamshire, UK). All other chemicals if not otherwise stated were from Sigma Co. (St. Louis, MO, USA).

Cell culture

NKR and HeLa cells were cultured in DMEM supplemented with 10% FCS and 1 mM l-glutamine at 37°C in a humidified atmosphere of 5% CO2.

Treatments with botulinum C2 and Shiga toxins, and latrunculin B

Botulinum C2 toxin (100 ng/ml of each C2I and C2II subunits, final concentration) was added to cells cultured in DMEM and supplemented with 0.5% FCS for at least 4 h at 37°C. Cy3-Shiga toxin B-fragment tagged with KDEL motif (4 μg/ml, final concentration) was added to cells preincubated for 30 min in binding medium, FCS-free DMEM) for 45 min at 4°C. After the withdrawal of unbound toxin by washing for 5 min in ice-cold PBS, cells were incubated with DMEM at 37°C. Latrunculin-B (500 nM, final concentration) was added to cells cultured in DMEM for 15 min before the experimental procedure.

Immunofluorescence

Indirect immunofluorescence was carried out as previously described (17) and the following antibody dilutions were used: anti-giantin, 1:500; anti-KDEL receptor, 1:1000; anti-Man II, 1:4000, and fluorescent secondary antibodies, 1:35. Microscopy and imaging were performed with an Olympus BX60 epifluorescence microscope with a chilled charge-coupled device (CCD) camera (Olympus DP50) or with a Leica TCS-NT confocal microscope. For the quantitative analysis of the morphological experiments, all images were obtained from randomly chosen fields and captured at nonsaturating integration levels with the CCD camera. At least 200 cells, randomly chosen, were counted per experimental condition. The images were processed on a PC using Adobe Photoshop 5.0.

Virus infection and VSV-G transport assays

The infection with the temperature-sensitive mutant ts45 VSV and the assay of the acquisition of the Endo H-resistance form of the G glycoprotein were performed as described (17). Indirect immunofluorescence transport of VSV-G from ER-to-Golgi complex was performed as described elsewhere (34).

Fluorescent phalloidin-binding assay

The F-actin content in untreated and C2 toxin- and cyD-treated cells was measured as previously described (18,24).

Retrograde transport assay of Shiga toxin B-fragment constructs

Mutant Shiga toxin B-subunits (ST-B-Glyc-KDEL) were iodinated as previously reported (29). HeLa cells were incubated with the iodinated B-subunits mutant (200 nM) in DMEM without FCS at 4°C. Cells were rinsed and incubated at 19.5°C in DMEM without FCS for 1 h, and then with C2 toxin (C1 and CII subunits, 2 μg/ml each) for a further 2 h. C2 toxin enters the cell and is functionally active at 19.5°C. F. Valdenarena and G. Egea, unpublished observations). Thereafter, cells were rinsed and incubated with DMEM containing 10% FCS and the mannosidase I inhibitor DMM (1 mM, final concentration) at 37°C. At the indicated time points, cells were lysed in SDS sample buffer. Samples were subsequently run on 10–20% polyacrylamide–SDS gradient gels, analyzed by autoradiography, and quantified with a PhosphorImager using the ImageQuant software (Molecular Dynamics). For the transport experiments with C2 toxin and nocodazole, HeLa cells were incubated with iodinated ST-B-Glyc-KDEL as described above, but at the 19°C blockade; besides C2 toxin, nocodazole (30 μM, final concentration) was also added.

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