NSF is required for the brefeldin A-promoted disassembly of the Golgi apparatus

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Abstract ***N*-Ethylmaleimide-sensitive factor (NSF) is required for multiple pathways of vesicle-mediated protein transport. Microinjection of a monoclonal anti-NSF antibody almost completely blocked brefeldin A-promoted Golgi disassembly without affecting the rapid release of β-COP, a subunit of the Golgi coat proteins (COPI), from the Golgi apparatus. Similar results were obtained using a dominant-negative NSF which is known to compete with endogenous NSF. The present results suggest that an NSF-mediated step is present in the brefeldin A-promoted disassembly of the Golgi apparatus.**

Key words: Brefeldin A; Golgi apparatus; Microinjection; Retrograde transport; *N*-Ethylmaleimide-sensitive factor

1. Introduction

The transport of proteins between compartments of the biosynthetic and endocytic pathways is mediated by vesicles that bud from the donor compartment and move to the target compartment, where the fusion of vesicles occurs. *N*-Ethylmaleimide-sensitive factor (NSF) is a key component for the fusion of transport vesicles [1–4]. Studies involving cell-free or permeabilized cell systems revealed that NSF is involved in many transport pathways [1] and in the formation of Golgi stacks from Golgi-derived vesicles [5,6].

NSF is associated with membranes as a 20S complex of proteins including soluble NSF attachment proteins (SNAPs) [7] and SNAP receptors (SNAREs) [8]. Disassembly of the 20S complex in vitro is probably evoked by the hydrolysis of Mg^{2+}-ATP by NSF, and thereby NSF and SNAPs are released from membranes [8,9]. Although it was proposed that the disassembly of the 20S complex triggers the fusion of the vesicles which have been docked with the target membrane [1], recent studies rather suggest that NSF has a predocking role [3,4].

Brefeldin A (BFA) induces remarkable morphological changes of organelles [10]. This compound promotes the rapid release of Golgi-coat proteins, COPI [11], and thereby causes Golgi components to be redistributed into the endoplasmic reticulum (ER) [12–14]. It also promotes the extensive tubulation of endosomes [15–17]. BFA inhibits the exchange of guanine nucleotide bound to ADP-ribosylation factor (ARF) [18,19]. Since the GTP-dependent interaction of ADP-ribosylation factor with Golgi membranes is required for the association of COPI with the membranes [20,21], inhibition of the exchange of guanine nucleotide onto ARF causes the release of COPI from the Golgi apparatus [11], which promotes the redistribution of Golgi components into the ER. However, dissociation of COPI from Golgi membranes is not sufficient for the disassembly of the Golgi apparatus. Calmodulin antagonists block BFA-promoted Golgi disassembly without affecting the rapid release of COPI [22]. It was recently shown that ADP-ribosylation [23] and protein prenylation [24] are required for BFA-promoted retrograde transport. Although the use of BFA has provided valuable information regarding the control of organelle structures as well as membrane traffic [10], the mechanism of the disassembly of the Golgi apparatus and the tubulation of endosomes is not fully understood.

To examine the involvement of NSF in BFA-promoted Golgi disruption, we used a microinjection method. Microinjection of antibodies into cells is a powerful method to study intracellular transport in vivo. In this paper we showed that mAb 2C8 and a dominant-negative NSF protein, both of which are known to inhibit endogenous NSF [25,26], block the disassembly of the Golgi apparatus induced by BFA.

2. Materials and methods

2.1. Materials

CELLocate 5245 (square size, 175 mm) was obtained from Eppendorf (Hamburg, Germany). BFA was purchased from Wako Chemicals (Osaka, Japan). IgG of mAb 2C8 was prepared as previously described [25]. Polyclonal antibodies against mannosidase II (man II) and β-COP were kindly donated by Drs. K. Moremen (University of Georgia) and Y. Ikehara (Fukuoka University), respectively. NSF(K266Q) and NSF(K549Q) in which Lys-266 and Lys-549 were individually replaced by glutamine were isolated as described [26].

2.2. Cell culture

Chinese hamster ovary (CHO) cells donated by Dr. H. Tanaka of this university, and normal rat kidney (NRK) cells obtained from the Riken Cell Bank (Wako, Japan) were grown in minimum essential medium (α-MEM) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin and 7.5∼10% fetal calf serum under a humidified atmosphere of 95% air and 5% CO_2.

2.3. Immunofluorescence and electron microscopic analyses

Immunofluorescence analysis was performed as previously described [27]. Briefly, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline containing 0.25 M sucrose, permeabilized with 0.2% Triton X-100, and then blocked with 2% bovine serum

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Abbreviations: BFA, brefeldin A; CHO, Chinese hamster ovary; COPI, Golgi coat proteins; ER, endoplasmic reticulum; mAb 2C8, monoclonal antibody named 2C8; man II, mannosidase II; NRK, normal rat kidney; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor

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albumin. Next, the cells were incubated with the primary and then the secondary antibodies.

For electron microscopic analysis, the cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight, and then post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 h. After washing, they were dehydrated in 50% ethanol for 10 min, and then block-stained with 2% uranyl acetate in 70% ethanol for 2 h. The cells were further dehydrated with a graded series of ethanol, and then embedded in epoxy resin. Ultrathin sections were doubly stained with uranyl acetate and lead citrate, and then observed under a Hitachi H7000 electron microscope.

2.4. Microinjection

Antibodies or NSF mutants were centrifuged, and then filtered through Micropure (Amicon, Beverly, MA, USA) before use. To identify injected cells, CELLocate was used as coverslips. The medium for cells contained 25 mM HEPES (pH 7.2) during microinjection. Microinjection was performed using an Eppendorf Microinjector 5242 with Femtotips (Eppendorf, Hamburg, Germany) with an injection time of 0.2 s and 0.6 s for antibodies and NSF mutants, respectively. At least more than 40 cells were injected.

3. Results

3.1. mAb 2C8 inhibits BFA-promoted Golgi disassembly

We first examined whether mAb 2C8 specifically recognizes NSF from CHO cells. Immunoprecipitation and immunofluorescence analyses revealed that the antibody specifically recognizes NSF from CHO cells (data not shown). To examine the involvement of NSF in the disassembly of the Golgi apparatus promoted by BFA, mAb 2C8 at a concentration of 4.0 mg/ml was injected into CHO cells, and then the cells were incubated with 10 μM BFA for 40 min. Fig. 1B,C show cells double-immunostained with antibodies against man II and mouse IgG (i.e. injected antibody), respectively. In injected cells (indicated by arrows), significant immunoreactivity for man II was still detected in the perinuclear region, although its shape became round. On the other hand, immunoreactivity for man II was dispersed throughout the cytoplasm in non-injected cells, reflecting the redistribution of man II into the ER. When the concentration of mAb 2C8 for injection was lower than 1.0 mg/ml, its effect was not remarkable. However, Golgi disassembly was blocked in almost all cells (at least 40 cells examined) when 4.0 mg/ml mAb 2C8 was injected. Electron microscopic analysis revealed that the Golgi apparatus consisting of several stacks is indeed present in mAb 2C8-injected cells in spite of incubation with BFA (Fig. 2).

Since NSF from NRK cells was not recognized by mAb 2C8, this cell line is suitable for evaluating the specificity of the inhibitory effect of mAb 2C8. When mAb 2C8 was injected, and then the cells were incubated with BFA, redistribution of man II into the ER occurred in injected cells as well as non-injected cells (data not shown), suggesting that the inhibitory effect of mAb 2C8 is specific.

One possibility to explain the blockage of BFA-promoted Golgi disassembly by mAb 2C8 in CHO cells is that the antibody suppressed the rapid release of COPI from Golgi membranes. However, this possibility was excluded by the finding that the release of β-COP, a subunit of COPI [28,29], from the Golgi apparatus occurred within 3 min in both injected (the cell indicated by an arrow in Fig. 1E) and non-injected cells. These results suggest that NSF is not involved in the binding of β-COP to the Golgi apparatus, but is required for BFA-promoted Golgi disassembly [30].

3.2. A dominant-negative NSF inhibits BFA-promoted Golgi disassembly

To confirm that NSF activity is required for BFA-promoted Golgi disassembly, we used a dominant-negative NSF. NSF contains the amino-terminal and two homologous nucleotide-binding domains [31] whose connecting regions are susceptible to proteolysis [25]. We previously constructed mutant NSF proteins in which Lys-266 and Lys-549 in the consensus sequences for nucleotide binding are individually replaced by glutamine, referred to as NSF(K266Q) and NSF(K549Q), respectively. NSF(K266Q) but not NSF(K549Q) inhibits an intra-Golgi transport assay [26].

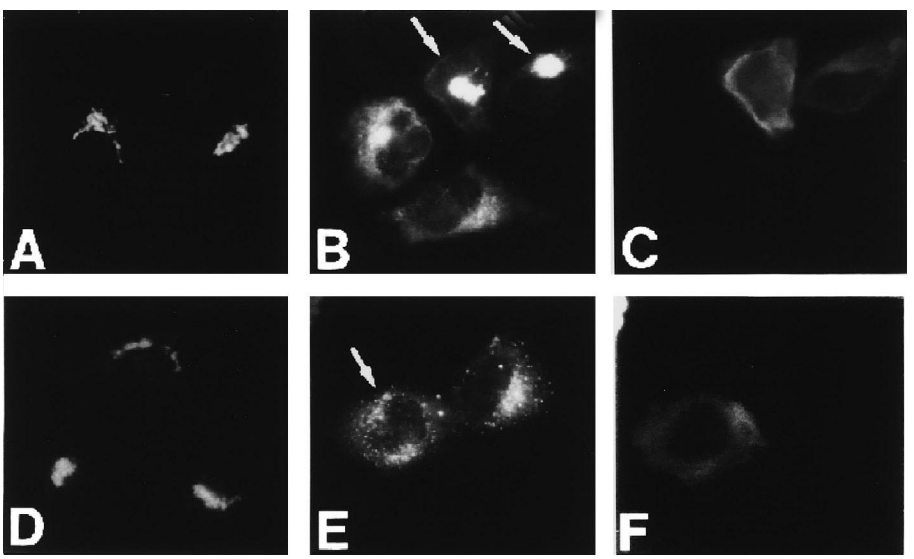

Fig. 1. mAb 2C8 inhibits BFA-induced disassembly of the Golgi apparatus without affecting the rapid release of β-COP in CHO cells. Immediately after injection of intact mAb 2C8 into CHO cells, the cells were incubated with 10 μM BFA for 40 min (B and C) or 3 min (E and F). Double-staining for man II (B) and the injected antibody (C), and double-staining for β-COP (E) and the injected antibody (F). Staining for man II (A) and β-COP (D) in untreated cells is shown. Arrows indicate injected cells.

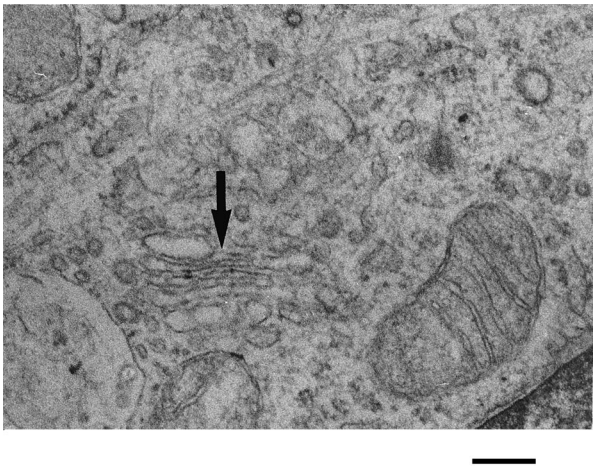

Fig. 2. The Golgi apparatus in CHO cells is not disassembled by BFA in cells injected with mAb 2C8. Immediately after injection of mAb 2C8 into CHO cells, the cells were incubated with 10 μM BFA for 40 min, fixed with glutaraldehyde, and then processed for electron microscopic analysis. The arrow indicate the position of the Golgi apparatus. Bar = 200 nm.

Whiteheart and colleagues [32,33] also showed that the NSF mutant in which Lys-266 is replaced by alanine has a similar effect.

NRK cells were chosen for the microinjection of NSF mutants because these cells express lower amounts of NSF than CHO cells (data not shown). To localize injected cells, mouse IgG was co-injected with NSF(K266Q). The injected cells were incubated with 10 μM BFA for 40 min, and then double-immunostained with antibodies against man II (Fig. 3B) and mouse IgG (Fig. 3C). In injected cells man II was not redistributed and still located in the perinuclear region, whereas it was redistributed into the ER in non-injected cells. We examined about 40 injected cells, and found that Golgi disassembly was prevented in almost all cells when the mutant at a concentration of 1.5 mg/ml or higher was injected. As in the case of microinjection of mAb 2C8 into CHO cells, the BFA-promoted release of β-COP from the Golgi apparatus occurred rapidly in both injected and non-injected NRK cells, although β-COP was not completely released by BFA in the case of this cell line (Fig. 3G,H). When NSF(K549Q), a mutant that does not compete with endogenous NSF [26], was used, the disassembly of the Golgi apparatus was caused by BFA in both injected and non-injected cells (Fig. 3D,E), reflecting the specificity of the inhibitory effect of NSF(K266Q).

4. Discussion

BFA promotes the Golgi apparatus to be disassembled into tubular structures. The tubules containing most Golgi components move to the ER, and then fuse with the membrane [12–14]. In the present study, we investigated the role of NSF in the action of BFA using a microinjection method. Disassembly of the Golgi apparatus was remarkably blocked when CHO cells were microinjected with mAb 2C8, although β-COP was rapidly released from the Golgi apparatus. Similar results were obtained with NSF(K266Q), a mutant NSF which inhibits an intra-Golgi transport assay by competing with endogenous NSF. In control experiments microinjection of mAb 2C8 or NSF(K549Q) into NRK cells did not inhibit the redistribution of man II into the ER. These results strongly suggest that NSF is required for BFA-promoted Golgi disassembly. This idea is consistent with the recent finding that *N*-ethylmaleimide blocks the movement of Golgi tubules induced by BFA and prevents the redistribution of Golgi components into the ER [34].

The Golgi apparatus is a highly dynamic organelle, in which the formation and/or detachment of tubules constantly occur among adjacent Golgi membranes [34,35]. In spite of this dynamics, the overall Golgi structure is fairly maintained during interphase. However, when coat proteins are removed by BFA, the organelle is redistributed into the ER via a ten-

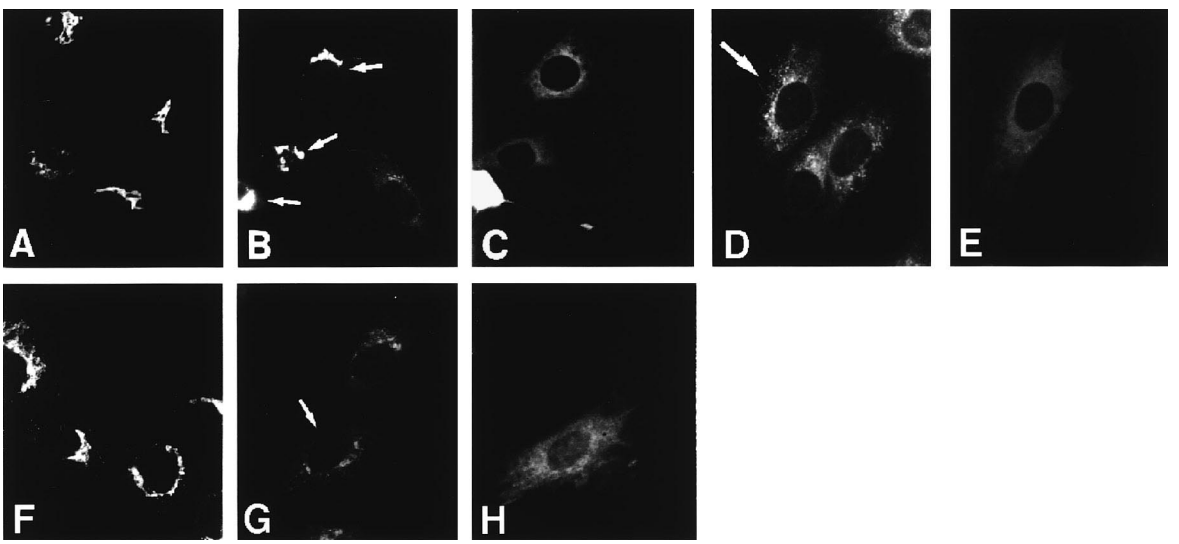

Fig. 3. Disassembly of the Golgi apparatus is inhibited by NSF(K266Q), but not by NSF(K549Q). Immediately after injection of NSF(K266Q) (B, C, G, and H) or NSF(K549Q) (D and E), the cells were incubated with 10 μM BFA for 40 min (B–E) or 3 min (G and H). To localize the injected cells, a control mouse IgG was co-injected with an NSF mutant. Double-staining for man II (B and D) and the injected antibody (C and E), and double-staining for β-COP (G) and the injected antibody (H). Staining for man II (A) and β-COP (F) in untreated cells is shown. Arrows indicate injected cells.

sion-driven membrane flow mechanism [34]. On the other hand, when the release of coat proteins is blocked by an antibody against β-COP, the Golgi apparatus is disassembled by BFA, but its components are not returned into the ER [36]. These findings may suggest that coat proteins blocks non-specific fusion by covering the fusion machinery such as the NSF-SNAP-SNARE complex. This idea is supported by the fact that isolated Golgi membranes are fused to form extensive tubular networks in a cytosol-, ATP- and NSF-dependent manner, when coat proteins are removed by BFA in vitro [37]. The present results are most likely relevant to these in vitro observations.

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