Regulation of the Golgi structure by the α subunits of heterotrimeric G proteins

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Abstract Disassembly of the Golgi apparatus is elicited by the action of nordihydroguaiaretic acid (NDGA) and this disassembly is prevented by the activation of heterotrimeric G proteins. In the present study we showed that overexpression of $G\alpha_z$ or $G\alpha_{i2}$ significantly suppresses the disassembly of the Golgi apparatus induced by NDGA. Overexpression of $G\beta_1\gamma_2$, on the other hand, had no effect on NDGA-induced Golgi disassembly. $G\alpha_z$ neither blocked Golgi disassembly induced by brefeldin A or nocodazole, nor interfered with protein transport, suggesting its specificity on the action of NDGA. Our results suggest that the α subunits of heterotrimeric G proteins are responsible for the maintenance of the Golgi structure.

Key words: Heterotrimeric G protein; Golgi apparatus; Nordihydroguaiaretic acid; Vesicular transport

1. Introduction

In mammalian cells the Golgi apparatus consists of interconnected and polarized stacks of flattened cisternae [1]. It receives secretory and membrane proteins that are transported from the endoplasmic reticulum (ER) via the anterograde pathway and sorts and distributes them to their final destinations [2]. ER-resident proteins that have been exported from the ER are transported back to the ER via the retrograde pathway [3,4]. These movements of proteins are mediated by membrane-surrounded vesicles or tubules that transit between organelles [5,6]. Thus, massive membrane flux in and out of the Golgi apparatus occurs for intracellular trafficking.

Disruption of membrane flow can be achieved with drugs such as brefeldin A (BFA) [7] and ilimaquinone [8] that specifically block protein transport. BFA promotes the rapid release of coat protein I (COPI) from Golgi membranes, which leads to Golgi components being redistributed to the ER [9]. Ilimaquinone causes the Golgi apparatus to be disassembled into small vesicles without affecting the association of COPI with Golgi membranes [8]. We screened transport inhibitors and found that nordihydroguaiaretic acid (NDGA), originally known as a dual inhibitor of lipoxygenases and cyclooxygenase, inhibits protein transport in vitro [10] and in vivo [11]. In certain cells this compound, like ilimaquinone, promotes Golgi disassembly without the release of COPI from Golgi membranes [12–14] and this disassembly is prevented by activators of heterotrimeric G proteins [12].

The heterotrimeric GTPases consist of α, β and γ subunits [15]. Upon the binding of signaling molecules, seven membrane span-cell surface receptors catalyze the replacement of GDP with GTP in Gα, which leads to the dissociation of the heterotrimer into GTP-bound Gα and Gβγ. Both Gα and Gβγ independently modulate the activities of certain enzymes and proteins [16]. Our finding that activators of heterotrimeric G proteins inhibit NDGA-induced Golgi disassembly suggests the requirement of GTP-bound Gα for the maintenance of the Golgi structure. However, Malhotra et al. [17] demonstrated that Gβγ per se is sufficient for the vesiculation of Golgi stacks in permeabilized cells. More recently, they showed that protein kinase D is a downstream effector for Gβγ [18].

To resolve this controversial issue we sought to determine the Gα subunit subtype involved in the blockage of NDGA-induced Golgi disassembly. We found that overexpression of $G\alpha_{i2}$ or $G\alpha_z$ significantly and specifically inhibited Golgi disassembly induced by NDGA. In contrast, overexpression of $G\beta_1\gamma_2$ did not cause the vesiculation of Golgi stacks or enhance the effect of NDGA.

2. Materials and methods

2.1. Antibodies and plasmids

A monoclonal antibody against mannosidase II (Man II) (clone 53FC3) was purchased from BAbCo. Polyclonal antibodies against $G\alpha_{i3}$, $G\alpha_s$, $G\alpha_{11}$, $G\alpha_z$, $G\alpha_{12}$ and Gβ were purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA) Mammalian expression plasmids for rat $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_z$ and $G\alpha_{12}$, mouse $G\alpha_{11}$ and bovine $G\alpha_s$ were prepared as described [19–23]. cDNAs for GTPase-deficient (constitutively active) mutants were generated by primer-mediated mutagenesis and inserted into pCMV5 [24]. cDNAs for β_1 and γ_2 were each subcloned into pCMV5 [25]. The plasmid for the green fluorescent protein-tagged glycoprotein of vesicular stomatitis virus ts045 (VSVG-GFP) [26] was kindly donated by Dr. J. Lippincott-Schwartz of the National Institute of Child Health and Human Development.

2.2. Treatment of cells with NDGA and immunofluorescence

For NDGA treatment, NRK cells were washed once with fetal calf serum-free medium and then incubated with 20 μM NDGA in the serum-free medium. Previously we reported that 100 μM NDGA is

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Abbreviations: ER, endoplasmic reticulum; BFA, brefeldin A; COPI, coat protein I; Man II, mannosidase II; NDGA, nordihydroguaiaretic acid; VSVG-GFP, green fluorescent protein-tagged glycoprotein of vesicular stomatitis virus ts045

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required for the disassembly of the Golgi apparatus [12]. The requirement of such a high concentration of NDGA was perhaps due to the adsorption of NDGA to fetal calf serum. Immunofluorescence analysis was performed as described previously [27].

2.3. Microinjection

Plasmids (0.1 mg/ml in phosphate-buffered saline) were microinjected into the nuclei of NRK cells grown on Cellocate coverslips (Eppendorf) using an Eppendorf Microinjector 5242 with Femtotips, with an injection time of 0.2 s.

3. Results

3.1. Overexpression of $G\alpha_z$ or $G\alpha_{i2}$ prevents NDGA-induced Golgi disassembly

Gα subunits can be classified into four subfamilies, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ [28]. To identify the α subunit subtype involved in the blockage of Golgi disassembly induced by NDGA, we examined the effects of overexpression of various

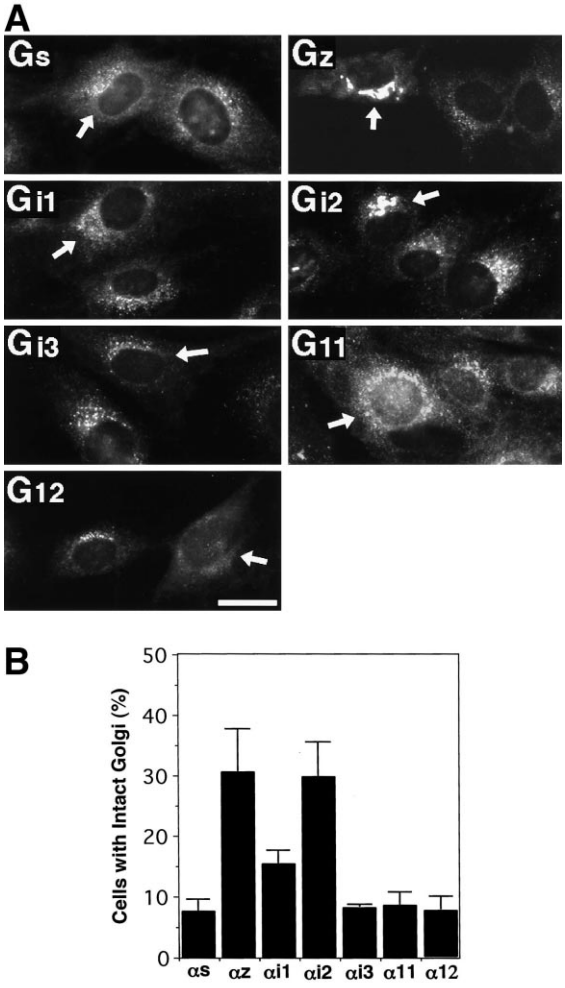

Fig. 1. Overexpression of wild-type $G\alpha_z$ or $G\alpha_{i2}$ inhibits Golgi disassembly induced by NDGA. A: Plasmids encoding various Gα proteins were microinjected into the nuclei of NRK cells. After incubation for 4 h at 37°C, the cells were incubated with 20 μM NDGA at 37°C for 10 min. Although Man II and Gα were double-immunostained, only Man II staining is shown. Arrows indicate cells overexpressing Gα. Bar, 20 μm. B: Quantitation of cells with an intact Golgi apparatus after NDGA treatment. The vertical axis shows the percentage of cells with an intact Golgi apparatus. More than 200 cells for each (up to 600 cells) were scored.

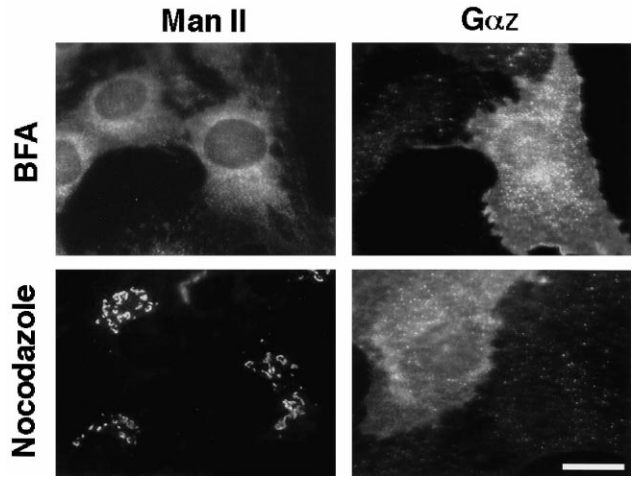

Fig. 2. Overexpression of $G\alpha_z$ does not affect BFA- or nocodazole-induced Golgi disassembly. The plasmid encoding $G\alpha_z$ was microinjected into the nuclei of NRK cells. After incubation for 4 h at 37°C, the cells were incubated with 5 μM BFA at 37°C for 10 min or 15 μg/ml nocodazole at 37°C for 20 min. Man II and $G\alpha_z$ were double-immunostained. Bar, 20 μm.

α subunits in NRK cells. A plasmid (0.1 mg/ml) carrying the cDNA for $G\alpha_s$, $G\alpha_z$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{11}$ ($G\alpha_q$ family) or $G\alpha_{12}$ was microinjected into the nuclei of NRK cells and then the cells were incubated to allow expression of the proteins for 4 h. The cells were then treated with 20 μM NDGA for 10 min and the Golgi apparatus was visualized by immunostaining with an anti-Man II antibody. To identify cells expressing the Gα subunits, the cells were also immunostained with anti-Gα antibodies. Although the antibodies against Gα subunits recognized endogenous proteins, the cells overexpressing Gα subunits were easily detectable from the high staining intensity.

Incubation of NRK cells with 20 μM NDGA for 10 min caused disassembly of the Golgi apparatus in 90–95% of the cells. When $G\alpha_z$ or $G\alpha_{i2}$ was overexpressed, Golgi disassembly was prevented in a significant fraction of the cells (Fig. 1A). Quantitative analysis revealed that Golgi disassembly was suppressed in about 30% of the cells expressing $G\alpha_z$ or $G\alpha_{i2}$ (Fig. 1B). Overexpression of the other Gα subunits, except that $G\alpha_{i1}$ had some effect, did not remarkably prevent Golgi disassembly. The suppressive effect was dependent on the level of expression. When the expression of $G\alpha_z$ at a lower level was achieved by the injection of a lower amount of the plasmid (0.01 mg/ml of plasmid), Golgi disassembly was not significantly blocked (data not shown). Perhaps expression of $G\alpha_z$ near the maximal level in living cells is required for the protective effect. When the expression of $G\alpha_z$ at a higher level was achieved, cells started to die. The requirement of the high expression of $G\alpha_z$ for the appearance of the suppressive effect may partly explain why Golgi disassembly was inhibited in only 30% of the cells expressing $G\alpha_z$. Another possibility to explain this limited effect is that the $G\alpha_z$ and $G\alpha_{i2}$ may function in cells that are in a certain phase of the cell cycle.

When GTPase-deficient (constitutively active) Gα mutants were overexpressed, the results were similar to those for the expression of the wild-type Gα subunits. Overexpression of $G\alpha_z$ (Q205L) or $G\alpha_{i2}$ (Q205L) suppressed Golgi disassembly in about 30% of the cells, whereas expression of the other mutants had little effect, if any.

The involvement of $G\alpha_z$ in this Golgi disassembly reaction is consistent with the previous finding that cholera toxin and pertussis toxin had no effect on NDGA-induced Golgi disassembly [12]. $G\alpha_z$ is a $G\alpha_i$ family protein, but is insensitive to pertussis toxin [29]. We therefore investigated the properties of cells overexpressing $G\alpha_z$ in more detail.

3.2. Overexpression of $G\alpha_z$ does not inhibit BFA- or nocodazole-induced Golgi disassembly

We wondered whether the overexpression of $G\alpha_z$ affects a specific step of NDGA-induced Golgi disassembly or generally stabilizes Golgi membranes. To examine these possibilities, cells overexpressing $G\alpha_z$ were incubated with BFA, a reagent which promotes the redistribution of Golgi components to the ER [7,9], or nocodazole, a compound which causes Golgi scattering by disrupting microtubules [30]. If the overexpression of $G\alpha_z$ merely stabilizes Golgi membranes, it is expected that the Golgi apparatus in cells expressing $G\alpha_z$ would not be disassembled by these reagents. To examine the initial changes in the Golgi morphology induced by these reagents, cells were incubated with 5 μM BFA for 10 min or 15 μg/ml nocodazole for 20 min. As shown in Fig. 2, disassembly of the Golgi apparatus by BFA or nocodazole occurred in cells expressing $G\alpha_z$ as well as non-transfected cells. These results strongly suggest that $G\alpha_z$ is specifically related to the action of NDGA.

3.3. Overexpression of $G\alpha_z$ does not interfere with protein transport from the ER to the plasma membrane

Overexpression of $G\alpha_{i3}$ retards the secretion of a heparan sulfate proteoglycan by LLC-PK_1 epithelial cells [31]. We next examined whether or not the overexpression of $G\alpha_z$ interferes with the transport of VSVG-GFP from the ER to the plasma membrane. This mutant VSV-G protein resides in the ER at a non-permissive temperature (40°C) and is exported from the ER at a permissive temperature (32°C) [26]. The plasmids for $G\alpha_z$ and VSVG-GFP were microinjected into cells, and then the cells were incubated at 40°C for 4 h. VSVG-GFP expression was observed in the ER (Fig. 3). Golgi disassembly induced by NDGA was significantly blocked even when VSVG-GFP was coexpressed with $G\alpha_z$ (data not shown). Upon a temperature shift to 32°C for 30 min in the presence of cycloheximide, VSVG-GFP was detected in the perinuclear Golgi region. By 60 min, it had been transported to the plasma membrane, although a considerable amount of VSV-G protein was still observed in the Golgi region. Comparison of the time course of protein transport between control cells and cells expressing $G\alpha_z$ suggested that expression of $G\alpha_z$ does not remarkably inhibit the transport of VSV-G protein from the ER to the plasma membrane via the Golgi apparatus.

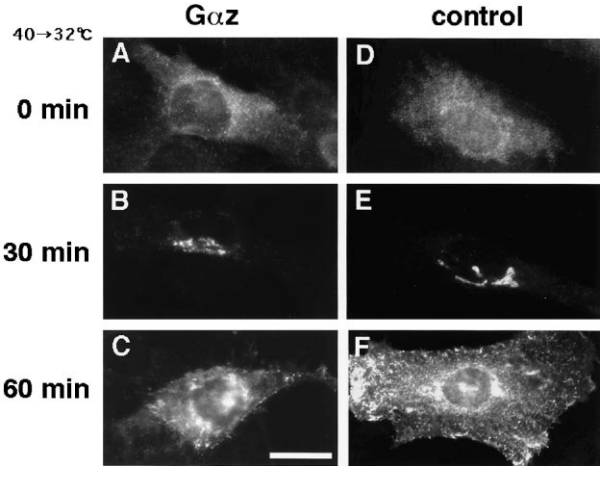

Fig. 3. $G\alpha_z$ does not remarkably block the intracellular transport of VSVG-GFP. The plasmids for VSVG-GFP and $G\alpha_z$ were co-microinjected into the nuclei of NRK cells. The cells were incubated at 40°C for 4 h, and then shifted to 32°C for 0 min (A), 30 min (B), or 60 min (C) in the presence of 20 μg/ml cycloheximide. Expression of $G\alpha_z$ was confirmed by immunostaining with an anti-$G\alpha_z$ antibody. As a control, only VSVG-GFP was expressed, and then the cells were shifted to 32°C for 0 min (D), 30 min (E), or 60 min (F) in the presence of 20 μg/ml cycloheximide. Fluorescence derived from GFP is shown. Bar, 20 μm.

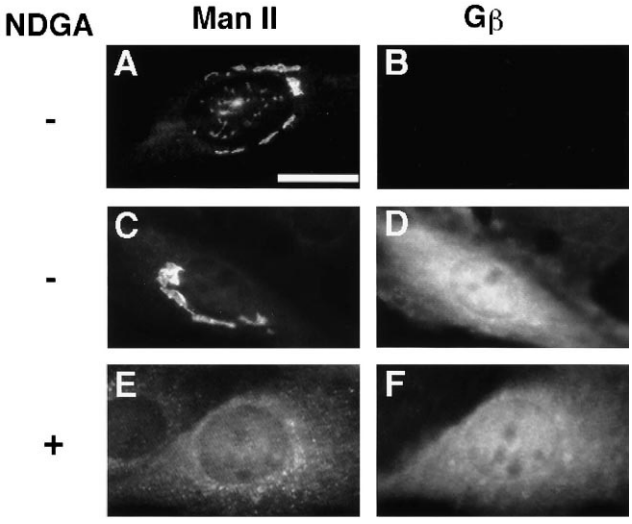

Fig. 4. Overexpression of $G\beta_1\gamma_2$ had no effect on the Golgi morphology. The plasmids encoding the β_1- and γ_2 subunits were co-microinjected into the nuclei of NRK cells, and then the cells were incubated for 4 h at 37°C. The cells were incubated without (A, B, C and D) or with (E and F) 20 μM NDGA at 37°C for 10 min. They were double-immunostained for Man II (A, C and E) and Gβ (B, D and F). An antibody against Gβ was diluted so as to stain cells overexpressing $G\beta_1\gamma_2$. Expression of $G\gamma_2$ was confirmed in a separate experiment (data not shown). A: Immunostaining for Man II in a non-transfected cell (control). Bar, 20 μm.

3.4. Overexpression of $G\beta_1\gamma_2$ does not cause Golgi disassembly

Malhotra et al. reported that Gβγ per se triggers Golgi disassembly in permeabilized NRK cells [17,18]. We examined whether or not overexpression of Gβγ in intact cells also causes Golgi vesiculation. The plasmids for β_1 and γ_2 were microinjected into NRK cells and then the cells were incubated for 4 h. $\beta_1\gamma_2$ is major species in brain [16]. As shown in Fig. 4, no morphological change of the Golgi apparatus was observed and NDGA was essential for Golgi disassembly in cells overexpressing $G\beta_1\gamma_2$.

4. Discussion

Our previous results using NDGA suggest that heterotrimeric G proteins play a role in the organization of the Golgi apparatus [12]. In the present study we attempted to determine which Gα subunit subtypes are involved in the organization of the Golgi structure. Our strategy was to overexpress

various Gα subunits in NRK cells, and to examine their effects on the disassembly of the Golgi apparatus induced by NDGA. The results showed that overexpression of $G\alpha_z$ or $G\alpha_{i2}$ significantly suppressed NDGA-induced Golgi disassembly. The effect of $G\alpha_z$ appears to be specific to the action of NDGA. Overexpression of $G\alpha_z$ had no effect on Golgi disassembly induced by nocodazole or BFA. In addition, protein transport within the secretory pathway was not retarded.

Malhotra et al. [17,18] found that Gβγ is sufficient for the vesiculation of Golgi stacks in permeabilized NRK cells. In the present work we found that overexpression of $G\beta_1\gamma_2$ did not cause disassembly of the Golgi apparatus. There are several possibilities for reconciling these apparently inconsistent results. Given the presence of diverse β and γ subunit types that may be responsible for the specificity of cell signaling [16], one possibility is that a specific subtype of βγ other than $\beta_1\gamma_2$ is involved in Golgi disassembly. Jamora et al. [17] used Gβγ isolated from bovine brain, which is obviously a mixture of various Gβγ subtypes. Another possibility is that the Golgi disassembly induced by Gβγ is due to an effect of free Gβγ at non-physiological concentrations in a semi-intact system. We previously showed that the incubation of permeabilized cells with Gβγ isolated from bovine brain did not cause Golgi disassembly when cytosol was included in the system [12]. A similar result was reported by Schwaninger et al. [32]. Perhaps proteins in the cytosol interact with the added Gβγ, which may suppress the Golgi-disrupting ability of Gβγ. At present we favor the latter possibility because one cannot explain why the activation of heterotrimeric G proteins by GTPγS prevents Golgi disassembly induced by ilimaquinone in the presence of cytosol in permeabilized cells [17]. It is believed that free Gβγ is formed as a consequence of the dissociation of the GTP-bound Gα from the trimeric complex [15]. If Gβγ has the ability to cause Golgi disassembly, activation of G proteins, which is supposed to cause an increase in the concentration of free Gβγ, would rather induce Golgi disassembly. Therefore, the involvement of Gβγ in Golgi disassembly contradicts the finding that ilimaquinone-induced Golgi disassembly is prevented by GTPγS.

Two lines of evidence strongly suggest that the active (GTP-bound) form of Gα plays a role in the maintenance of the Golgi structure. First, activators of heterotrimeric G proteins suppressed Golgi disassembly induced by NDGA ([12] and this study). Second, the constitutively active form of $G\alpha_z$ or $G\alpha_{i2}$ was effective in protecting the Golgi structure. Since overexpression of the wild-type Gα abrogated the effect of NDGA, one can assume that the wild-type Gα is in the active (GTP-bound) form in the absence of extracellular signals such as hormones. This assumption is surprising from the standpoint of classical heterotrimeric G proteins, but not from that of an endomembrane system. Overexpression of $G\alpha_{i3}$ retards the secretion of a heparan sulfate proteoglycan in LLC-PK_1 epithelial cells, and this effect was reversed by pertussis toxin, which causes functional uncoupling of $G\alpha_{i3}$ on Golgi membranes [31]. These results can be interpreted by the idea that expressed $G\alpha_{i3}$ bound to Golgi membranes is in the active form due to coupling with unknown signaling protein(s) in Golgi membranes and uncoupling by the toxin renders it inactive.

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