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Inhibition of Vesicle-Mediated Protein Transport by Nordihydroguaiaretic Acid[1]

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Nordihydroguaiaretic acid (NDGA) blocks intra-Golgi protein transport in a cell-free system and prolactin secretion from GH_3 cells [Tagaya, M., Henomatsu, N., Yoshimori, T., Yamamoto, A., Tashiro, Y., and Fukui, T. (1993) *FEBS Lett.* 324, 201-204]. To determine which intracellular secretory pathway(s) is inhibited by NDGA, we investigated its effect on the transport of the vesicular stomatitis virus-encoded glycoprotein in BHK-21 cells. NDGA blocked protein transport from the endoplasmic reticulum to the Golgi apparatus, and from the *trans*-Golgi network to the plasma membrane. In addition, it retarded the brefeldin A-induced retrograde transport of mannosidase II to the endoplasmic reticulum. Although NDGA had an inhibitory effect on protein synthesis, it induced the expression of BiP, a chaperone located in the endoplasmic reticulum. The induction of BiP may be a consequence of the inhibition of protein transport by NDGA.

Key words: brefeldin A, nordihydroguaiaretic acid, protein transport, secretion, vesicular stomatitis virus.

Vesicle-mediated protein transport consists of several steps including the formation of vesicles, and then targeting and fusion to receptor membranes. Each step is mediated by a few or more proteins (*1-4*). Transport inhibitors could be very powerful tools for analyzing this complicated process. Indeed, *N*-ethylmaleimide was used for the identification of NSF, that is involved in the multi-pathways of vesicle-mediated protein transport (for a review, see Ref. *5*). GTPγS, which inhibits intra-Golgi protein transport through binding to ADP-ribosylation factors (*6*), was employed to isolate Golgi-derived non-clathrin-coated vesicles (*7*). The retrograde pathway from the Golgi apparatus to the ER was discovered by the use of BFA (for a review, see Ref. *8*). Ilimaquinone (*9*) and caffeine at 20°C (*10*) were found to promote the vesiculation of Golgi membranes, although the mechanism remains to be elucidated.

With the hope of finding new factors involved in protein transport, we started screening transport inhibitors by using a well-established intra-Golgi transport assay in a cell-free system (*11*), and found that several types of phospholipase A_2 inhibitors inhibit this transport (*12*). This suggests that phospholipase A_2 or proteins having properties similar to phospholipase A_2 are involved in this protein transport system. NDGA, a membrane-permeable phospholipase A_2 inhibitor, blocked prolactin secretion from GH_3 cells (*12*), reflecting the physiological relevance to the inhibition of protein transport *in vitro*. In the present study we investigated the effect of NDGA on the intracellular transport of the VSV-G protein in BHK-21 cells. We found that NDGA inhibits the transport of the VSV-G protein from the ER to the Golgi apparatus, and from the TGN to the plasma membrane. In addition, it retarded the retrograde transport of man II to the ER.

EXPERIMENTAL PROCEDURES

Materials—NDGA was purchased from Biomol Research Laboratories and dissolved in dimethylsulfoxide. BFA and actinomycin D were purchased from Wako Chemicals. End H was purchased from Genzyme. EN^{3}HANCE was obtained from DuPont-New England Nuclear. [^{35}S]Methionine was obtained from ICN Biomedicals. A prestained molecular weight marker (high molecular weight range) and reagents for tissue culture were obtained from Gibco BRL. A monoclonal anti-man II antibody (clone 53FC3) was obtained from BAbCo. An anti-Hsp 70 (clone BRM-22) was purchased from Sigma. Polyclonal antibodies against man II and β-COP were generous gifts from Dr. K. Moremen at University of Georgia and Dr. Y. Ikehara at Fukuoka University, respectively. A polyclonal anti-BiP was kindly donated by Drs. K. Mihara and M. Sakaguchi at Kyushu University.

Cell Culture and Virus Infection—BHK-21 cells, cultured in MEM medium (α-MEM supplemented with 7.5%

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Abbreviations: BFA, brefeldin A; endo H, endoglycosidase H; ER, endoplasmic reticulum; man II, mannosidase II; MEM, minimum essential medium; NDGA, nordihydroguaiaretic acid; NSF, *N*-ethylmaleimide-sensitive factor; PBS, phosphate-buffered saline; TGN, *trans*-Golgi network; VSV, vesicular stomatitis virus; VSV-G protein, vesicular stomatitis virus-encoded glycoprotein.

fetal calf serum, 50 μg/ml streptomycin, 50 IU/ml penicillin, and 10 mM Hepes, pH 7.2), were washed once with 1 ml of PBS, and then incubated with 1 ml of MEM medium containing 5-10 pfu/cell VSV. Actinomycin D at 2 μg/ml was added throughout infection and pulse labeling-chase experiments. This reagent was omitted for morphological experiments. After 1 h, the infection medium was removed, and the cells were washed twice with 1 ml of PBS and then incubated with 1 ml of MEM medium.

Pulse-Labeling with [^{35}S]Methionine—At 3-4 h after infection, the medium was removed, and the cells were washed twice with 1 ml of PBS and then pulse-labeled with 0.5 ml of methionine-free α-MEM containing 20 μCi of [^{35}S]methionine for 10 min. For the chase, 0.5 ml of α-MEM containing 15% fetal calf serum and 0.2 mM methionine, with or without NDGA, was added. Since NDGA has an inhibitory effect on protein synthesis, it was added after the pulse-labeling. The final concentration of dimethylsulfoxide in the medium was 0.2-0.5%. For endo H treatment, the medium was aspirated off, and the cells were washed twice with 1 ml of cold PBS and then solubilized with 0.5 ml of 1% SDS, 1% mercaptoethanol, and 50 mM Tris-HCl, pH 7.5. The cell lysate was heated and stored at −20°C until use.

Endo H Treatment—To 10 μl of a cell lysate was added 70 μl of 0.125 M sodium citrate, pH 5.5. Endo H was added to a final concentration of 25 mIU/ml, and then the mixture was incubated at 37°C for 16-20 h. The proteins were precipitated with 10% trichloroacetic acid, washed in cold acetone, and then subjected to SDS-polyacrylamide gel electrophoresis.

Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (*13*) using a mini-electrophoresis apparatus with 10% gels. For maximal resolution of the unprocessed and processed forms of the VSV-G protein, electrophoresis at 150 V was continued for 30-40 min after bromophenol blue had reached the bottom of the gel. A prestained molecular weight marker was used to confirm maximal resolution. For fluorography, the gel was stained with Coomassie Brilliant Blue R-250, destained, and then treated with EN^{3}HANCE. After drying, the gel was exposed to Kodak X-OMAT X-ray

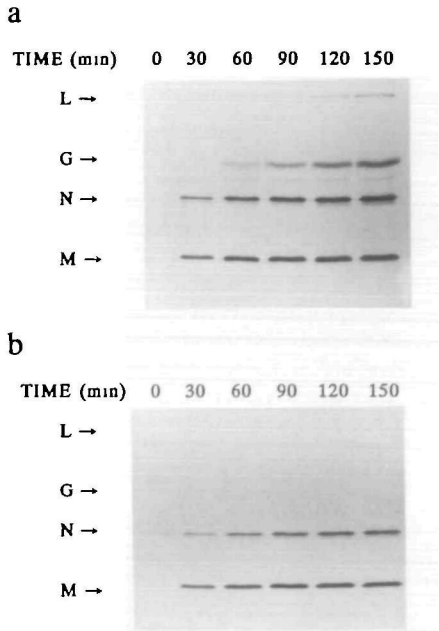

Fig 1 **NDGA inhibits the secretion of the VSV-G protein into the medium.** BHK-21 cells were infected with VSV, pulse-labeled with [^{35}S]methionine, and then chased at 37°C in the absence (a) or presence (b) of 100 μM NDGA. After the indicated chase times, portions of the medium were withdrawn and subjected to SDS-polyacrylamide gel electrophoresis L (large protein), G (glycoprotein), N (nucleocapsid protein), and M (matrix protein) indicate the positions of individual VSV proteins

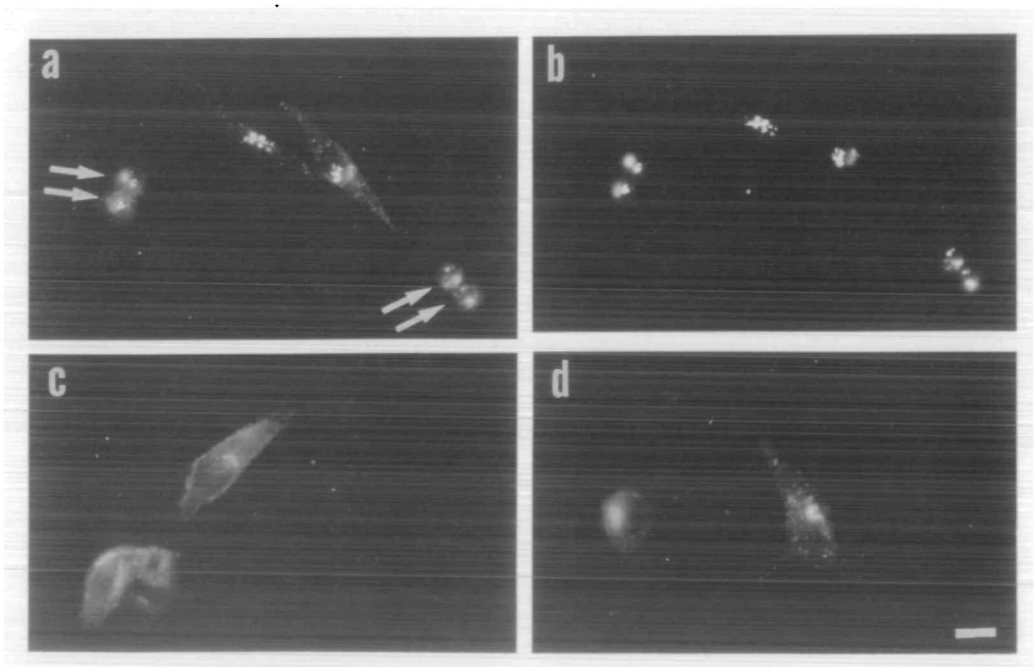

Fig 2 **Effect of NDGA on the transport of the VSV-G protein from the TGN to the plasma membrane.** The VSV-G protein was transiently synthesized in VSV-infected BHK-21 cells as described under "EXPERIMENTAL PROCEDURES." The cells were then incubated at 20°C for 2 h in the presence of 20 μg/ml cycloheximide to allow the movement of the VSV-G protein from the ER to the TGN. The cells were fixed, permeabilized with Triton X-100, and then double-immunolabeled for the VSV-G protein (a) and man II (b). Alternatively, after 2-h incubation at 20°C, the cells were incubated at 37°C with cycloheximide in the absence (c) or presence (d) of 100 μM NDGA. After 45 min, the cells were fixed, permeabilized with Triton X-100, and then immunolabeled for the VSV-G protein. Arrows indicate round cells. Bar, 15 μm.

film for an appropriate time.

Preparation of VSV-Infected Cells for Immunofluorescence Analysis—At 1 h after infection, cycloheximide was added to cells at a final concentration of 20 μg/ml to stop protein synthesis, and then the incubation was continued for another 1 h. The medium was aspirated off, and the cells were washed twice with PBS and then incubated with MEM medium to allow synthesis of the VSV-G protein. After 10 min, cycloheximide was added again to stop further protein synthesis. The transport of the VSV-G protein synthesized in 10 min was monitored by immunofluorescence microscopy as described previously (*14*). When incubation was conducted at 20°C, the concentration of Hepes in the medium was raised to 25 mM.

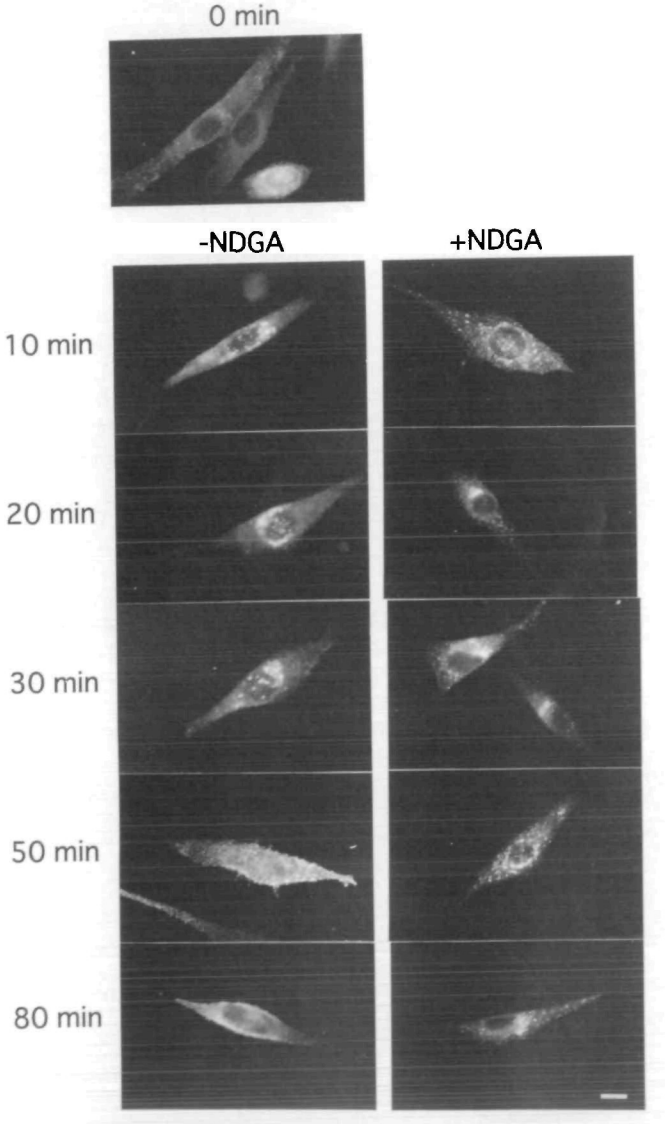

Fig. 3. **Effect of NDGA on the transport of the VSV-G protein from the ER to the Golgi apparatus.** The VSV-G protein was transiently synthesized in VSV-infected BHK-21 cells as described under "EXPERIMENTAL PROCEDURES." The cells were then incubated with cycloheximide in the absence or presence of 100 μM NDGA. After the indicated chase times, the cells were fixed, permeabilized with Triton X-100, and then immunolabeled for the VSV-G protein. Bar, 15 μm.

When cells were infected with VSV, many of them became round. However, the location of organelles such as the nucleus and Golgi apparatus was more easily recognizable in spread cells than round cells. Therefore, the immunostaining patterns of spread cells are shown in most figures. The immunostaining patterns for the VSV-G protein in spread cells were essentially similar to those in round cells.

RESULTS

Secretion of the VSV-G Protein into the Medium Is Blocked by NDGA—To investigate the effect of NDGA on the secretion of the VSV-G protein from BHK-21 cells into the medium, cells were infected with VSV, pulse-labeled for 10 min with [^{35}S]methionine, and then chased in the absence or presence of 100 μM NDGA. In the absence of NDGA, the VSV-G protein, in addition to the VSV-L, VSV-N, and VSV-M ones, appeared in the medium and accumulated with time (Fig. 1a). In contrast, little VSV-G protein appeared, if any, in the medium up to 2.5 h in the presence of NDGA, whereas the VSV-N and VSV-M proteins were secreted (Fig. 1b). The amount of the VSV-L protein secreted into the medium also decreased significantly. A similar defect of secretion of the VSV-L protein was observed when the transport and synthesis of the VSV-G protein were blocked by the lysosomotropic agent, primaquine (*15*), and by interferon (*16*), respectively. These results suggest a correlation between the two proteins in the incorporation into virus particles, although the mechanism is not known.

The inhibitory effect of NDGA on the secretion of the VSV-G protein into the medium was dose-dependent. Twenty to 30 μM NDGA was required for 50% inhibition of the VSV-G protein secretion (data not shown). This concentration range was similar, in order of magnitude, to that required for 50% inhibition of intra-Golgi protein transport in a cell-free system (9 μM), and of prolactin secretion from GH_3 cells (60 μM) (*12*).

NDGA Blocks Transport from the TGN to the Plasma Membrane—To determine which intracellular secretory pathway(s) is inhibited by NDGA, we first examined the effect of NDGA on the transport of the VSV-G protein from the TGN to the plasma membrane as described below. At 1 h after infection of cells with VSV, cycloheximide was added to stop protein synthesis. At this time, most cells (93-95%) had not started to synthesize the VSV-G protein (data not shown). After 1-h incubation with cycloheximide, the cells were washed with PBS and then incubated for 10

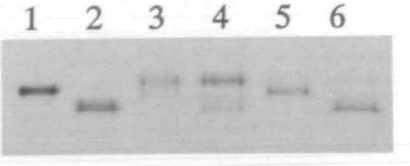

Fig. 4. **Effect of NDGA on acquisition of endo H-resistance of the VSV-G protein.** BHK-21 cells were infected with VSV, pulse-labeled with [^{35}S]methionine, and then chased at 37°C for 0 min (lanes 1 and 2) or 30 min in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of 100 μM NDGA. Mock- (lanes 1, 3, and 5) and endo H- (lanes 2, 4, and 6) treated samples were subjected to SDS polyacrylamide gel electrophoresis.

min without cycloheximide to allow synthesis of the VSV-G protein. Although the amount of VSV-G protein synthesized in 10 min varied somewhat from one cell to another, this transient synthesis allowed us to monitor the transport of the VSV-G protein from the ER by means of immunofluorescence microscopy because the protein had not been synthesized in most cells before the 10-min incubation without cycloheximide. After the transient synthesis of the VSV-G protein, the cells were incubated at 20°C for 2 h in the presence of cycloheximide. This incubation allows the transport of secretory proteins from the ER to the Golgi apparatus, but not from the TGN to the plasma membrane (*17*). As shown in Fig. 2a, the VSV-G protein accumulated at the perinuclear region after incubation at 20°C for 2 h. This region corresponds to the Golgi region, as revealed by the co-localization of the VSV-G protein with a Golgi-resident protein, man II (Fig. 2b). Upon incubation at 37°C for 45 min, the VSV-G protein was transported from the TGN to the plasma membrane in most cells (Fig. 2c). On the other hand, it did not reach the plasma membrane when cells were incubated in the presence of 100 μM NDGA (Fig. 2d). These results suggest that NDGA blocks protein transport from the TGN to the plasma membrane.

In a minor fraction of cells (5-7%), the VSV-G protein reached the plasma membrane before incubation at 37°C for 45 min (data not shown). This probably reflects the fraction of cells that had synthesized the VSV-G protein before the addition of cycloheximide at 1 h after infection.

NDGA Blocks Transport from the ER to the Golgi Apparatus—We next examined the effect of NDGA on the transport of the VSV-G protein from the ER to the Golgi apparatus. As shown in Fig. 3, the VSV-G protein synthesized on 10-min incubation was exclusively located in the ER. In the absence of NDGA, significant vesicle-like staining was observed at 10 min after labeling. After 20-30 min, staining was mainly observed in the perinuclear region, which corresponds to the Golgi region. After 50-80 min, the VSV-G protein reached the plasma membrane. On the other hand, although vesicle-like staining was observed at 10 min after pulse-labeling and changed to Golgi-like staining at 20-30 min in the presence of NDGA, the VSV-G protein had not reached the plasma membrane by 50-80 min.

To determine whether or not the VSV-G protein reached the Golgi apparatus in the presence of NDGA, the VSV-G protein was pulse-labeled with [^{35}S]methionine for 10 min and then chased in the presence of NDGA. Figure 4 shows that most of the pulse-labeled VSV-G protein acquired endo H-resistance during a 30-min chase in the absence of NDGA, whereas all the VSV-G protein was sensitive to endo H in the presence of NDGA. Endo H cleaves high-mannose-type but not complex-type oligosaccharide chains of glycoproteins (*18*). When high-mannose-type oligosaccharides attached in the ER are converted to complex-type ones in the *medial* Golgi by processing enzymes including man II (*19*), the resultant VSV-G protein is resistant to endo H treatment. Therefore, the results in Fig. 4 imply that NDGA inhibits protein transport from the ER to the *medial* Golgi.

NDGA Does Not Cause Disassembly of Microtubules—Previous studies revealed the involvement of microtubules in some transport pathways (*20-24*). We examined whether or not NDGA inhibits protein transport by disrupting microtubules. As shown in Fig. 5, microtubules appeared to be intact when BHK-21 cells were incubated with 100 μM NDGA for 60 min. The Golgi apparatus might be slightly disintegrated. However, it was not clear whether this integration is significant or not, because some cells showed a slightly dispersed Golgi pattern without NDGA.

Redistribution of Man II into the ER Induced by BFA Is Retarded by NDGA—BFA promotes disassembly of the Golgi apparatus and causes Golgi-resident proteins to be redistributed into the ER (*25-27*). The earliest detectable effect of BFA (within 30 s) is the release of β-COP, a component of Golgi-derived coated vesicles (*28, 29*), from the Golgi apparatus (*30*). *In vitro* studies revealed that BFA blocks the formation of transport vesicles by inhibiting the binding of β-COP to Golgi membranes (*31*).

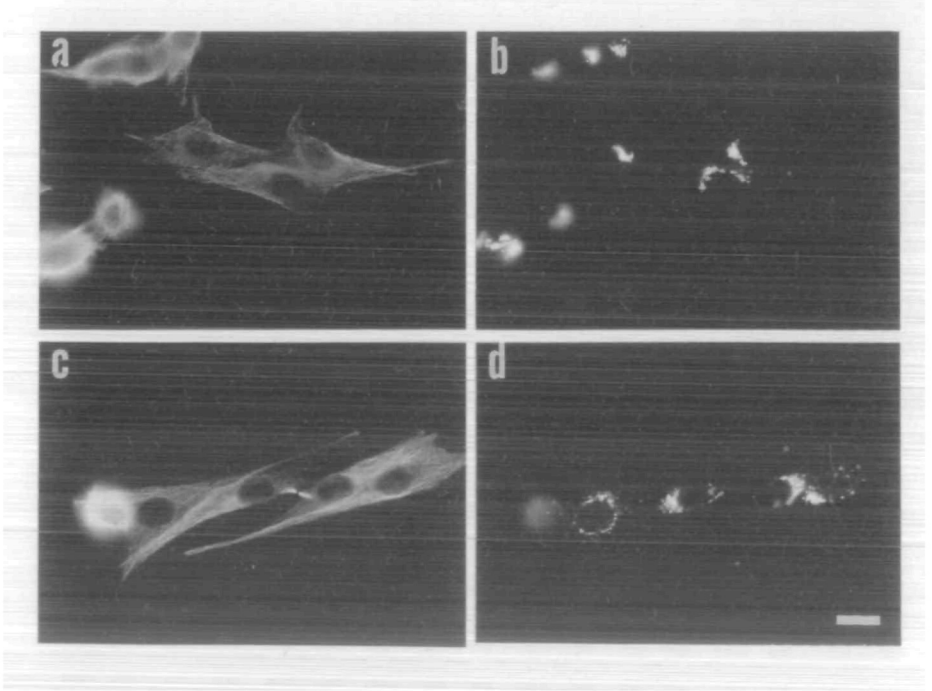

Fig. 5 **NDGA does not induce the disassembly of microtubules.** BHK-21 cells were incubated without (a and b) or with (c and d) 100 μM NDGA at 37°C. After 1-h incubation, the cells were fixed, permeabilized, and then double-labeled for α-tubulin (a and c) and man II (b and d). Bar, 15 μm.

Following the release of β-COP, Golgi-resident proteins such as man II are redistributed into the ER (within 15 min) in a microtubule-dependent manner (*27*). The immunostaining pattern for man II changes from a perinuclear one, characteristic of a Golgi distribution, to a reticular one, characteristic of an ER distribution, through "necklace-like" projections (*27*). We first investigated the effect of NDGA on BFA-mediated release of β-COP from the Golgi apparatus and found that the compound did not prevent the rapid release of β-COP from the apparatus (data not shown). Next, we examined whether NDGA inhibits retrograde transport or not. BHK-21 cells were incubated with BFA in the presence or absence of NDGA for 5, 30, and 60 min, and then fixed, permeabilized, and stained with an anti-man II antibody. Before the addition of BFA, man II was distributed in the perinuclear region (Fig. 6a). As observed by Lippincott-Schwartz *et al.* (*27*), the addition of BFA resulted in a reticular pattern of man II (Fig. 6c), characteristic of an ER distribution, by 30 min through necklace-like projections by 5 min (Fig. 6b). In the presence of NDGA, the necklace-like projections were observed after 5 min BFA incubation (Fig. 6e), as observed in the absence of NDGA (Fig. 6b). However, the necklace-like projections were still present after 30 min (Fig. 6f). Even after 1 h, they were still significant in some cells (Fig. 6g). These results suggest that NDGA retarded the retrograde transport of man II to the ER, although the inhibition was not complete.

Induction of BiP by NDGA—We noticed that NDGA strongly inhibits protein synthesis by BHK-21 cells (Fig. 7). It seemed that NDGA does not inhibit the uptake of [^{35}S]methionine from the medium because radioactivity detectable in the supernatant on trichloroacetic acid treatment was not significantly decreased by NDGA up to 60 μM. The inhibitory effect of NDGA on protein synthesis was reversible. As shown in Fig. 8, cells started protein synthesis after treatment with 30 μM NDGA for 5 h.

It is known that some transport inhibitors, such as BFA, induce BiP (*32*). We therefore examined whether or not BiP is induced by NDGA. As shown in Fig. 9, 30 μM NDGA caused the expression of a 78-kDa protein, although total protein synthesis was inhibited by 90%. This protein was recognized by a monoclonal anti-Hsp 70 antibody (clone BRM-22) and a polyclonal anti-BiP antibody, suggesting that it is Bip. It is noteworthy that Hsp 70 is not induced by NDGA.

DISCUSSION

We previously showed that NDGA inhibits *in vitro* intra-Golgi protein transport and prolactin secretion from GH_3 cells (*12*). Our final goals are to isolate the protein(s) inhibited by this compound and to reveal its/their role(s) in protein transport. The present study aimed to reveal which

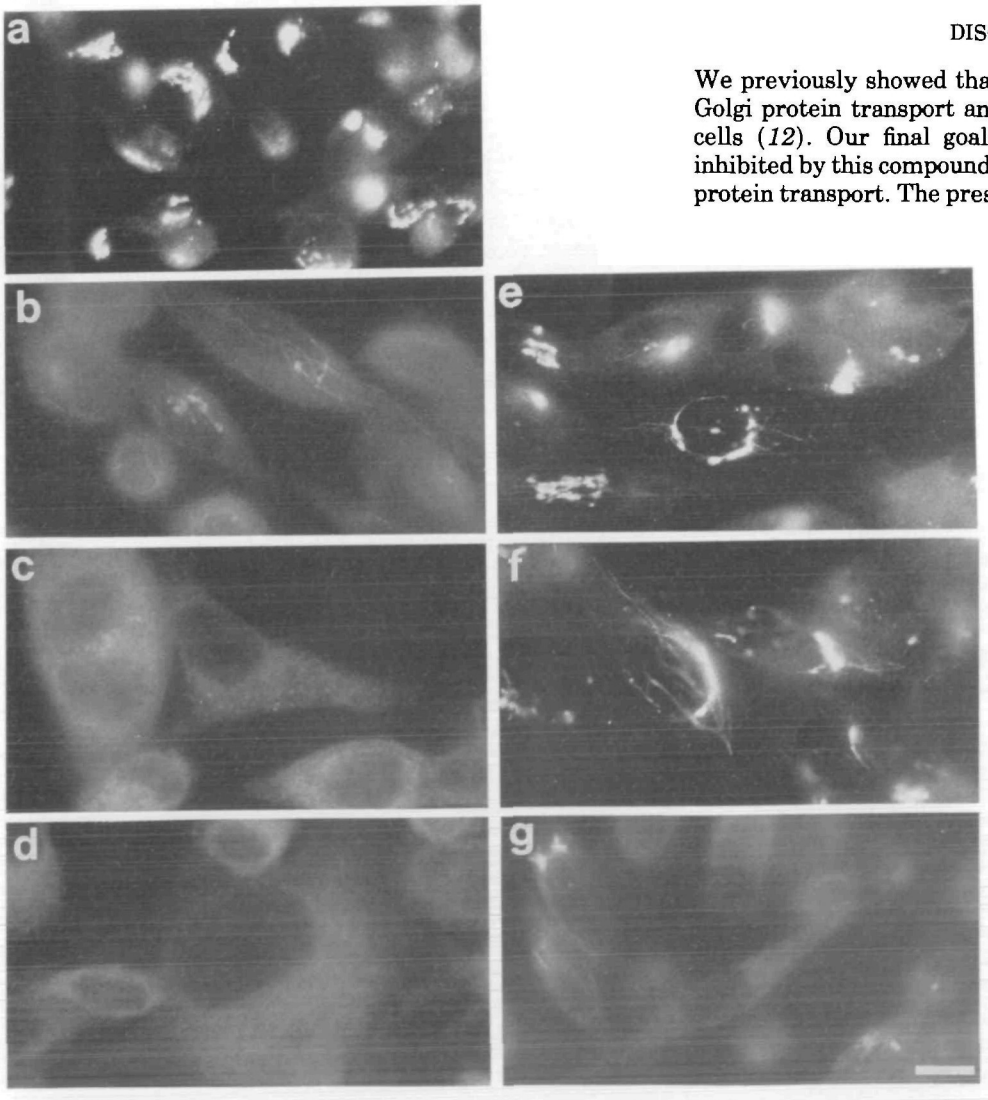

Fig. 6. **Effect of NDGA on retrograde transport.** BHK-21 cells were incubated at 37°C for 0 min (a), 5 min (b and e), 30 min (c and f), and 1 h (d and g) in the presence of 36 μM BFA alone (b-d) or 36 μM BFA and 100 μM NDGA (e-g). The cells were fixed, permeabilized, and then labeled for man II. Bar, 24 μm

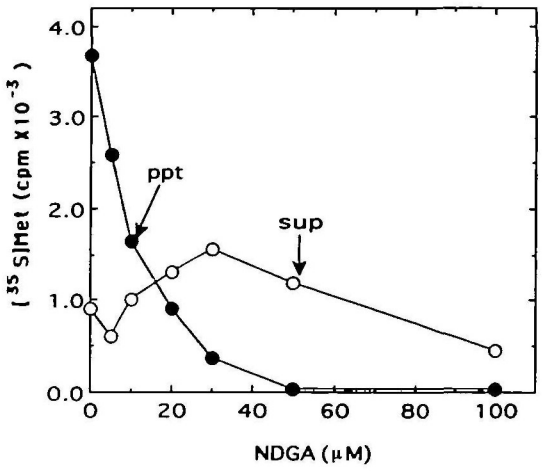

Fig. 7. **Inhibition of protein synthesis by NDGA.** BHK-21 cells were incubated with 50 μCi of [^{35}S]methionine in the presence of various concentrations of NDGA. After 10 min at 37°C, the cells were washed with PBS three times, and then solubilized with 0 5 ml of 150 mM NaCl, 1% Triton X-100, 0.2 mM protease inhibitor mixture (leupeptin, pepstatin, chymostatin, and antipain), 1 mM phenylmethylsulfonyl fluoride, and 25 mM Tris-HCl, pH 7.5 (lysis buffer). Proteins in the lysate were precipitated with 6% trichloroacetic acid and 0.02% deoxycholic acid The radioactivity of the supernatant (sup) and precipitate (ppt) was measured.

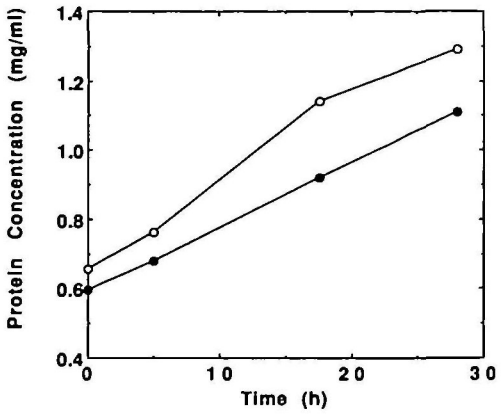

Fig. 8. **Inhibition of protein synthesis by NDGA is reversible.** BHK-21 cells (roughly 30–40% confluent) were incubated with (●) or without (○) 30 μM NDGA. After 5-h incubation, NDGA was removed (time 0), and the cells were further incubated. At the indicated times, the cells were washed with PBS twice, and then solubilized with 0 5 ml of lysis buffer. The protein concentration of the lysate was determined.

pathway(s) in intracellular traffic is inhibited by NDGA. NDGA inhibited the transport of the VSV-G protein from the ER to the Golgi apparatus, and from the TGN to the plasma membrane. In addition, it retarded the BFA-induced retrograde transport of man II to the ER.

The effect of NDGA on protein transport is different from those of other transport inhibitors so far discovered. Among conventional inhibitors, GTPγS and aluminum fluoride inhibit a late step of protein transport, *i.e.*, the step of uncoating of transport vesicles (*33, 34*). These inhibitors are different from NDGA in terms of the effect on the binding of vesicle coat proteins. GTPγS and aluminum

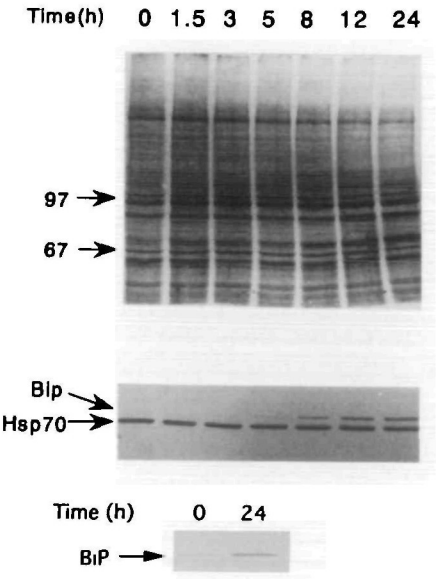

Fig. 9. **Induction of BiP by NDGA.** BHK-21 cells were incubated with 30 μM NDGA At the indicated times, the cells were solubilized with 0.5 ml of lysis buffer. Proteins in the lysate were subjected to SDS polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue R-250 (top panel), or immunostaining with anti-Hsp 70 (middle panel) or anti-BiP (bottom panel) Arrows in the top panel indicate the positions of molecular weight marker proteins.

fluoride promote the binding of β-COP (*35*) and the ADP-ribosylation factor (*36, 37*) to the Golgi apparatus. On the other hand, NDGA has no effect on the binding of these coat proteins to the Golgi apparatus. Although monensin (*38*), bafilomycin A_1 (*39*), and BFA (*8*) block specific pathways of protein transport, NDGA blocks multi-pathways in intracellular transport. It is interesting that NDGA does not inhibit endosome fusion, although phospholipase A_2 activity is required for the fusion (*40*).

NDGA induced the expression of BiP, but not Hsp 70 in BHK-21 cells. It is well known that the accumulation of malfolded proteins in the ER induces BiP (*41*). In addition, the induction of *Kar2*p, the yeast homologue of BiP, occurs when protein transport is impaired in a *SEC18* mutant strain (*42*). Although the precise mechanism underlying the induction of BiP by NDGA is unclear, it is reasonable to assume that the inhibition of protein transport is somehow related to it.

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