Geldanamycin-associated Inhibition of Intracellular Trafficking Is Attributed to a Co-purified Activity*

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Geldanamycin, an ansamycin antibiotic that specifically inhibits heat-shock protein-90 (HSP90) and its endoplasmic reticulum homologue, glucose-regulated protein-90 (GRP94), accelerates the degradation of selected cellular proteins. We showed previously that geldanamycin inhibits maturation and transport of the epidermal growth factor receptor in addition to accelerating its degradation (Supino-Rosin, L., Yoshimura, A., Yarden, Y., Elazar, Z., and Neumann, D. (2000) J. Biol. Chem. 275, 21850–21855). Here we demonstrate that the additional activities of geldanamycin on intracellular transport and protein maturation are related to its supply source. By combining chemical separation of Streptomyces hygroscopicus var. geldanus extracts and biological screens, we show that the geldanamycin-associated effects on intracellular transport and protein maturation are not mediated by geldanamycin itself but are due to the presence of an additional component(s). Chromatography of S. hygroscopicus var. geldanus extracts on a silica-gel column allowed separation between the inhibition of intracellular trafficking and geldanamycin-mediated degradation. One fraction that was devoid of geldanamycin blocked secretion of a soluble form of the erythropoietin receptor, retarded maturation of the epidermal growth factor receptor without enhancing its degradation, and blocked anterograde transport of a temperature-sensitive mutant of the vesicular stomatitis virus G protein (VSVGtsO45) from the early Golgi cisternae. This fraction was enriched (>95%) in 17-demethylgeldanamycin. However, as synthetically derived 17-demethylgeldanamycin did not inhibit intracellular trafficking, we concluded that 17-demethylgeldanamycin is not the active component. We thus propose that a compound(s) that co-purifies with benzoquinone ansamycins inhibits intracellular transport. Taken together, our data demonstrate that the inhibitory effects on protein maturation and intracellular trafficking, previously attributed to geldanamycin, are mediated by another distinct moiety.

Benzoquinone ansamycins (BAs)† are a family of antibiotics, isolated from Streptomyces hygroscopicus broth (1), that specifically inhibit the cytosolic chaperone heat-shock protein-90 (HSP90) and its endoplasmic reticulum homologue, glucose-regulated protein-90 (GRP94) (2, 3). Inhibition of these chaperones by geldanamycin (GA) and herbimycin A, the most prominent BAs, promotes the degradation of HSP90 substrates. Among these are Tyr kinases (4) such as epidermal growth factor receptor (EGF-R) (5), p185erbB2 (6, 7), and viral Src (8), as well as other proteins such as the cystic fibrosis transmembrane conductance regulator (CFTR) protein (9), mutated p53 (10), Raf-1 (11), Cdk4 (12), and steroid hormone receptors (13). The fact that many HSP90 substrates are involved in cell proliferation and malignant transformation renders BAs attractive candidates for clinical development. 17-allylamino-17-demethoxygeldanamycin (17-AAG), a GA derivative showing promising results in preclinical studies, is currently being used in clinical trials (3, 14). Whereas GA-mediated enhanced degradation of certain HSP90 client proteins is consistently observed (reviewed in Refs. 2, 3, 15, and 16), GA-mediated inhibition of intracellular trafficking of EGF-R (5, 17), p185erbB2 (18), platelet-derived growth factor receptor (5), viral Src (5), the cystic fibrosis transmembrane conductance regulator (9), and the mammalian prion protein (19) has only been noted in a few reports. Here, we demonstrate that different GA preparations differ in their ability to inhibit intracellular trafficking and that chemical separation of ethyl-acetate extracts of Streptomyces hygroscopicus var. geldanus broth distinguishes between GA-mediated degradation and inhibition of intracellular trafficking. We thus propose that the inhibition of intracellular transport and protein maturation by certain GA preparations is mediated by a co-purified component(s).

EXPERIMENTAL PROCEDURES
Materials were obtained from previously listed sources (17, 20).
BAs—GA was obtained from both Invitrogen (formerly Gibco/Life Technologies) and the National Cancer Institute (NCI, National Institutes of Health) in Rockville, MD. Synthetic 17-demethylgeldanamycin (17DMG) was kindly provided by L. Neckers, NCI, National Institutes of Health. Ethyl-acetate extracts of S. hygroscopicus var. geldanus broth were generously supplied by D. Newman, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health.

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¶ Materials were obtained from previously listed sources (17, 20).
** The abbreviations used are: BA, benzoquinone ansamycin; GA, geldanamycin; 17DMG, 17-demethylgeldanamycin; EGF-R, epidermal growth factor receptor; Ex EGF-R, extracellular EGF-R; EPO-R, erythropoietin receptor; sEPO-R, soluble EPO-R; Endo H, endoglycosidase H; PNGase F, N-glycosidase F; GalT, galactosyltransferase; VSVG, vesicular stomatitis virus G protein; DMEM, Dulbecco’s modified Eagle’s medium; CFP/GFP/YFP, cyan/green/yellow fluorescent protein.
Antibodies—Mouse monoclonal antibodies directed against the extracellular part of EGF-R were used at a 1:2,500 dilution for immunoprecipitation (monoclonal antibody 199.12, Neo Markers, Fremont, CA) and at a 1:1,000 dilution for immunoblotting (monoclonal antibody 111.6, Neo Markers). Polyclonal antibodies directed against the extracellular part of the erythropoietin receptor (EPO-R) (20) were used at a 1:1,000 dilution for immunoblotting. Mouse monoclonal antibodies against green fluorescent protein (GFP; clones 7.1 and 13.1, Roche Diagnostics) were used at a 1:1,000 dilution for immunoblotting.

Plasmids—An EPO-R mutant containing only the extracellular domain in pXCM (sEPO-R) was employed (21). The EGF-R mutant containing the extracellular and transmembrane domains (Ex EGF-R) in pCB6+ (22) was a generous gift from Dr. C. Carlin (Case Western Reserve University, Cleveland, OH). Plasmids expressing the transmembrane domain of galactosyltransferase (GalT) fused to a cyan fluorescent protein (GaT-CFP) and a temperature-sensitive (ts) VSVG mutant fused to a yellow fluorescent protein (VSVGts045-YFP) (23) were employed.

Cell Culture and Transfection—COS 7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum. Cells were cultured to 60% confluence and transiently transfected using the DEAE-dextran/chloroquine method (24) with 5 µg of plasmid containing the appropriate cDNA.

VSVG expression—COS 7 cells transiently transfected with sEPO-R cDNA were cultured in 24-well plates. Confluent cell cultures (2.5 × 10⁵ cells) were incubated at 37 °C for 4 h in 200 µl of DMEM supplemented with 0.2% fetal calf serum in the presence or absence of added compound. Aliquots of media (40 µl) and cell lysates (8 × 10⁵ cells) were subjected to Western blot analysis with anti-EPO-R antibodies.

EGF-R Metabolism Assay—COS 7 cells were cultured in 24-well plates and incubated at 37 °C for 18 h in DMEM supplemented with 10% fetal calf serum in the presence or absence of added compound.

EGF-R content in the cell lysate was assessed by Western blot analysis.

Membrane Labeling and Immunoprecipitation—COS 7 cells transiently transfected with Ex EGF-R cDNA (2 × 10⁵ cells each for time point) were labeled with [³⁵S]methionine and chased as described previously (20). Cell solubilization and immunoprecipitation were performed as described previously (20, 25). Compounds were added to the cells during the period of methionine and cysteine starvation and were also present during the pulse-labeling and chase incubations.

Endoglycosidase H (Endo H) Digestion—Digestion with Endo H (Endo H, New England Biolabs, Inc.) following immunoprecipitation was performed as described previously (20, 25). For Endo H digestion of cell extracts, 10⁵ cells were lysed in 30 µl of phosphate-buffered saline containing 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, and 5 mM EDTA supplemented with protease inhibitors (Complete protease inhibitors, Roche Diagnostics) and denatured in 0.5% (w/v) SDS for 5 min at 100 °C prior to the addition of 3 µl of 0.5 M sodium citrate and incubation with or without 500 units of Endo H for 1 h at 37 °C. Samples were then resolved on 7.5% SDS-PAGE, prior to Western blot analysis.

N-Glycosidase F (PNGase F) Digestion—Cell lysis was performed in phosphate-buffered saline containing 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, and 5 mM EDTA supplemented with protease inhibitors (Complete protease inhibitors, Roche Diagnostics). Cell lysates obtained from 10⁶ cells were denatured with 0.5% (w/v) SDS at 100 °C for 5 min. Subsequently, samples were incubated with or without 0.6 units of PNGase F (Roche Diagnostics) for 2 h at 37 °C, prior to Western blot analysis.

VSVG trafficking—COS 7 cells co-expressing VSVGts045-YFP and GaT-CFP, cultured on glass coverslips, were incubated for 24 h at 40 °C. Compounds (1 µg/ml) were added upon temperature shift to 32 °C for 2 h. Slides were fixed in 3% (w/v) paraformaldehyde immediately before the temperature shift and 2 h after it. Images were captured using the Zeiss LSM PASCAL laser-scanning confocal microscope with 458- and 514-nm laser lines. For biochemical analysis, cells were lysed and subjected to Endo H digestion prior to Western blot analysis with anti-GFP antibodies.

Purification of GA Crude Extracts—Ethyl-acetate extracts of S. hygroscopicus var. geldanatus were chromatographed on a methanol-washed silica-gel column, and six fractions were collected. GA was eluted with ethyl acetate alone, whereas the fractions that inhibited intracellular trafficking were slightly more polar and were eluted with ethyl acetate containing 5–10% methanol.

RESULTS

Secretion of sEPO-R Is Inhibited by Certain GA Preparations—To evaluate the inhibition of intracellular transport by GA, we utilized COS 7 cells transfected with a truncated EPO-R, designated sEPO-R, that lacks the cytosolic and transmembrane domains. As degradation of sEPO-R is not enhanced in the presence of GA (17), it was used to assess the effect of the compounds on constitutive secretion. Two sources of GA were compared with respect to their ability to inhibit sEPO-R secretion. Following a 4-h incubation period with the compounds (at 1 µg/ml each), aliquots of media from sEPO-R-expressing cells were subjected to Western blot analysis. Whereas GA obtained from Invitrogen, designated GA(Invitrogen) throughout the text and GA(Gibco) in Figs. 1, 4, and 5, blocked secretion of sEPO-R, GA obtained from NCI, National Institutes of Health, designated GA(NCI), did not affect this process (Fig. 1A). It should be noted that even 10 µg/ml GA(NCI) did not inhibit sEPO-R secretion (data not shown). Inhibition of sEPO-R secretion did not result in a measurable increase in intracellular sEPO-R (Fig. 1B), as only a small fraction of the sEPO-R molecules are secreted (21). The different activities of these GA preparations raised the possibility that GA(Invitrogen) contains an additional co-purified component(s) that inhibits sEPO-R secretion. Theoretically, this difference may also result from a variation in chemical structure. However, this possibility is less likely, as NMR analyses of the two GA preparations were similar and did not reveal co-purified compounds (data not shown). As GA content in both preparations exceeded 95%, the putative co-purified agent should represent <5% of GA(Invitrogen).

Degradation Is Mediated by GA, whereas Inhibition of sEPO-R Secretion Is via a Co-purified Component(s)—To determine whether inhibition of sEPO-R secretion was mediated by an activity that co-purified alongside GA, we examined whether GA and the inhibition of intracellular trafficking could be chemically separated. We chromatographed ethyl-acetate extracts of S. hygroscopicus var. geldanatus broth on a silica-gel column. Purification steps were monitored by NMR analysis as well as by assessing the capacity to promote EGF-R degradation and inhibit sEPO-R secretion. The results, depicted in Fig. 2, show that GA-mediated degradation of EGF-R and inhibition of sEPO-R secretion are conferred by distinct entities. Although there was a degree of overlap between the fractions that inhibited sEPO-R secretion (Fig. 2, fractions 4 and 5) and those that promoted EGF-R degradation (Fig. 2, fractions 3 and 4), fractions 3 and 5 (Fig. 2) were each active only in the degradation of EGF-R or the inhibition of sEPO-R secretion, respectively. NMR analysis revealed that fraction 3 (Fig. 2) contained mainly GA. Fraction 4 (Fig. 2) contained a mixture of GA and...
with sEPO-R cDNA were incubated with 1 μg/ml H9262 calf serum in the presence of 10% fetal calf serum in the presence of 1 μg/ml each eluted fraction and further processed as described in the Fig. 1 legend. Top panel, COS 7 cells were incubated for 18 h in DMEM supplemented with 10% fetal calf serum. The residual EGF-R detected in cells treated with fraction 4 (Fig. 2A) also displayed faster mobility in the gel. This fraction contained GA and 17DMG and was active in both promoting EGF-R degradation and inhibiting sEPO-R secretion. 17DMG Fraction (Fig. 2A, fraction 5) inhibited sEPO-R secretion, implying that it impedes intracellular transport. We thus questioned whether the inhibitory effects of the 17DMG fraction on intracellular transport are manifested in modified sugar processing of the EGF-R, thus culminating in its accelerated mobility in SDS-PAGE. To address this issue, cells were treated for 18 h in the presence of the 17DMG fraction or synthetic 17DMG. Fig. 3B demonstrates that the 17DMG fraction (Fig. 3B, lane 2, arrowhead) but not synthetic 17DMG (Fig. 3B, lane 3) slightly increased the mobility of EGF-R. PNGase F digestion of the cell lysates abolished the above-mentioned mobility shift of EGF-R (Fig. 3B, lanes 4–6), indicating that this shift can be attributed to aberrant sugar processing of EGF-R.

**GA(Invitrogen) but Not GA(NCI) Inhibits Maturation of Ex EGF-R**—We showed previously that, in addition to accelerating its degradation, GA inhibits maturation and transport of EGF-R (17). Because GA(Invitrogen) was used in those experiments, here we compared the two GA preparations as well as the 17DMG fraction and synthetic 17DMG with respect to their ability to inhibit maturation of an EGF-R mutant that lacks the cytosolic domain, designated Ex EGF-R. To enable a comparison of EGF-R and Ex EGF-R in the same cell, we transiently expressed Ex EGF-R in COS 7 cells that also express endogenous EGF-R; 48 h after transfection, the cells were metabolically labeled in medium containing [35S]cysteine-methionine and chased for 4 and 6 h (Fig. 4). In control, non-treated cells, or cells treated with either the 17DMG fraction or synthetic...
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17DMG, newly synthesized EGF-R was stable throughout the chase and acquired resistance to Endo H (Fig. 4, A, D, and E, respectively), whereas in the presence of either GA(Invitrogen) or GA(NCI) it was rapidly degraded (Fig. 4, B and C, respectively). Newly synthesized Ex EGF-R was metabolically stable under all the treatments, because GA does not promote Ex EGF-R degradation (17). In GA(NCI)-treated and synthetic 17DMG-treated cells, as well as in control cells, two forms of Ex EGF-R were discerned after 4 and 6 h of chase (Fig. 4, A, D, and E; arrow). In contrast, in GA(Invitrogen)-treated cells as well as in cells treated with the 17DMG fraction, the slower migrating band of Ex EGF-R was absent (Fig. 4, B and D). Endo H-resistant forms were barely detected, and partially processed Ex EGF-R forms were observed as a smear (Fig. 4, B and D). Thus, although GA(Invitrogen) and GA(NCI) both promoted degradation of EGF-R, only GA(Invitrogen) inhibited Ex EGF-R maturation. The fact that both the 17DMG-enriched fraction and GA(Invitrogen) inhibited Ex EGF-R maturation suggests that the same co-purified agent is present in both preparations. In contrast to the effect of the 17DMG fraction on Ex EGF-R processing, EGF-R acquired Endo H resistance (Fig. 4). Despite the apparent Endo H-resistant profile of EGF-R, the PNGase F-sensitive mobility shift of EGF-R from the 17DMG fraction-treated cells indicates that the 17DMG fraction affects EGF-R N-glycan processing (Fig. 3B).

**The 17DMG Fraction Inhibits VSVG Trafficking**—To study the effect of the 17DMG fraction on trafficking of VSVG along the secretory pathway, we employed a temperature-sensitive mutant of VSVG, VSVGtsO45, which is misfolded and accumulates in the endoplasmic reticulum when incubated at 40 °C. Following a temperature shift to 32 °C, it exits the endoplasmic reticulum and transports via the Golgi complex to the plasma membrane (26). COS 7 cells were transiently transfected with-VSVGtsO45-YFP, and maturation of VSVG was assessed using Endo H digestion. While VSVG from cells at 40 °C was completely Endo H-sensitive (Fig. 5A, lane 2), 2 h after the temperature shift was shifted to 32 °C Endo H-resistant forms of VSVG could be detected (Fig. 5A, lane 3; empty arrowhead). When the 17DMG fraction or GA(Invitrogen) was added at the time of temperature shift, VSVG maturation was blocked, the corresponding Endo H-resistant forms could not be seen, and Endo H-sensitive VSVG appeared as a thicker band (Fig. 5A, lanes 4 and 5, respectively). Incubation with GA(NCI) or synthetic 17DMG (Fig. 5A, lanes 6 and 7, respectively) did not interfere with VSVG maturation; Endo H susceptibility of VSVG was similar to that of VSVG from control non-treated cells. To identify where in the cell VSVG is accumulated, COS 7 cells were transiently transfected with VSVGtsO45-YFP as well as with GalT-CFP as a Golgi marker. Two hours after the temperature shift, VSVGtsO45-YFP could be identified on the plasma membrane in control non-treated cells. However, when the 17DMG fraction was added to the cells at the time of the temperature shift, VSVGtsO45-YFP was not detected at the plasma membrane, and it accumulated in the Golgi apparatus instead (Fig. 5B). Accumulation of VSVGtsO45-YFP in the Golgi apparatus was also evident in GA(Invitrogen)-treated cells but not in those treated with GA(NCI) or synthetic 17DMG (data not shown).

**DISCUSSION**

Here we demonstrate that the activities of GA on intracellular transport and protein maturation are related to its source of supply. Chromatography of *S. hygroscopicus* var. *geldanus* broth enabled us to separate the effects on intracellular trafficking from GA-mediated degradation. Inhibition of intracellular trafficking was evident in fractions slightly more polar than GA that contained 17DMG. To our knowledge, this is the first demonstration of naturally occurring 17DMG. Methylation of 17DMG may generate GA, or, alternatively, both GA and 17DMG may originate from another distinct precursor. The inability of 17DMG to promote EGF-R degradation (Fig. 4) is consistent with its inability to mediate p185<sup>16–18</sup> degradation (27) and allowed us to discriminate between the GA-mediated degradation and the BA-associated inhibition of intracellular trafficking. The inactivity of GA(NCI) as well as that of GA(Invitrogen) and GA(NCI) both promoted
resenting cleavage of some but not all N-glycan residues. The same explanation could be valid for the thicker Endo H-sensitive VSVG band observed in cells treated with the 17DMG fraction and GA(Invitrogen). The effect of these compounds on N-glycan processing remains to be elucidated. Accumulation of VSVGtsO45 in the Golgi in the presence of the 17DMG fraction (Fig. 5) and the presence of GA(Invitrogen) (data not shown) indicates that folding and assembly of VSVG are not affected and that the block in transport occurs in an early Golgi compartment.

The fact that GA(Invitrogen) and the 17DMG fraction-inhibited transport of EGF-R, sEPO-R, and VSVG suggests a general effect on the protein transport machinery. GA(Invitrogen) however, does not inhibit transport of all proteins (5, 17). Assuming that most proteins are delivered to the cell surface via a common pathway, the differential effect of GA(Invitrogen) on protein transport suggests that it alters specific sorting processes. Thus, analysis of intracellular trafficking of different cargo molecules (membrane-bound and soluble) in the presence of this drug may provide means for further dissecting these processes.

In conclusion, we show that GA preparation obtained from two different sources, namely Invitrogen, whose product has been previously used by us (17) and others, e.g. (5, 19), and the Natural Products Branch of the NCI, National Institutes of Health, differ with respect to their ability to inhibit maturation of Ex EGF-R, secretion of sEPO-R, and trafficking and maturation of VSVG. Although both GA preparations mediated EGF-R degradation, only GA(Invitrogen) inhibited intracellular trafficking.

The inhibitory effects of GA(Invitrogen) and of the 17DMG fraction render them powerful tools for elucidating transport mechanisms within the Golgi apparatus and from the Golgi to other organelles. Identification of the active component in su-

Fig. 5. Intracellular transport of VSVG protein is blocked by the 17DMG fraction. A, COS 7 cells expressing VSVGtsO45-YFP were incubated at 40 °C for 24 h. Compounds (1 μg/ml) were added upon shift of the temperature to 32 °C and allowed to incubate for 2 h prior to cell lysis, Endo H digestion, and Western blot analysis with anti-GFP antibodies. Empty arrowheads indicate the Endo H-resistant forms. B, COS 7 cells co-expressing VSVGtsO45-YFP and GalT-CFP were incubated at 40 °C for 24 h. The 17DMG fraction was added for an additional 2 h upon temperature shift to 32 °C, followed by fixation in 3% paraformaldehyde. Bar, 10 μm.
pertinants of S. hygroscopicus var. geldanus and in the 17DMG fraction is currently underway. Finally, the results described herein shed light on the inconsistencies in the literature with respect to the effects of GA on intracellular trafficking. Previous publications concerning BAs should hence be reviewed and the possible involvement of other compounds considered.

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