

Intra-Golgi Transport Inhibition by Megalomicin*

(Received for publication, June 29, 1995, and in revised form, November 1, 1995)

Pedro Bonay‡, Sean Munro§, Manuel Fresno‡, and Balbino Alarcón‡¶

From the ‡Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Madrid 28049, Spain and the §MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom

Megalomicin (MGM) is a macrolide antibiotic which has been demonstrated previously to cause an anomalous glycosylation of viral proteins. Here we show that MGM produces profound alterations on Golgi morphology and function. The addition of MGM at 50 μ M for 1 h caused a dilation of the Golgi detected by immunofluorescence staining for medial- and trans-Golgi markers. The effect of MGM was clearly more intense on the trans-side of the Golgi, as evidenced in electron microscope preparations. The effect on Golgi morphology was reversible and correlated with an impairment of glycoprotein processing in the trans-Golgi. Thus, although the vesicular stomatitis virus G protein was processed in the presence of MGM to an endoglycosidase H-resistant form, it was poorly sialylated. The sialylation of cellular proteins was also inhibited, resulting in cells with low level of sialylation on the cell surface. However MGM did not inhibit the activities of the galactosyl- or sialyltransferase as measured *in vitro*. MGM inhibited cis- to medial-, and more strongly, medial- to trans-Golgi transport of vesicular stomatitis virus G protein in an *in vitro* system, suggesting that the impairment in glycoprotein maturation observed *in vivo* is the result of intra-Golgi transport inhibition.

Proteins which enter the central vacuolar system are synthesized in the endoplasmic reticulum (ER)¹ where they acquire N-linked glycans which are then processed and matured as they are sequentially transported through the cis-, medial-, and trans-cisternae of the Golgi complex. From here they move to an array of tubulo-vesicular structures, the trans-Golgi network (TGN) from where sorting occurs to lysosomes, regulated secretory vesicles, and to the plasma membrane.

The use of specific inhibitors has been highly valuable for cellular biologists, allowing the study of intracellular transport processes in intact cells in a convenient way. Nocodazole and other agents that depolymerize microtubules have been widely used to study the role of the cytoskeleton in intracellular transport (Kelly, 1990). In the last years, brefeldin A (BFA) has

attracted the attention of many researchers for its effects on intracellular transport (Pelham, 1991). It was first documented that BFA inhibited the secretion of proteins at an early step. In the presence of BFA, secretory proteins were retained in the endoplasmic reticulum (Lippincott-Schwartz *et al.*, 1989, 1990, 1991). It was found that even Golgi enzyme markers were located in the ER after a few minutes of treatment with BFA (Misumi *et al.*, 1986; Fujiwara *et al.*, 1988; Doms *et al.*, 1989). In addition, no recognizable Golgi stacks were seen in BFA-treated cells. These data suggested that BFA promoted the redistribution of Golgi stacks into the ER. Markers of the TGN did not redistribute into the ER and were, however, found to fuse with endosomes. BFA did not affect the cycling between the plasma membrane and endosomes, although the traffic between endosomes and lysosomes was impaired (Lippincott-Schwartz *et al.*, 1991). BFA produced the tubulation of Golgi stacks, endosomes, and lysosomes, and it has been suggested that these tubules may target membrane fusions (Pelham, 1991). The redistribution of Golgi markers to the ER produced by BFA is microtubule-mediated, requires ATP, and is suppressed by the addition of nonhydrolyzable GTP analogs.

Other compounds that have been lately used as probes of intracellular processes are the macrolide antibiotics bafilomycin (Bowman *et al.*, 1988; Yoshimori *et al.*, 1991; Johnson *et al.*, 1993; Pakolungas *et al.*, 1994) and concanamycin (Yilla *et al.*, 1993). These antibiotics are highly selective and specific inhibitors of vacuolar proton ATPases (V-H⁺-ATPases) (Nelson and Taiz, 1989) identified in organelles belonging to the central vacuolar system such as lysosomes (Moriyama and Nelson, 1989a) and the Golgi complex (Moriyama and Nelson, 1989b), as well as in coated vesicles (Xie and Stone, 1986; Arai *et al.*, 1987). Those ATPases are likely responsible for the generation and maintenance of the acidity in those organelles. The acidic luminal environment has been suggested to be important to assure the fidelity of vesicular transport. In this sense, Yilla *et al.* (1993), showed that concanamycin B significantly impaired intra-Golgi trafficking and plasma membrane delivery in HepG2 cells without affecting the endoplasmic reticulum to Golgi transport. Bafilomycin A₁ (Yoshimori *et al.*, 1991) and concanamycin B (Woo *et al.*, 1992) have been found to inhibit *in vivo* lysosomal protein degradation through inhibition of the ATP-dependent acidification of endosomes and lysosomes, without an apparent inhibition of the intracellular protein transport, including the endocytic pathway. However other authors have shown an inhibition of protein transport in the endocytic pathway (Oda *et al.*, 1991; Pakolungas *et al.*, 1994; Johnson *et al.*, 1993). In addition, Xu and Shields (1994) provided evidence that prosomatostatin processing in the TGN is inhibited by bafilomycin A₁.

Two other types of compound have been added to the list of inhibitors of vesicular transport. The sponge metabolite ilimaquinone (IQ) caused the breakdown of the Golgi into small vesicles, allowing the transport of secretory vesicle proteins to

* This work was supported by grants from the Fondo de Investigaciones Sanitarias (94/0280), the Comunidad de Madrid (AE13/95), the European Union Biotech Program (BIOCT920164), and from the Fundación Ramón Areces. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Centro de Biología Molecular, Universidad Autónoma de Madrid, Cantoblanco, Madrid 28049, Spain. Tel.: 341-3978049; Fax: 341-3978344.

¹ The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; β -COP, coatamer protein β ; endo-H, endoglycosidase H; HSV, herpes simplex virus; IQ, ilimaquinone; MGM, megalomicin; NRK, normal rat kidney cells; VSV, vesicular stomatitis virus; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; BHK, baby hamster kidney.

the cis-Golgi, but not further (Takizawa *et al.*, 1993). Interestingly, IQ inhibited the association of β -COP to transport vesicles but, unlike BFA, did not produce the fusion of the Golgi into the ER. In addition, a product isolated from the culture broth of *Streptomyces* sp. originally described as an inhibitor of the intracellular transport of VSV G glycoprotein (Seog *et al.*, 1994), although there is no indication on the mechanism or site of action.

Here we describe that megalomicin (MGM), a macrolide antibiotic with wide antibacterial spectrum (Weinstein *et al.*, 1972) and antiviral activity (Alarcón *et al.*, 1988), produces profound morphological and functional effects on the Golgi complex of cultured cells, causing the inhibition of the last steps of glycoprotein processing in the Golgi without an apparent effect on the processing enzymes (glycosyltransferases) themselves, but more probably affecting the intra-Golgi transport.

EXPERIMENTAL PROCEDURES

Materials

Drugs—MGM was obtained from cultures of *Micromonospora megalomicea* (ATCC 27598) by a procedure described (Weinstein, 1972). Briefly, 50 ml of medium 172 were inoculated with the spores of the actinomycete and incubated at 27 °C with high aeration for 5 days and was then used as an inoculum for a 400-ml culture. After 3 days this culture was used to inoculate 4 liters of medium 172 in the same conditions. 5 days later, the pH of the cultures was raised to 9.5 with sodium hydroxide, and the culture was extracted with an equal volume of ethyl acetate. The ethyl acetate extract was evaporated to 0.01 of the initial volume and was then extracted twice with 0.14 N hydrochloric acid. The acid extract was brought to pH 9.5 and extracted twice with equal volumes of ethyl acetate. The ethyl acetate extracts were pooled and evaporated completely, dissolved in acetone, and precipitated by rapid addition to 1 liter of distilled water brought to pH 9.5 with sodium hydroxide. The precipitate was collected by filtration through Whatman paper.

Bafilomycin A₁ was a kind gift of Dr. L. Carrasco (Centro de Biología Molecular, Madrid, Spain) and BFA was from Epicentre Technologies (Madison, WI). Erythromycins A, B, and C were a generous gift of Dr. J. Corbalan, J. (Lilly).

Cells—COS-7, CHO 15B cells, and normal rat kidney cells (NRK), wild type and ricin-resistant mutant 17 (Ric 17), were grown in DMEM supplemented with 5% fetal bovine serum.

Plasmids—A vector encoding human sialyltransferase tagged with a c-Myc epitope (SSST) was constructed as described (Munro, 1991). Plasmid HE22M encoding human ERD2 tagged with the c-Myc epitope was generously given by Dr. H. Pelham (MRC, Cambridge).

Antibodies and Lectins—The mouse monoclonal antibody against the c-Myc epitope was kindly given by Dr. G. Evan (Evan *et al.*, 1985). The mouse monoclonal anti-cis-Golgi antibody MG-160 was kindly given by Dr. N. K. Gonatas (Mourelatos *et al.*, 1990). The mouse anti- β -COP monoclonal M3A5 was kindly given by Drs. V. Allen (MRC, Cambridge) and T. Kreis (EMBL, Heidelberg, Federal Republic of Germany). The rabbit antiserum anti-mannosidase II was obtained from Dr. K. W. Moremen (Moremen *et al.*, 1991). The anti-VSV G protein monoclonal antibody P5D4 was purchased from Sigma. Fluorescein and rhodamine-labeled goat antibodies specific for mouse and for rabbit immunoglobulins were purchased from Southern Biotechnology (Birmingham, AL).

Transfection

COS cells were transfected by the DEAE-dextran method as described (Alarcón *et al.*, 1991). Subconfluent Petri dishes were washed with 10 ml of PBS and covered with 2 ml of transfection medium: DME supplemented with 10% Nuserum (Collaborative Research, Bedford, MA) and 100 μ M chloroquine diphosphate (Sigma). Then 10 μ g of plasmid were added and gently mixed by swirling the plate. An aliquot of 0.5 ml of 2 mg/ml DEAE-dextran in transfection medium was added dropwise, and the plates were incubated for 4 h at 37 °C. Then the medium was removed, and the cells were trypsinized, counted, and plated in 24-well plates (Nunc, Roskilde, Denmark) containing round coverslips (Deckglasser, Mülheim, Germany).

Immunofluorescence and Immunoelectron Microscopy

Cell monolayers were washed twice with PBS, fixed for 20 min with 2% paraformaldehyde (Merck, Darmstadt, Germany) in PBS at room temperature, and permeabilized by incubation with 1% BSA (Sigma) and 0.1% saponin (Serva) in PBS for 1 h at room temperature. After this step the coverslips were incubated for 30–60 min upside down on a drop of antibody solution (2–5 μ g/ml) in PBS-BSA-saponin. The coverslips were extensively washed with PBS and incubated on a drop of the second fluoresceinated or rhodaminated antibody in PBS-BSA-saponin for 30 min. Finally, the coverslips were extensively washed with PBS and mounted on slides with a mowiol solution prepared as described (Heimer and Taylor, 1974). The preparations were examined in a Zeiss Axioskop microscope and photographed on Kodak TMAX 400 or Kodak Ektachrome P800/1600 film. Immunoelectron microscopy was carried out essentially as described (Bonay *et al.*, 1992).

Flow Cytometry

Fluorescein isothiocyanate labeling of *Limulus polyphemus* agglutinin, *Maackia amurensis* agglutinin, and *Sambucus nigra* agglutinin was carried out as described (Kaku *et al.*, 1993). The unlabeled lectins were obtained from Sigma. The molar ratio of fluorescein isothiocyanate to protein was 0.8, 1.2, and 1.1, respectively. Cells (control and MGM-treated) detached off the plate with 50 mM EDTA were stained with the fluorescein isothiocyanate-labeled lectins at 4 °C during 1 h, washed three times with PBS/BSA, and fixed with 2% paraformaldehyde. The stained cells were analyzed on a Coulter Profile Cytofluorimeter.

Glycan Analysis

Cells were metabolically labeled with [³H]mannose (50 μ Ci/ml, Amersham, Amersham, Ibérica, Madrid, Spain) in glucose-free DMEM, 5% dialyzed fetal calf serum for 16 h. The preparation of the glycopeptides and analysis by lectin affinity chromatography on concanavalin A-Sepharose and lentil lectin-Sepharose was carried out as described previously (Monis *et al.*, 1987; Bonay and Hughes, 1991). Analysis of anionic oligosaccharides was carried out essentially as described (Varki and Kornfeld, 1983).

Analysis of Anionic (Sialylated) Oligosaccharides of VSV Protein G

NRK cells were infected with VSV and 4 h after infection were metabolically labeled with [³H]mannose (10 μ Ci/ml in low glucose DMEM) for 4 h. The medium was removed, the cells were harvested in 1 ml of PBS, lysed in lysis buffer containing 1% Nonidet P-40, and immunoprecipitation with antibody P5D4 was performed as described previously. The immunoprecipitates were resuspended in 75 μ l of citrate-phosphate buffer, pH 5.5, containing 0.1% SDS and then heated for 5 min at 100 °C. After cooling, samples were treated with 2 milliunits of endo-H (Oxford Glycosystems, Oxford) and 5 milliunits of N-glycanase (Oxford Glycosystems) at 37 °C for 20 h. The digest was boiled for 5 min and diluted to 500 μ l with 2 mM Tris-HCl, pH 8.2. The number of negative charges on the released oligosaccharides was determined by chromatography on a 1-ml Q-Sepharose FF column (Pharmacia Biotech Inc., Uppsala, Sweden) equilibrated in 2 mM Tris-HCl, pH 8.2. The bound material was eluted with increasing concentrations of NaCl in the same buffer. Under these conditions, oligosaccharides with one, two, and three net negative charges elute at 20 mM, 75 mM, and 120 mM NaCl, respectively. Fractions of 0.75 ml were collected and the radioactivity monitored. To exclude the putative presence of phosphate residues that could be contributing with negative charges, a control sample was treated with alkaline phosphatase prior to Q-Sepharose chromatography. The results obtained indicated that phosphate residues were not mediating binding to the Q-Sepharose column.

Glycosyltransferase Assays

The assays for the Golgi enzyme UDP-galactose:glycoprotein galactosyltransferase and CMP-sialic acid:glycoprotein sialyltransferase activities were performed as described (Vischer and Hughes, 1981).

Intra-Golgi Transport Assay

The *in vitro* cis- to medial-Golgi transport assay was carried out essentially as described by Rothman (Balch *et al.*, 1984; Beckers and Rothman, 1992) using donor Golgi membranes from VSV-infected CHO-15B cells (defective in N-acetylglucosaminyltransferase I) and acceptor membranes obtained from uninfected CHO wild type cells. For the medial- to trans-Golgi transport the method described by Rothman (1987) was employed with slight modifications. Briefly, addition of

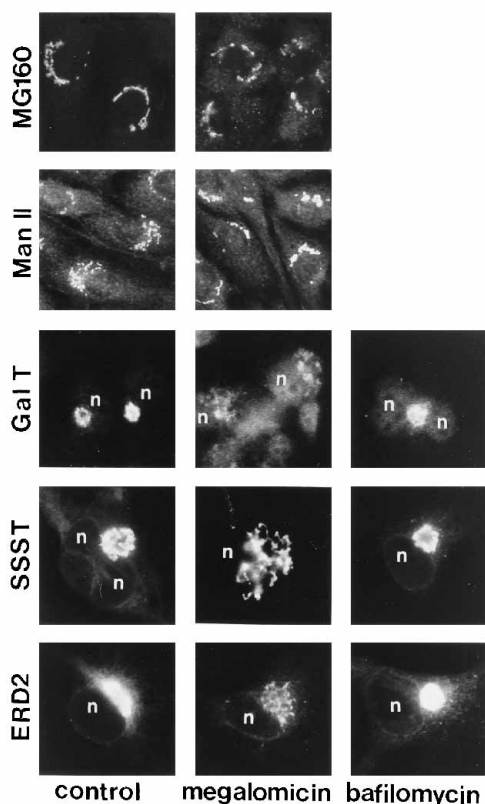


FIG. 1. Distribution of Golgi markers in COS and NRK cells upon MGM treatment. Cells were treated with 50 μ M MGM or 1 μ M bafilomycin for 2 h and stained with the cis-Golgi marker MG-160 (MG-160), with an anti-mannosidase II antibody (*Man II*) as a medial-Golgi marker and with antibodies to the trans-Golgi markers galactosyl-transferase (*GalT*) and sialyltransferase (*SSST*). *ERD2* is a marker distributed along the ER, intermediate compartment, and Golgi stacks. NRK cells were used for MG-160 and *Man II* stainings and COS cells for *GalT* staining. COS cells transfected with the corresponding plasmids were used for the *SSST* and *ERD2* markers. All photographs were taken at $\times 630$.

[3 H]galactose to VSV G protein in a transport-dependent manner was monitored instead of G protein sialylation. Donor Golgi membranes were obtained from VSV-infected BHK ricin-resistant mutant 17 (defective in galactosyltransferase (32) and UDP-[3 H]galactose substituted for CMP-[3 H]sialic acid in the transport reaction.

Golgi ATPase Assay

Proton translocation by V-type ATPases was monitored by the quinacrine's fluorescence quenching in a 1.5-ml reaction volume containing 15 μ M quinacrine, 1 mM MgSO_4 , 0.1 mM Na_3VO_4 , 100 mM KCl, 25 mM HEPES, pH 7.5, an enriched fraction of Golgi membranes, prepared as described (Balch *et al.*, 1984), containing 50 μ g/ml of protein and variable concentrations of megalomicin or bafilomycin. The reaction was initiated by the addition of 1 mM ATP (adjusted at pH 7.5). Nigericin (5 μ M) was added at some point to dissipate the pH gradient. Quinacrine fluorescence was measured with excitation and emission wavelengths of 420 and 496 nm, respectively.

RESULTS

Morphological Alterations of the Golgi Apparatus Induced by MGM—We have reported previously that MGM, a macrolide antibiotic, produces anomalous glycosylation of herpes simplex virus (HSV) proteins (Alarcón *et al.*, 1988). This led us to examine if this effect was due to drug-induced alterations of the Golgi apparatus, where maturation to complex carbohydrates takes place. Fig. 1 shows the immunofluorescence patterns of normal and MGM-treated NRK and COS cells stained for specific Golgi subcompartmental markers. The perinuclear, punctuated, and compact pattern, revealed in control NRK cells by

the cis- and medial-markers MG-160 (a membrane protein) and Golgi mannosidase II (a luminal Golgi protein), respectively (Fig. 1), was shifted in MGM-treated cells to a more loose and dilated structure that remained in the vicinity of the nucleus. The effect of MGM treatment on Golgi morphology was more evident when the trans-Golgi markers galactosyl- and sialyltransferase were tested. The compact appearance of the trans-Golgi in COS cells was changed upon treatment with MGM to a large vesicular, swollen structure that, sometimes, spread out through a large portion of the cytoplasm (Fig. 1). The Golgi morphology, as surveyed when ERD2, the KDEL receptor (Lewis and Pelham, 1992), was used as a marker, was also modified after drug treatment. No effect was, however, detected on the morphology of the ER upon MGM treatment (data not shown). These data, together with previously described data in which MGM was shown to affect protein glycosylation but not protein synthesis (Alarcón *et al.*, 1988), suggest that MGM specifically acts on the Golgi complex. The specific effect of MGM is further supported, because another macrolide antibiotic, bafilomycin A_1 , which inhibits vacuolar H^+ -ATPases (Bowman *et al.*, 1988), did not have any detectable effect on Golgi's morphology (Fig. 1). Moreover, erythromycins A, B, and C, which are structurally related to MGM, did not produce any alteration on the morphology of the Golgi apparatus (data not shown). The main structural difference between erythromycin and MGM is the presence in the latter of an uncommon sugar, D-rhodamine which seems, therefore, to participate in MGM activity.

Confocal laser scanning and electron microscopy were performed to evaluate the effect of MGM on Golgi morphology. Confocal microscopy of COS cell samples stained for the trans-Golgi marker sialyltransferase showed that upon incubation with MGM for periods as short as 1 h, the distribution of the marker was well spread and a dilation and engorging of the compartment became evident as noticed by the larger size of the stained vesicles extending through a reticular-like structure (Fig. 2, compare *a* with *b* and *c*). Fig. 2 also shows that the effect of MGM on the trans-Golgi is reversible, returning, although slowly, to normal morphology after a 1–3 h washout (*d* and *e*).

The immunofluorescence data presented above showed that MGM caused a more intense alteration on the trans-Golgi. This was confirmed by electron microscopy of NRK cells treated with MGM and stained with a Golgi mannosidase II-specific antibody by the immunoperoxidase method. As shown in Fig. 3, the stacks of flattened cisternae seen in the control sample (*left panel*) were dramatically altered in MGM-treated cells (*right panel*). In these cells, the Golgi apparatus appeared to be formed by stacks of normal size cisternae, mostly located on the cis-side of the Golgi, which were infiltrated by dilated cisternae that were specially abundant on the trans-side.

MGM Disrupts the Normal Processing of Carbohydrates in the Golgi Apparatus—As described above, MGM had dramatic effects on the morphology of the Golgi apparatus causing a dilation of trans-Golgi stacks. To determine whether MGM could be distorting the normal processing of glycoproteins, NRK cells were infected with VSV, and the processing of the viral G glycoprotein was followed by pulse-chase labeling with [35 S]methionine. As shown in Fig. 4, VSV G protein was processed to an endo-H-resistant form after a 30-min chase in control cells as well as in cells treated with 50 μ M MGM. Although MGM caused an expansion of the medial-Golgi in NRK cells (Figs. 1 and 2), the VSV G protein data indicate that, in the presence of MGM, glycoproteins could be almost normally transported to the medial-Golgi, but does not allow to conclude anything about further transport. Somewhat different

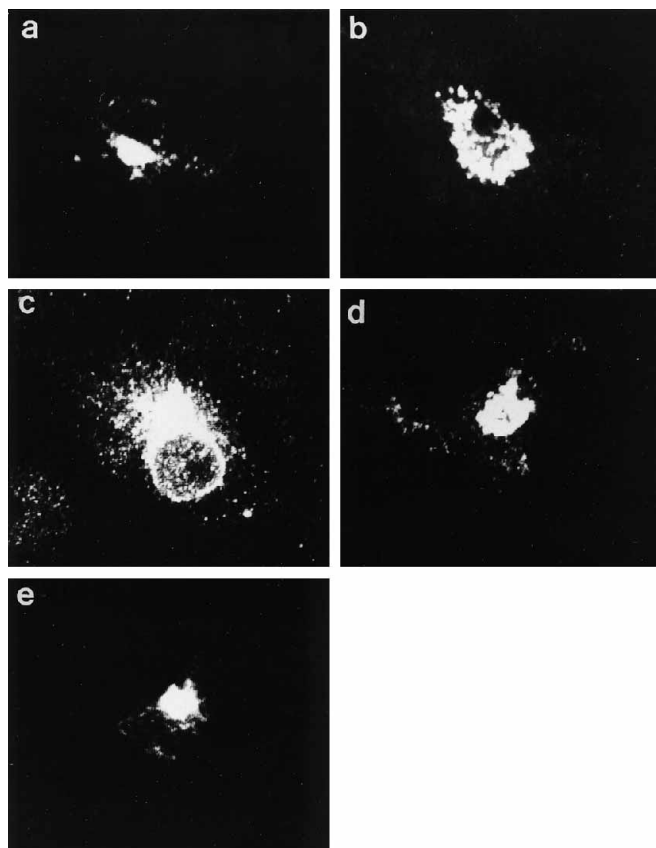


FIG. 2. **The effect of MGM on the trans-Golgi is reversible.** COS cells transfected with SSST were treated with 50 μ M MGM for 1 h (b), for 3 h (c), or left untreated (a). Alternatively, COS cells treated with 50 μ M MGM for 3 h were washed and incubated in the absence of MGM for 1 h (d) or 3 h (e). Photographs were taken in a confocal microscope.

results were obtained when total oligosaccharides from [3 H]mannose-labeled, uninfected NRK cells were analyzed by lectin chromatography. A preparation of total oligosaccharides was subjected to chromatography on a concanavalin A column to separate them according to their mannose content. As shown in Fig. 5A, MGM inhibited, to a certain degree, the processing of glycans to complex type in cell lysates (11% of the total pool of oligosaccharides in MGM-treated cells *versus* 23% in control cells, representing a decrease of about 50%). The inhibition in the conversion to the complex type was paralleled by an increase in the absolute amount of high mannose oligosaccharides (60% in nontreated cells *versus* 88% upon drug treatment). Interestingly, the fraction corresponding to hybrid-type oligosaccharides, although being a minor component in control cells (6% of the total distribution of oligosaccharides), was completely absent in lysates from MGM-treated cells. It is important to highlight that MGM incubation only had a marginal effect on the total incorporation of [3 H]mannose, [3 H]galactose, and [3 H]*N*-acetylglucosamine (8, 13, and 11% inhibition, respectively).

Concomitantly to the inhibition in the formation of complex type oligosaccharides, MGM caused a lower secretion of glycoproteins containing this type of sugars (Fig. 5B). In this figure, it can be clearly seen that the composition of the secreted glycoproteins from MGM-treated cells was richer in the forms with a higher content in mannose (hybrid and mannose-rich). Interestingly, the addition of MGM resulted in the secretion of proteins containing high mannose oligosaccharides, which were not detected in culture supernatants from untreated cells.

The apparent discrepancy between the endo-H sensitivity data of VSV G protein and the concanavalin A chromatography

data of total NRK glycoprotein glycans could be due to different times of exposure to the drug or to the different sensitivity of the labeling techniques. Furthermore, VSV G protein could be converted to an endo-H-resistant form in the medial-Golgi, but still be defectively matured in the presence of MGM as their transport to the trans-cisternae of the Golgi is impaired.

The addition of sialic acid is the last step in the maturation of glycoproteins taking place in the trans-Golgi. To determine how MGM affected this process, oligosaccharide preparations of MGM-treated VSV-infected NRK cells were subjected to chromatography on Q-Sepharose. This system allows to distinguish between oligosaccharides that have incorporated different number of sialic acid residues. As shown in Fig. 5C, the fractions corresponding to oligosaccharides containing a low number of sialic acids (0–1) were higher in MGM-treated cells than in control samples, and conversely, MGM inhibited the formation of oligosaccharides with a higher level of sialylation. These data suggest that MGM is acting preferentially on the trans-side of the Golgi or just before it.

The MGM effect on the sialylation of surface proteins was also evaluated by staining NRK cells with sialic acid-specific lectins, such as *M. amurensis*, *L. poliphemus*, and *S. nigra* agglutinins. After an overnight treatment with MGM, the lectin staining of the cell surface was reduced to a 13–24% of the staining in control cells (Fig. 6), further supporting the idea that MGM reduces the sialylation of glycoproteins.

MGM Inhibits Intra-Golgi Transport—Up to this point, the presented evidence indicated that the morphological alterations induced by MGM were paralleled by structural changes in the glycan moiety of the newly synthesized glycoproteins, particularly, by a decreased sialylation. These alterations are consistent with an impairment in the transport of glycoproteins from the medial-Golgi to the trans-Golgi. The results are also consistent with an inhibition of the trans-Golgi located glycosyltransferases. However, MGM did not inhibit the activities of the enzymes galactosyl- and sialyltransferases in *in vitro* assays (Table I). In order to gain further insight into the mechanism of action of MGM, *in vitro* intra-Golgi transport assays of protein G in VSV infected cells (cis- to medial-Golgi and medial- to trans-Golgi) were carried out. The specificities of the transport systems were assessed by the dependence on the presence of ATP and added cytosol and by the sensitivity to *N*-ethylmaleimide (NEM) (Fig. 7). The results showed that while MGM inhibited the transport of VSV G protein on both systems, the inhibition was stronger on the medial to trans-Golgi transport assay than in the cis- to medial-Golgi assay (50 *versus* 25% inhibition at 5 μ M). Because MGM did not have a direct effect on the activity of the galactosyl- or sialyltransferase in an *in vitro* assay (Table I), we can exclude the possibility that the reduced transport observed were the result of lower enzymatic activity.

Effect of MGM on Golgi Acidification—Although MGM did not have a direct inhibitory effect on galactosyl and sialyltransferases activity in an *in vitro* assay, it is possible that MGM could inhibit them indirectly by altering the milieu optimal for these enzymes via the disruption of the pH gradient in the trans-Golgi stacks. To test this hypothesis, the effect of MGM on the H^+ pumping activity of vacuolar ATPases was assayed in Golgi-enriched membrane preparations. As shown in Fig. 8, MGM had only a marginal effect (23%) on vesicular H^+ pumping activity at the active concentration of 50 μ M. By contrast, bafilomycin A_1 , which has been shown to be an inhibitor of vacuolar H^+ -ATPases (Bowman *et al.*, 1988; Crider *et al.*, 1994; Xu and Shields, 1994; Zhang *et al.*, 1994), completely inhibited the acidification of Golgi-enriched vesicles at a concentration of 5 nM. The results shown here suggest that the inhibitory effects

FIG. 3. **Electron microscopy structure of the Golgi apparatus in MGM-treated NRK cells.** NRK cells were stained with the antibody to mannosidase II by the immunoperoxidase method and contrasted with uranyl and lead salts. In control cells (*left panel*), the Golgi is seen as a stack of flattened cisternae (*arrow-head*) whereas in MGM-treated cells (*right panel*), the Golgi is seen as a series of swollen cisternae (*arrow*) intercalated among flattened cisternae (*arrowheads*). Both pictures were taken at $\times 30,000$. N indicates the position of the nucleus.

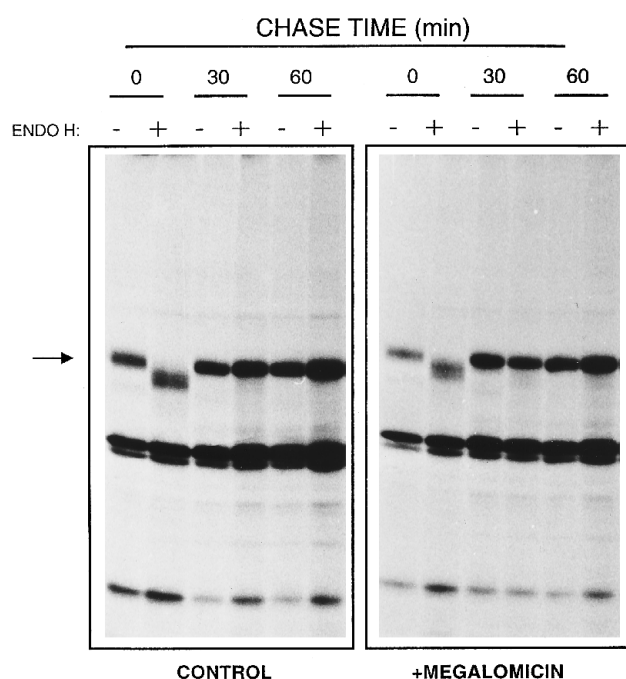
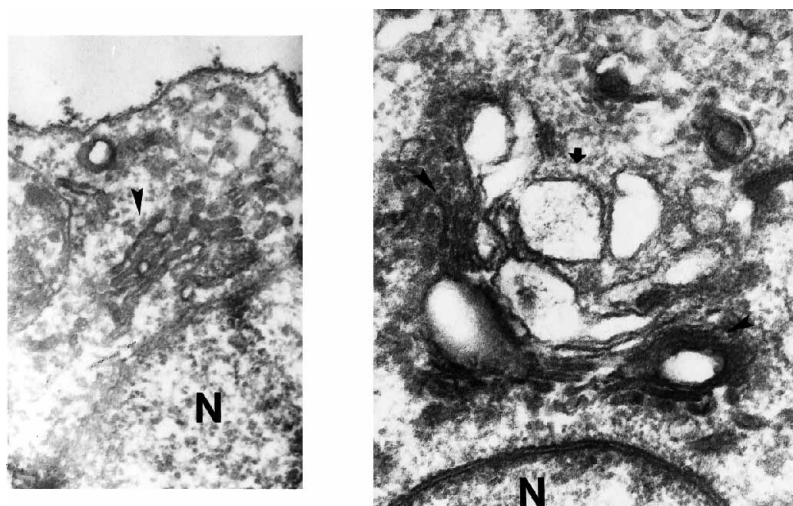


FIG. 4. **Acquisition of endo-H resistance of VSV G protein in MGM-treated cells.** NRK cells were infected with VSV at a multiplicity of infection of 20 and labeled 4 h later with [35 S]methionine for 10 min. After pulse labeling, cells were chased for the times indicated. MGM (50 μ M) was added from time 0 of infection. An acetone precipitate of each sample was obtained and treated (+) with endo-H or left untreated (-). Samples were run by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel. The position of VSV G glycoprotein is indicated with an arrow.

of MGM detected as a blockade in the final steps of glycoprotein processing are due to a direct inhibition of intra-Golgi transport and not indirectly to an inhibition of the Golgi enzymes or proton gradient-forming ATPases.

DISCUSSION

The results presented in this paper show that MGM causes profound effects on the Golgi apparatus morphology and function. Standard fluorescent and confocal laser scanning microscopy showed that MGM produced a general dilation of the Golgi cisternae, although the effect was most dramatic on the trans-Golgi. Electron micrographs showed the presence of swollen cisternae of the Golgi mostly located on the trans-side, coexisting with cis-located cisternae of normal appearance. The morphological alteration of MGM on the Golgi was reflected in an

impaired function, producing an anomalous glycosylation of VSV G protein and cellular glycoproteins. Interestingly, the MGM effects on glycosylation correlated well with its morphological effects, causing a higher inhibition of the later processes of glycan maturation, which take place on the trans-side of the Golgi. Thus, MGM caused an inhibition in the formation of complex-type oligosaccharides paralleled to an increase of the high mannose type. In addition, after shorter incubations, MGM had no detectable effect on the maturation of VSV G protein to an endo-H-resistant form, a process that is dependent on the activity of the medial-Golgi located enzyme mannosidase II, indicating that MGM does not inhibit, to a substantial degree, the access to the medial-Golgi compartment. Furthermore, in the presence of MGM, VSV G protein was undersialylated. A possible explanation for these results could be that MGM inhibits the activities of either sialyltransferase or the previous acting galactosyltransferase that would generate the substrate for the sialyltransferase. However, MGM did not inhibit the activities of galactosyl- and sialyltransferases *in vitro* (Table I). An alternative non-excluding explanation would be that the swelling induced by the drug could result in a decreased active luminal concentration of the sugar nucleosides and hence reduced modification rates. MGM could directly inhibit sugar nucleotide transporters which would account for some of the MGM effects described. Such idea would imply that MGM preferentially inhibits the UDP-Gal and/or CMP-NeuAc transporters but not the GDP-Man or the UDP-GlcNAc transporters. However, as far as the literature surveyed (for review, see Hirschberg and Snider (1987) and Milla *et al.* (1989)), all the sugar nucleoside transporters described so far share the same biochemical properties, and there is no structural homology between MGM to any sugar nucleotide to act as a selective inhibitor. It is clear that direct testing of MGM in a sugar nucleotide transport assay will answer that point. However, we have tested CHO mutant cell lines lacking UDP-Gal (CHO Lec 2, Deutscher *et al.*, 1984) or CMP-NeuAc (CHO Lec 8, Deutscher and Hirschberg, 1986) transporters for their MGM sensitivity. Both cell lines behave like the parental cell line in the sense that MGM induces the cisternal swelling to the same extent.² Finally, MGM could be inhibiting the access of the proteins to the compartments where the glycosyltransferases are located. This possibility is strongly supported by the effect of MGM in an established *in vitro* intra-Golgi transport assay. Although in the *in vitro* assay, MGM inhibited cis- to medial- and medial- to trans-Golgi transport of VSV G

² P. Bonay and B. Alarcón, unpublished results.

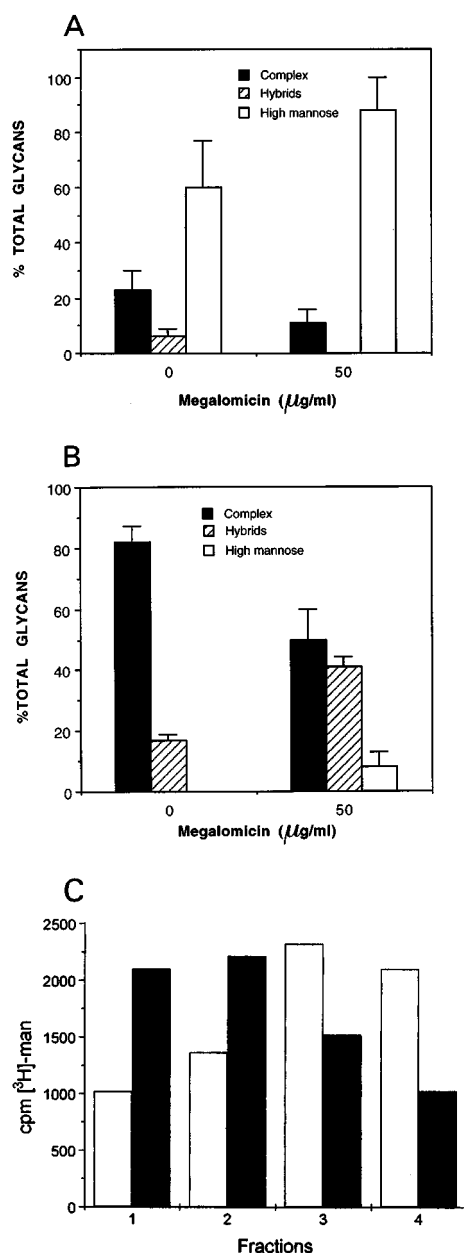


FIG. 5. Analysis of glycans synthesized in the presence of MGM. A and B, oligosaccharides were isolated from COS cells that had been labeled overnight with [³H]mannose in the presence of 50 μM MGM or mock-treated. The oligosaccharides from the cell lysates (A) or from the culture supernatants (B) were fractionated by concanavalin A-Sepharose chromatography. Complex type corresponded to the unbound material. Hybrid type make the major fraction eluted with 10 mM α-methylglucoside, and the high mannose fraction was eluted with 500 mM α-methylmannoside. C, VSV-infected NRK cells were labeled for 4 h with [³H]mannose in the presence (closed bars) or absence (open bars) of 50 μM MGM. Cell lysates were immunoprecipitated with VSV G protein antibody P5D4, and oligosaccharides on the VSV G protein were fractionated on a Q-Sepharose column. Fraction 1 was the unbound material and corresponded to neutral oligosaccharides. Fractions 2, 3, and 4 were obtained by elution with increasing concentrations of NaCl (20, 75, and 120 mM) and corresponded to monosialylated, disialylated, and trisialylated oligosaccharides, respectively.

protein, only this last effect was correlated with the effect of MGM *in vivo*. This is easily explained, considering that the maturation of glycans is a sequential process relying on the sequential action of compartmentalized enzymes, and therefore, the effects of transport inhibition accumulate through successive compartments.

The inhibition of glycoprotein sialylation was in agreement

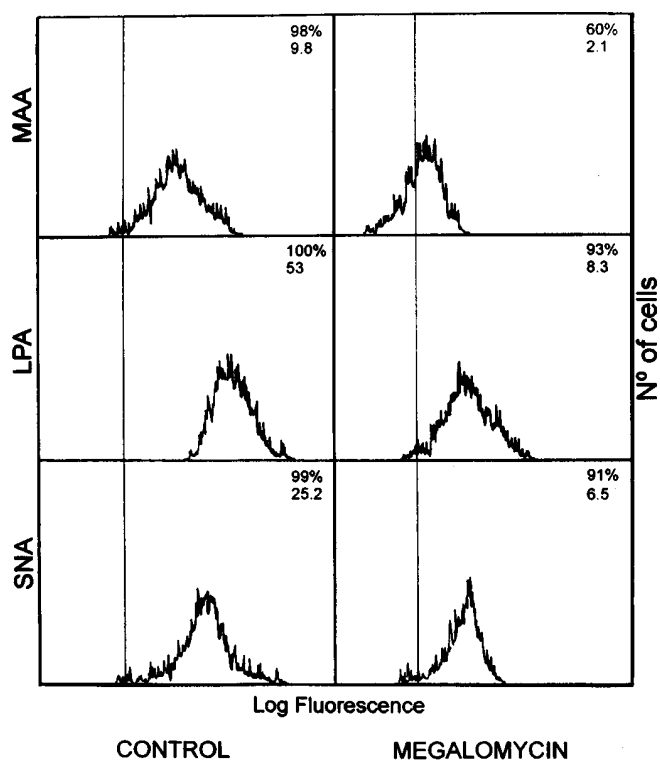


FIG. 6. Inhibition of glycoprotein sialylation in MGM-treated cells. Flow cytometry analysis of NRK cells stained with fluoresceinated lectins *M. amurensis* agglutinin (MAA), *S. nigra* agglutinin (SNA) and *L. polyphemus* agglutinin (LPA). The bar represents the point of maximum fluorescence for 98% of the cell population in unstained samples used as controls. The numbers in each quadrant represent the percentage of fluorescence positive cells (numbers on top) and the mean fluorescence intensity (numbers at bottom).

TABLE I
Effect of MGM on the *in vitro* activities of galactosyl- and sialyltransferases

	Activity	
	GalT	ST
	nmol/mg/h	
Homogenate		
Control	6.21 ± 0.45	0.98 ± 0.11
50 μM MGM	5.99 ± 0.64	1.02 ± 0.15
Golgi-enriched fraction		
Control	111.4 ± 9.78	14.7 ± 1.34
50 μM MGM	118.9 ± 12.74	13.2 ± 2.09

with previous data showing that MGM inhibited the addition of galactose to HSV glycoproteins (Alarcón *et al.*, 1988). The intra-Golgi transport inhibition shown in this paper could explain the activity of MGM as an antiviral agent. MGM inhibited normal glycosylation of HSV, but not the synthesis of viral proteins nor the formation of viral particles, which were, nevertheless, non-infectious (Alarcón *et al.*, 1988). The effect of MGM on glycoprotein maturation could also explain why was MGM active against enveloped viruses such as HSV, VSV, and Semliki forest virus, but not against naked ones, such as polio or encephalomyocarditis virus (Alarcón *et al.*, 1984). The antiviral effect of MGM is nevertheless difficult to explain, because, as shown in this paper, MGM was not selective for viral glycoproteins, causing the secretion of incompletely processed glycoproteins and an inhibition in the sialylation of cell membrane glycoproteins. Although dramatic, the effects of MGM on the Golgi of noninfected cells were not correlated with an apparent toxicity to cells grown with MGM added once a week (data not shown). The observed effect could be due to an adaptation of the

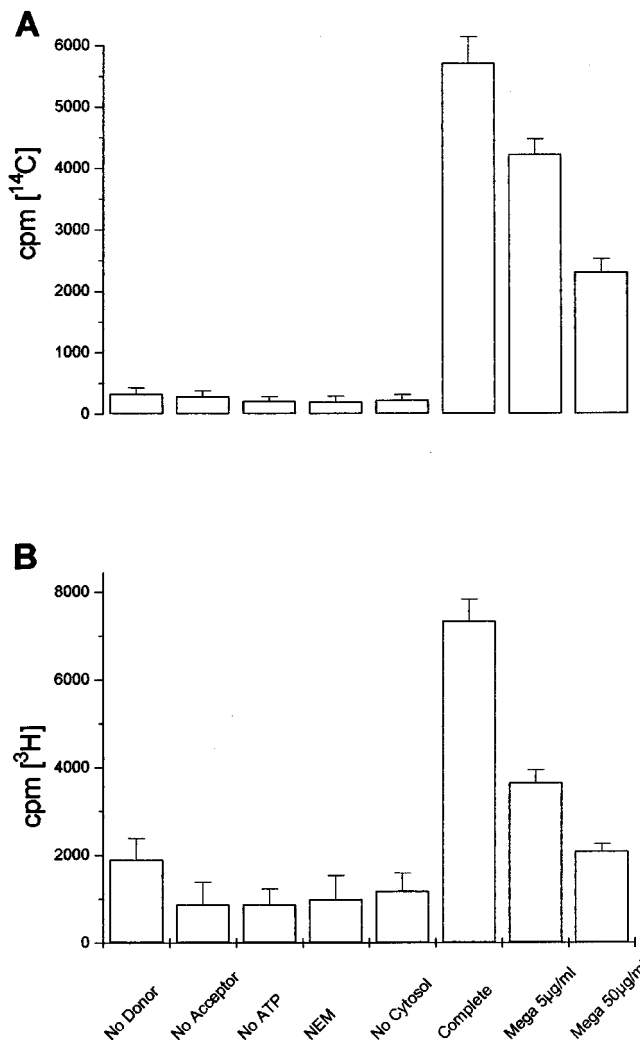


FIG. 7. **Intra-Golgi transport inhibition by MGM.** Standard transport reactions were carried out as described under "Materials and Methods." Where indicated the reactions were performed in the absence of one of the components of the reaction. In the other samples the assays were performed with all the components in the presence of the indicated concentrations of MGM or of 1 mM NEM. A shows the effect of MGM on cis- to medial-Golgi transport where the donor membranes were from CHO 15B cells, and the acceptor membranes were from CHO wild type cells. B shows the effect on medial- to trans-Golgi transport, where the donor membranes were obtained from BHK ricin-resistant mutant 17 and the acceptor membranes from BHK wild type cells. 1 μ g/ml of MGM roughly equals 1 μ M.

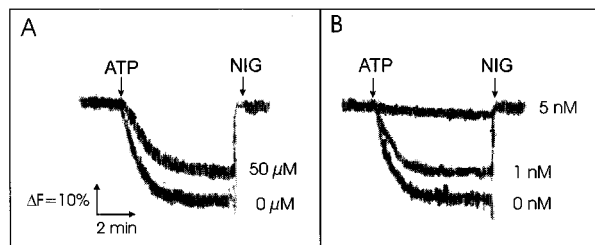


FIG. 8. **Effect of MGM on Golgi acidification.** H⁺ pumping activity was determined by fluorescence quenching of quinacrine with Golgi-enriched membrane vesicles as described under "Materials and Methods." MGM (A) or bafilomycin A₁ (B) were added at the concentrations indicated, and the reaction was started by addition of ATP. Finally, 5 mM nigericin was added to reverse the acidification of the Golgi vesicles.

mycin B (Yilla *et al.*, 1993), where an acidification inhibition of the TGN could cause a default pathway to be utilized. The authors propose that proteins would be shunted into a different pathway causing proteins to reach the cell surface and to be secreted although at a lower rate.

The effects of MGM on the Golgi complex organization were different to those seen with BFA and IQ, which inhibit vesicular transport by acting on early steps of vesicle formation. BFA causes the cis-, medial-, and trans-Golgi to redistribute into the ER by blocking the anterograde vesicular transport, whereas the trans-Golgi network is fused with endosomes (for a review, see Klausner *et al.* (1992)). IQ, on the other hand, causes the fragmentation and vesiculation of the Golgi and inhibits the transport beyond the cis-Golgi (Takizawa *et al.*, 1993). The molecular target of MGM is at present unknown. It seems clear that it is different to the target recognized by BFA, because β -COP is not dissociated from the Golgi upon MGM treatment (data not shown). In addition, transport took place at a normal extension when cytosol from MGM-treated cells was used in the *in vitro* transport assay (data not shown). This suggested that MGM does not inhibit intra-Golgi transport by binding to a soluble component.

On the other hand, it is difficult to understand how the swelling of Golgi cisternae may impair the access of docking vesicles from earlier Golgi compartments. In this regard, the effect of MGM would be similar to the effects of mastoparan and ARFp13, which have been recently described to inhibit intra-Golgi transport by damaging Golgi membranes (Weidman and Winter, 1994). However, unlike these peptides, the effect of MGM was reversible and affected specifically the last steps of glycoprotein maturation.

In accordance with the effect on intra-Golgi transport, MGM at 50 μ M produced a partial inhibition (25–35%) of total protein secretion (data not shown), pointing to an impairment in the transport of secretory proteins beyond the trans-Golgi. The presence of undersialylated glycoproteins in the secreted proteins may be due to sialylation by sialyltransferases in earlier Golgi compartments, perhaps due to mislocalization of the enzymes caused by MGM. This would not be surprising since other drugs, like BFA, produce anomalous processing of glycoproteins, due to the accumulation of Golgi enzymes in the ER (Chawla and Hughes, 1991).

The effect of concanamycin B, a macrolide antibiotic, on the Golgi apparatus has been described (Yilla *et al.*, 1993). Concanamycin B, as well as bafilomycins, is a V-H⁺-ATPase inhibitor (Bowman *et al.*, 1988; Woo *et al.*, 1992). The almost complete blockade of glycoprotein secretion by concanamycin B (Yilla *et al.*, 1993) and the impairment in the transport of viral particles to the membrane by bafilomycin (Pakolungas *et al.*, 1994) suggested that V-H⁺-ATPases maintain a low pH in the trans-Golgi, which is fundamental for protein trafficking through the trans-Golgi and for the activity of sialyltransferases (Yilla *et al.*, 1993). The undersialylation of proteins induced by MGM also could be explained by an indirect inhibition of the sialyltransferase(s), due to an alteration of the intra-Golgi milieu essential to the enzymatic activity caused by the drug. In this regard, as concanamycin B inhibits sialylation (Yilla *et al.*, 1993), it could be argued that MGM could also act by raising the intraluminal pH of the Golgi through an inhibition of the V-H⁺-ATPases. An efficient recognition of the transported proteins (soluble and membrane) by the components of the sorting machinery may rely on the compartment pH. However, in contrast to concanamycin and bafilomycin, MGM inhibited poorly the V-type H⁺-ATPase of the Golgi in an *in vitro* test at the concentrations that were inhibitory in the intra-Golgi transport assays. In addition, bafilomycin A₁, which did

cells to the continuous presence of the inhibitor and also to a possible loss of activity of MGM in culture conditions. A possible mechanism of adaptation has been suggested for concana-

inhibit Golgi acidification, had no detectable effects on Golgi morphology as concanamycin and MGM do. The swelling of the Golgi induced by MGM could be due to the alteration of other ion gradients, different from proton, across the Golgi membrane. In this regard, monensin has been shown to cause a swelling of the trans-Golgi stacks that result from the dissipation of Na^+ gradients (for review, see Mollenhauer *et al.* (1990)). The swelling of the Golgi cisternae is produced because there is a net flow of water into the Golgi stacks to compensate for the H^+ gradient that is still maintained in the presence of monensin. Thus, the addition of a proton ionophore prior to monensin results in the abrogation of monensin-caused swelling (Mollenhauer *et al.*, 1993). By contrast, the addition of the proton ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide did not prevent the swelling effects of MGM on the trans-Golgi (data not shown), suggesting that the mechanism of MGM-induced swelling is different from that of monensin. Monensin, on the other hand, totally inhibits the acquisition of endo-H resistance, while MGM did not.

So, at this point, the subtle differences between other macrolide antibiotics and MGM are more interesting than their similarities, and one relevant issue raised is that they are possibly revealing some hitherto unappreciated complexity in the family of vesicular ATPases not equally susceptible to inhibition by the drugs, with a defined substrate specificity or acting on unrelated processes, located in all of the Golgi compartments. It is important to mention here that the vacuolar H^+ -ATPase is a minor component of the total ATPase activity on Golgi fractions (Moriyama and Nelson, 1989b). Furthermore, in a recent report it has been shown that even after bafilomycin A_1 inhibition of the V-H^+ -ATPase, the pH of the endosome/late endosome was not completely neutral (van Weert *et al.*, 1995), and Yoshimori *et al.* (1991) have reported a lysosomal pH of 6.3 in the presence of Bafilomycin indicating that V-H^+ -ATPase may not be the only factor responsible for pH homeostasis in late endosomes/lysosomes and probably also in the Golgi.

Finally, the use of drugs like MGM can be useful tools to dissect the complexity of the mechanisms responsible of maintaining the homeostasis of the Golgi.

Acknowledgments—We thank Drs. Vicky Allan, Eric Berger, Luis Carrasco, N. K. Gonatas, Thomas Kreis, Mark Marsh, K. Moremen, and Hugh Pelham for kindly providing reagents. We also acknowledge the expertise of Drs. Gustavo Egea, Carmen San Martín, Inmaculada Herrera, and Ismael Santamaría, and we thank Gemma Rodríguez-Tarduchy and Jorgina Satrustegui for helpful discussions.

REFERENCES

- Alarcón, B., Lacal, J. C., Fernández-Sousa, J. M., and Carrasco, L. (1984) *Antiviral Res.* **4**, 231–243.
- Alarcón, B., González, E., and Carrasco, L. (1988) *FEBS Lett.* **231**, 207–211.
- Alarcón, B., Ley, S. C., Sánchez-Madrid, F., Blumberg, R., Ju, S. T., Fresno, M., and Terhorst, C. (1991) *EMBO J.* **10**, 903–912.
- Arai, H., Berne, M., Terres, G., Terres, H., Puopolo, K., and Forgac, M. (1987) *Biochemistry* **26**, 6632–6638.
- Balch, W. E., Dunphy, W. G., Braell, W. A., and Rothman, J. E. (1984) *Cell* **39**, 405–416.
- Beckers, C., and Rothman, J. E. (1992) *Methods Enzymol.* **219**, 5–12.
- Bonay, P., and Hughes, R. C. (1991) *Eur. J. Biochem.* **197**, 229–238.
- Bonay, P., Roth, J., and Hughes, R. C. (1992) *Eur. J. Biochem.* **205**, 399–407.
- Bowman, E. J., Siebers, A., and Attendorf, K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7972–7977.
- Chawla, D., and Hughes, R. C. (1991) *Biochem. J.* **279**, 159–165.
- Crider, B. P., Xie, X. S., and Stone, D. K. (1994) *J. Biol. Chem.* **269**, 17379–17381.
- Deutscher, S. L., and Hirschberg, C. B. (1986) *J. Biol. Chem.* **261**, 96–100.
- Deutscher, S. L., Nuwayhid, N., Stanley, P., Briles, E. B., and Hirschberg, C. B. (1984) *Cell* **39**, 295–299.
- Doms, R. W., Russ, G., and Yewdell, J. W. (1989) *J. Cell Biol.* **109**, 61–72.
- Donaldson, J. G., Kahn, R. A., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E., and Klausner, R. D. (1990) *J. Cell Biol.* **111**, 2295–2306.
- Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) *Mol. Cell. Biol.* **5**, 3610–3616.
- Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. (1988) *J. Biol. Chem.* **263**, 18545–18552.
- Heimer, G. V., and Taylor, C. E. D. (1974) *J. Clin. Pathol.* **27**, 254–256.
- Hirschberg, C. B., and Snider, M. D. (1987) *Annu. Rev. Biochem.* **56**, 63–87.
- Johnson, L. S., Dunn, K. W., Pytowski, B., and McGraw, T. E. (1993) *Mol. Biol. Cell* **4**, 1251–1266.
- Kaku, H., Mori, Y., Goldstein, I. J., and Shibuya, N. (1993) *J. Biol. Chem.* **268**, 13237–13241.
- Kelly, R. B. (1990) *Cell* **61**, 5–7.
- Klausner, R. D., Donaldson, J. C., and Lippincott-Schwartz, J. (1992) *J. Cell Biol.* **116**, 1071–1080.
- Lewis, M. J., and Pelham, H. R. B. (1992) *Cell* **68**, 353–364.
- Lippincott-Schwartz, J., Yuan, L. C., Bonifacio, J. S., and Klausner, R. D. (1989) *Cell* **56**, 801–813.
- Lippincott-Schwartz, J., Donaldson, J. C., Schweizer, A., Berger, E. G., Hauri, H. P., Yuan, L. C., and Klausner, R. D. (1990) *Cell* **60**, 821–836.
- Lippincott-Schwartz, J., Yuan, L. C., Tipper, C., Amherdt, M., Orci, L., and Klausner, R. D. (1991) *Cell* **67**, 601–616.
- Milla, M., Capasso, J., and Hirschberg, C. B. (1989) *Biochem. Soc. Trans.* **17**, 447–448.
- Misumi, Y., Misumi, Y., Miki, Y., Takatsuki, A., Tamura, G., and Ikehara, Y. (1986) *J. Biol. Chem.* **261**, 11398–11403.
- Mollenhauer, H. H., Morre, D. J., and Rowe, L. D. (1990) *Biochim. Biophys. Acta* **1031**, 225–246.
- Monis, E., Bonay, P., and Hughes, R. C. (1987) *Eur. J. Biochem.* **168**, 287–294.
- Moremen, K. W., Touster, O., and Robbins, P. W. (1991) *J. Biol. Chem.* **266**, 16876–16885.
- Moriyama, Y., and Nelson, N. (1989a) *Biochim. Biophys. Acta* **980**, 241–247.
- Moriyama, Y., and Nelson, N. (1989b) *J. Biol. Chem.* **264**, 18445–18450.
- Mourelatos, Z., Adler, H., Hirano, A., Donnenfeld, H., Gonatas, J. O., and Gonatas, N. K. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4393–4395.
- Munro, S. (1991) *EMBO J.* **10**, 3577–3588.
- Nelson, N., and Taiz, I. (1989) *Trends Biochem. Sci.* **14**, 113–116.
- Oda, K., Nishimura, Y., Ikehara, Y., and Kato, K. (1991) *Biochem. Biophys. Res. Commun.* **178**, 369–377.
- Palokangas, H., Metsikko, K., and Vaananen, K. (1994) *J. Biol. Chem.* **269**, 17577–17585.
- Pelham, H. R. B. (1991) *Cell* **67**, 449–451.
- Rothman, J. E. (1987) *J. Biol. Chem.* **262**, 12502–12510.
- Seog, D. H., Yamasaki, M., and Takatsuki, A. (1994) *Biochem. Biophys. Res. Commun.* **199**, 1073–1080.
- Takizawa, P. A., Yucel, J. K., Veit, B., Faulkner, D. J., Deerinck, T., Soto, G., Ellisman, M., and Malhotra, V. (1993) *Cell* **73**, 1079–1090.
- van Weert, A. M. W., Dunn, K. W., Geuze, H. J., Maxfield, F. R., and Stoorvogel, W. (1995) *J. Cell Biol.* **130**, 821–834.
- Varki, A., and Kornfeld, S. (1983) *J. Biol. Chem.* **258**, 2808–2818.
- Vischer, P., and Hughes, R. C. (1981) *Eur. J. Biochem.* **117**, 275–284.
- Weidman, P. J., and Winter, W. M. (1994) *J. Cell Biol.* **127**, 1815–1827.
- Weinstein, M. J., Luederman, G. M., Ridge, G., Wagmar, G. M., and Marquez, J. A. (January 7, 1972) U. S. Patent 3,632,750.
- Woo, J., Shinihara, C., Sakai, K., Hasumi, K., and Endo, A. (1992) *Eur. J. Biochem.* **207**, 383–389.
- Xie, X. S., and Stone, D. K. (1986) *J. Biol. Chem.* **261**, 2492–2495.
- Xu, H., and Shields, D. (1994) *J. Biol. Chem.* **269**, 22875–22881.
- Yilla, M., Tan, A., Ito, K., Miwa, K., and Ploegh, H. L. (1993) *J. Biol. Chem.* **268**, 19092–19100.
- Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M., and Tashiro, Y. (1991) *J. Biol. Chem.* **266**, 17707–17712.
- Zhang, J., Feng, Y., and Forgac, M. (1994) *J. Biol. Chem.* **269**, 23518–23523.