Megalomicin disrupts lysosomal functions

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SUMMARY

Megalomicin (MGM) has been shown to cause a dilation of the most distal cisternae of the Golgi complex. The effects of MGM on Golgi morphology correlated with an inhibition of protein transport to the trans-Golgi resulting in an accumulation of poorly sialylated glycoproteins. Here we show that the addition of 50 μM MGM caused a rapid swelling of lysosomes in cultured cells and inhibited the degradation of the newly synthesized T cell antigen receptor CD3δ subunit. Although MGM did not affect the uptake of fluid phase markers, it prevented their degradation. Interestingly, endocytosed ovalbumin did not colocalize with lysosomes in MGM-treated cells, suggesting an MGM-induced impairment in the delivery to lysosomes. This was confirmed by Percoll density gradients, where the fluid phase marker remained in endosomal fractions, even after long chase times, whereas in control cells the endocytosed marker was located in lysosomes. The effect of MGM was not confined to soluble proteins since it did also inhibit the delivery of the membrane-bound epidermal growth factor receptor to lysosomes. Finally, MGM strongly inhibited the ATP-dependent acidification of lysosomes in vitro, suggesting a possible mechanism for its in vivo activity.

Key words: Megalomicin, Intracellular trafficking, Endosome, Lysosome, Endosomal/lysosomal fusion, V-H⁺-ATPase inhibitor

INTRODUCTION

The central vacuolar system includes the membrane organelles that constitute the endo- and exocytic pathways: the endoplasmic reticulum, the Golgi apparatus including both the cis- and the trans-Golgi network, secretory vesicles, endosomes (both sorting and recycling), lysosomes and the plasma membrane (Klausner, 1989).

The mechanisms underlying the assembly, intracellular retention and surface expression of the T cell receptor (TCR)/CD3 complex have been extensively studied as a model for these cellular processes. The TCR/CD3 complex is composed of at least 6 subunits: TCR-α and TCR-β (or TCR-γ and TCR-δ in γδ T lymphocytes) which are responsible for antigen recognition, and CD3-γ, CD3-δ, CD3-ε and CD3-ζ which are responsible for signal transduction (reviewed by Clevers et al., 1988; Klausner and Samelson, 1991). The surface expression of the TCR/CD3 complex is regulated in order to guarantee the expression of its different components in a coordinated fashion. Thus, when expressed in isolation, each subunit is either retained in the ER or rapidly degraded. While some subunits (TCR-α, TCR-β, CD3-δ and murine, but not human, CD3-γ) are apparently degraded in an endo-H sensitive form, others (CD3-ε and CD3-ζ) seem to be stably retained in the ER (Wileman et al., 1990a; Chen et al., 1988; Bonifacino et al., 1989). Interestingly, when an unstable subunit associates with a stable one, the resulting dimer is not degraded and remains in the ER, suggesting that the association with a stable subunit either prevents accessibility of the sensitive subunit to ER located proteases or prevents its export and degradation outside of the ER (Bonifacino et al., 1989; Wileman et al., 1990a). Compelling evidence showed that degradation of newly synthesized TCR-α, TCR-β and CD3-δ occurred in the ER since it was insensitive to a wide array of lysosomotropic drugs and endo-H resistant forms of these subunits were never found (reviewed by Klausner and Sitia, 1990). Nevertheless, a partial temperature dependence of CD3-δ degradation suggested that, at least for this subunit, degradation was in part lysosomal (Wileman et al., 1990b).

The regulated and highly controlled vectorial transport of proteins between their site of synthesis and their final destination relies on the communication among the membrane compartments by means of shuttling transport vesicles containing the sorted proteins, which bud from and then fuse to successive compartments in the corresponding pathway. As the components in each separate compartment are fairly unique serving specific functions, it is clear that sorting and targeting mechanisms most efficiently act to preserve the composition of each organelle (Pelham and Munro, 1993). Internal ionic environment plays an important role on cellular sorting and targeting and the acidification of some components in the intracellular membranous system is suggested to play an important role in such processes (Mellman et al., 1986). At present, the recently described vacuolar type H⁺-ATPases are the most probable candidates for generating and maintaining a low pH in the central vacuolar system (Bowman et al., 1988) as it has been shown that plasma membrane, Golgi apparatus, endosomes and lysosomes possess unique electrogenic and non-electrogenic proton pumping ATPases which lower intraluminal pH. Certain macrolide antibiotics which selectively act on the V-H⁺-ATPases have frequently been used to assert the effect of internal pH in protein trafficking along the...
endocytic pathway without interference of the side effects induced by acidotropic agents and ionophores (van Weert et al., 1995; Benaroch et al., 1995). Recently our group (Bonay et al., 1996), showed that megalomicin (MGM) produces profound morphological and functional alterations on the Golgi complex of cultured cells, causing the inhibition of the last steps of glycoprotein processing in the Golgi by blocking intra-Golgi transport without any substantial effect on the Golgi V-H+-ATPase. Here we report that the effects of MGM are not restricted to the Golgi apparatus but extend to the lysosomal/endosomal system causing an enlargement of lysosomes and inhibiting the delivery of endocytosed material to lysosomes. MGM also inhibits degradation of newly synthesized proteins in lysosomal compartments. Unlike Golgi V-H+-ATPase, the lysosomal enzyme seems to be sensitive to inhibition by MGM.

MATERIALS AND METHODS

Cells and reagents

MGM was obtained and purified from cultures of *Micromonaspora megalomicea* (ATCC no. 27598) as described before (Bonay et al., 1996). COS-7 cells, kindly given by Dr B. Seed (Massachusetts General Hospital, Boston), and normal rat kidney cells (NRK) were grown in DME supplemented with 5% fetal bovine serum. Mycoplasma testing was routinely performed. Plasmids capable of expressing human CD4 and CD4 tagged with 10 amino acids from CD3-ε were constructed in the pSR-α vector as described (Mallabiaabarrena et al., 1992). Human CD3-δ cDNA was inserted in MNC8 plasmid as described (Alarcón et al., 1991). A plasmid encoding human transferrin receptor (pCD-TR1) was generously made against a cathepsin-enriched fraction was generated by one of us (P. Bonay) and mainly appeared to recognize cathepsin D by immunoblot analysis. The anti-CD63 monoclonal antibody 1B5 was kindly donated by Dr M. Marsh (University College, London) Epidermal growth factor (EGF) was purchased from Calbiochem (AMS Biotecnología, Spain) and the monoclonal antibody against the EGF-receptor was purchased from Transduction Laboratories (Lexington, KY).

Transfections and immunofluorescence microscopy

COS cells were transfected and stained for immunofluorescence microscopy as previously described (Salmerón et al., 1991).

Acidification assay

NRK cells were incubated at 37°C with FITC-dextran (5 mg/ml) for 30 minutes followed by a 1 hour chase in the absence of FITC-dextran to allow labeling of lysosomes. The ATP-dependent acidification assay of isolated lysosomes was carried out essentially as described (Galloway et al., 1983; Schmid et al., 1988), by the fluorescence quenching of FITC (ex= 485 nm, em= 515 nm).

Internalization of fluid phase markers and subcellular fractionation

NRK cells were incubated in the absence or presence of MGM (50 μM) with 1 μCi of a non-binding 125I-labeled antibody (SF34, specific for the human T cell protein CD3ε, which is not expressed in NRK cells) for 15 minutes to label endosomes or for 15 minutes and then chased for 90 minutes to label lysosomes. At the incubation and chase periods, the cells were washed twice with cold PBS plus 2% BSA and once with 250 mM sucrose in 10 mM Hepes, pH 7.4. A 25% cell suspension was homogenized by 20 strokes using a tight fitting pestle. A post-nuclear supernatant was prepared by centrifugation at 800 g for 10 minutes, then 2.5 ml of the supernatant was overlayed on top of 9 ml of 17% Percoll and centrifuged at 33,000 g in a 50Ti rotor for 15 minutes as described (Nolan, 1992). Fractions (600 μl) were collected from the bottom and assayed for radioactivity and β-hexosaminidase activity. Ovalbumin was labeled with rhodamine isothiocyanate (Sigma) as described (Harlow and Lane, 1988).

Radiolabeling and immunoprecipitation

For metabolic labeling a 100 mm Petridish of confluent CD3-δ transfected COS cells were washed once with 10 ml of PBS and covered with 2 ml of DME without methionine or cysteine and incubated for 30 minutes at 37°C. Then 0.5 mCi of a [35S]methionine and [3H]cysteine mixture (3,000 Ci/mmol; Amersham, UK) was added for 30 minutes and, afterwards, the cells were lysed in immunoprecipitation buffer (1% Nonidet P40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.8, 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, PMSF, and 1 μg/ml each of leupeptin and pepstatin). Alternatively, after the pulse-labeling, 10 ml of complete DME medium were added and the cells were incubated at 37°C for different chase times.

For immunoprecipitation, detergent cell lysates were centrifuged at 12,000 g for 15 minutes to pellet nuclei and cell debris and the supernatant was preclared 3 times by incubation for 1 hour each with Protein G-Sepharose beads (Sigma) previously coated with non-immune murine serum. The supernatant from the last precleration was incubated for 4 hours with 10 μl of Protein G-Sepharose beads previously coated with the anti-CD3-δ antibody. After immunoprecipitation, the beads were washed 5 times with 1 ml of immunoprecipitation buffer and boiled in 5 μl of 1% SDS for 3 minutes. The samples were cooled and 100 μl of 150 mM sodium citrate buffer, pH 5.5, plus 1 mM PMSF and 1 μg/ml each of leupeptin and aprotinin, were added. Half of each sample was then incubated overnight with 1 μl of Endo-H (Boehringer-Mannheim, Germany) at 37°C, or left untreated. The samples were afterwards prepared for electrophoresis as previously described (Alarcón et al., 1991).

Glycan analysis

The analysis of N-linked high-mannose glycans was done on microsomal fractions of control or MGM-treated NRK cells as described before (Bonay and Hughes, 1991).

Autoradiography and densitometry

For autoradiography, Kodak X-Omat XAR5 films were used and densitometry was performed in a Computing Densitometer model 300A. For densitometry, a curve with known amounts of a [35S]methionine-labeled standard was utilized to calibrate the response of the film. To calculate the amount of CD3-δ, adequate exposures were utilized that fit in the linear response region of the film.

EGF degradation

NRK cells were grown in 24-well culture plates and were pretreated or not with 50 μM MGM for at least 5 hours and with 10 mM NH4Cl for 1 hour (to prevent degradation of EGF during uptake) and 125I-labeled EGF was added at 25 ng/ml for 1 hour. At time 0 the cells were washed to remove excess EGF and ammonium chloride and the incubation was continued in the presence of either 50 μM MGM or 10 mM NH4Cl or incubated in the absence of drugs. At different times the medium was removed, cells were solubilized in 5 mM NaOH and 10% TCA-insoluble radioactivity was counted.

Subcellular localization of EGF-R

NRK cells were incubated in the presence or absence of 50 μM MGM.
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for 6 hours at 37°C, and with EGF at 10 ng/ml for the last 2 hours of preincubation. Cells were subsequently washed twice in 0.25 M sucrose, pH 7.3, and scraped off the plate in 2 ml of the same buffer and collected by centrifugation. The pellet was homogenized by 10 strokes in a loose-fitting Dounce homogenizer, and centrifuged at 800 g for 10 minutes. The supernatant was overlayed onto a 9 ml Percoll cushion (3 ml of stock Percoll solution diluted with 7 ml of 0.25 M sucrose solution, density 1.07 g/ml) and centrifuged at 33,000 g for 50 minutes. Fractions of 1 ml were collected from the top. A sample of each fraction was mixed with Laemmli SDS sample buffer, vortexed for 5 minutes and centrifuged at 100,000 g for 30 minutes to pellet the silica particles. The supernatants were separated by SDS-PAGE on a 6.5% acrylamide gel and transferred to PVDF membrane for immunoblotting. The membrane was blocked with 10% Blotto for 2 hours at room temperature and incubated with 1 μg/ml of the anti-EGF-R monoclonal antibody for 2 hours at room temperature. The membrane was washed, probed with a second peroxidase-labeled antibody and developed by the ECL method (Amersham) according to standard procedures. In these conditions lysosomes appear at the bottom of the gradient, in fractions 6, 7 and 8, as measured by the presence of acid hexosaminidase (Bonay and Hughes, 1991). The endosomal fractions corresponded to the top of the gradient (fractions 1, 2 and 3) as measured in a parallel gradient where cells had been incubated with [125]labeled EGF for 30 minutes.

RESULTS

MGM causes the accumulation in swollen lysosomes of proteins in the secretory route

When COS cells that had been transfected with plasma membrane receptors such as CD4 were incubated overnight with 50 μM MGM, these proteins were observed to accumulate in large vesicles (Fig. 1b), in sharp contrast to their normal intracellular location which is mostly in the Golgi area (Fig. 1a). The distribution of a CD4 chimera tagged with an ER retention signal from CD3ζ was, however, not affected by MGM (Fig. 1c, control; 1d, MGM-treated). Interestingly, another protein, CD3ζ, which is supposed to be retained and subsequently degraded in the ER (Klausner and Sitia, 1990), was also located in large vesicles in MGM-treated cells (Fig. 1e, control; 1f, MGM treated). Strikingly, these vesicles were co-stained with antibodies against lysosomal markers (data not shown). As shown in Fig. 2, an extensive colocalization of CD4 with the lysosomal marker CD63 was observed after a 3 hour and an overnight incubation with MGM. Although the detection of secretory proteins in large vesicles usually needed overnight incubations with MGM (Fig. 1b and f), an incubation of transferrin receptor (TfR) transfected cells for as short as 1 hour (Fig. 1h, treated, vs 1g, untreated), was sufficient to detect the TfR in large vesicles which were located in a juxtanuclear position. It is known that MGM inhibits intra-Golgi transport at the latter steps of the Golgi apparatus (Bonay et al., 1996), so the results shown in Fig. 1 are consistent with the notion that MGM could induce a missorting of proteins from the medial-Golgi to lysosomes. This possibility prompted us to study the effect of MGM on lysosomes. To this end, NRK cells were incubated for various times in the presence of 50 μM MGM and stained with a monoclonal antibody specific for the

Fig. 1. Effect of MGM on the distribution of transfected proteins in COS cells. COS cells which had been transfected 48 hours earlier with 10 μg of plasmids encoding wild-type CD4 (a,b); a chimeric CD4 form which is retained in the ER, CD4e10(c,d); CD3-ζ (e,f) or transferrin receptor, TfR (g,h), were incubated overnight (b,d,f) or 1 hour (h) with 50 μM MGM (b,d,f,h) or left untreated (a,c,e,g). After treatment, cells were fixed and stained for indirect immunofluorescence with specific antibodies. Note the characteristic ER distribution of CD4e10 which is not affected by MGM and the Golgi-like distribution of CD4 and TfR which is changed to a swollen vesicular aspect. CD3-ζ produces a mixed ER plus Golgi pattern which changes to a macrovesicular appearance when treated with MGM. A 630x magnification is shown in this and following figures. When the position of the nucleus is not clear, it is indicated with an n.
lysosomal membrane protein LIMP I (Barriocanal et al., 1986). As shown in Fig. 3, after a 30 minute incubation, lysosomes appeared two- to threefold larger than control ones, although they still maintained a perinuclear location. After a 1 hour treatment, lysosomes had become approximately 5-fold larger, and after 2.5 hours the lysosomes appeared as large swollen spheres (up to 1 µm diameter), with the fluorescence staining clearly defining the periphery of the organelle leaving a stain-free lumen. Staining with an antibody specific for the lysosome lumenal protein cathepsin D resulted in the same effect (Fig. 3, bottom). This morphology remarkably resembles that of ‘sucrosomes’, lysosomes of cells which have taken up sucrose (Ferris et al., 1987). By contrast, no apparent effect of MGM was shown when cells were stained with an antibody against the cation independent mannose-6-phosphate receptor (results not shown). These results suggest that the large vacuoles could come from the swelling or fusion of lysosomes but not from PLC/late endosomes. The pattern of swollen lysosomes was also obtained in COS cells treated with 50 µM MGM albeit with a slower kinetics than in NRK cells (data not shown).
MGM inhibits the lysosomal degradation of proteins in the secretory route

To determine whether the MGM-induced morphological alteration of lysosomes was accompanied by an impairment in their function (i.e., degradation of proteins), COS cells were transfected with CD3-δ, labeled with [35S]methionine for 30 minutes and chased for the indicated times. The acquisition of resistance to endo-H was observed in CD3-δ as well as in the albumin contaminant which specifically binds to Protein G (Kronvall et al., 1979; Akerström et al., 1987). As shown in Fig. 4a, the endo-H resistant form of CD3-δ was undetectable in control cells. By contrast, the endo-H resistant form was clearly present in MGM-treated cells after a 4 hour chase. Furthermore, as shown in Fig. 4b, CD3-δ degradation seemed to be a two-step phenomena. The first phase takes place during the first hour of chase and results in the degradation of approximately 40% of CD3-δ. This phase is insensitive to MGM as occurs with the same kinetics in both control and drug-treated cells. However, the second phase of degradation is almost completely prevented by MGM. After a 6 hour chase, 50% of the original protein was still present in treated cells, whilst more than 80% was degraded in control cells. Since after a 4 hour chase mostly all CD3-δ detected in MGM-treated cells is in an endo-H resistant form (Fig. 4a), the most likely explanation for those results is that MGM does not prevent transport to the medial-Golgi although it inhibits the degradation of CD3-δ in

Fig. 3. Lysosomal swelling induced by MGM. NRK cells were treated with 50 μM MGM for the indicated times and stained with an anti-lysosomal membrane protein LIMP I antibody (a) and anticithepsin D antibody 1C2 (b). Lysosomes appear increasingly swollen and unstained in the center.

Fig. 4. MGM inhibits the degradation of proteins in the exocytic route. (a) COS cells were transfected with 10 μg of the CD3-δ encoding plasmid MNC8-δ. At 48 hours after transfection the cells were pulse-labeled with [35S]methionine and subsequently chased in complete medium for the indicated times (hours) in the absence or the presence of 50 μM MGM. Once labeled, cells were lysed and immunoprecipitated with anti-CD3-δ antibody APA1/2. The immunoprecipitates were then digested with endo-H (+) or left untreated (−). The positions of undigested (δ) and digested CD3-δ (δ0) are indicated. The effect of MGM on the degradation and acquisition of endo-H resistance by the contaminant albumin is also shown. (b) The effect of MGM on CD3-δ degradation was evaluated as in a. The amount of remaining CD3-δ after each chase time point was estimated by densitometry. Untreated cells (open squares), cells treated with 50 μM MGM (filled squares).
Mannose residues per oligosaccharide unit.

Paper strips were cut at 1 cm intervals, eluted with water and counted.

NRK control cells or MGM-treated (50 μM) cells were metabolically labeled with [3H]mannose (50 μCi/ml) in glucose-free DMEM 5% dialyzed FCS for 16 hours. Glycopeptides from the microsomal fraction were prepared and fractionated by affinity chromatography on Con A-Sepharose. The high-mannose pool was then treated with a mixture of Endo D and Endo H and the deglycosylated material was fractionated by paper chromatography. Paper strips were cut at 1 cm intervals, eluted with water and counted in scintillation liquid. The x-axis represents the average number of mannose residues per oligosaccharide unit.

MGM has been previously shown to inhibit processing of N-linked oligosaccharides, resulting in an accumulation of glycoproteins with high-mannose containing oligosaccharides (Bonay et al., 1996). Due to medial- to trans-Golgi transport inhibition by MGM, it could be thought that glycoproteins accumulated in a medial or pre-medial Golgi location. We decided to examine the composition of high-mannose N-linked glycans in the microsomal fraction of MGM-treated cells as a means to determine the possible localization of the accumulating glycans. As shown in Fig. 5, whereas in control cells most of the high-mannose N-linked glycans were 6-9-mannose oligomers, suggesting an ER and/or early Golgi processing (Daniel et al., 1994), in MGM-treated cells there was a high level of oligosaccharides containing branched 3 and 4 mannose residues and also some linear 4-mannose oligomers. Those are characteristic products of the lysosomal acid mannosidase (Tulsiani and Touster, 1987), suggesting that a fraction of glycoproteins synthesized in the presence of MGM are accumulating in lysosomes.

Fig. 5. Analysis of high-mannose glycan composition upon MGM treatment. NRK control cells or MGM-treated (50 μM) cells were metabolically labeled with [3H]mannose (50 μCi/ml) in glucose-free DMEM 5% dialyzed FCS for 16 hours. Glycopeptides from the microsomal fraction were prepared and fractionated by affinity chromatography on Con A-Sepharose. The high-mannose pool was then treated with a mixture of Endo D and Endo H and the deglycosylated material was fractionated by paper chromatography. Paper strips were cut at 1 cm intervals, eluted with water and counted in scintillation liquid. The x-axis represents the average number of mannose residues per oligosaccharide unit.

The above data suggest that MGM is able to inhibit protein degradation of newly synthesized proteins. In addition, MGM inhibited the degradation, although not the uptake, of proteins used as fluid phase endocytic tracers (Table 1), suggesting that MGM either inhibited lysosomal degradation or the transport of endocytosed proteins to lysosomes.

Table 1. Effect of MGM on endocytosis and degradation of internalized protein

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control</th>
<th>MGM 50 μM</th>
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<tr>
<td>0</td>
<td>97</td>
<td>96</td>
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<tr>
<td>0.5</td>
<td>90</td>
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<td>1</td>
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<td>2</td>
<td>61</td>
<td>76</td>
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<td>3</td>
<td>31</td>
<td>78</td>
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*COS and NRK cells were preincubated with the indicated concentrations of MGM for 3 hours, 5 mg/ml horseradish peroxidase was added and incubated for 30 minutes at 37°C. Cell plates were rinsed and the amount of endocytosed peroxidase was evaluated and referred to the total amount of protein as described in Materials and Methods.

MGM prevents endocytosed fluid phase markers from reaching lysosomes

The above data suggest that MGM is able to inhibit protein degradation of newly synthesized proteins. In addition, MGM inhibited the degradation, although not the uptake, of proteins used as fluid phase endocytic tracers (Table 1), suggesting that MGM either inhibited lysosomal degradation or the transport of endocytosed proteins to lysosomes.

An immunofluorescence approach was carried out in order to demonstrate colocalization of endocytosed material with lysosomal markers. To this end, NRK cells were incubated with RITC-ovalbumin for 1 hour to trace the endocytic pathway, fixed and then stained with an FITC-conjugated antibody against cathepsin D. As shown by confocal microscopy, in control cells most vesicles appeared double labeled, showing that both the fluid phase (red) and lysosomal (green) markers colocalized (Fig. 6a). However, in MGM-treated cells, there was a clear distinction between green-stained swollen lysosomes and small red-stained endosomes. Most of the vesicles were not double stained and appeared either red or green. The few yellow vesicles that are shown in the sample are due to superposition at different levels of an endosome and a lysosome as suggested by the small size of the yellow spots which does not match the size of swollen lysosomes.

To further study the delivery of endocytosed material to lysosomes, trafficking of a fluid-phase endocytic marker (SP34, a T cell-specific antibody), was examined. 125I-IgG was either allowed to be internalized for 15 minutes to label endosomes or internalized (15 minutes) and chased for an ad-
Additional 90 minutes to wash the endocytic route and label lysosomes, in the presence or absence of MGM. The cell lysates were fractionated on Percoll density gradients and fractions were analyzed for $^{125}$I-labeled protein. In addition, fractions were analyzed for β-hexosaminidase activity to locate lysosomes. The distribution of endosomes and lysosomes was defined in control experiments. Endocytosed $^{125}$I-IgG (endosomes) peaked in fractions 14-16 (Fig. 6b), whereas lysosomes peaked around fraction 6. After the 90 minute chase, radioactivity peaked in lysosomal fractions in untreated cells, showing that endosomes had transferred their contents to lysosomes. However, in pulse-chased MGM-treated cells radioactivity still peaked in endosomal fractions whereas lysosomal markers were located in a broad peak correspond-
ing to fractions 5 to 9 (Fig. 6b and c). The broadening of the lysosome peak may be due to changes in hydrodynamic properties of the organelle as a consequence of the MGM-induced swelling. This result confirms that transport from endosomes to lysosomes was inhibited in the presence of MGM.

The inhibition by MGM of fluid phase marker transport into lysosomes contrasts with the accumulation of newly synthesized proteins in lysosomes (Figs 1 and 4) as well as of membrane receptors such as CD4 (Figs 1 and 2). It would appear as if MGM causes the accumulation of membrane proteins in lysosomes whereas it inhibits the entry of soluble proteins into them. To determine whether MGM has such a selective effect on soluble versus membrane proteins, the effect of MGM on the transport to lysosomes of a well documented membrane receptor/ligand system was studied. To this end, both the effect of MGM on the degradation of EGF and on the transport of EGF-receptor to lysosomes were investigated. As shown in Fig. 7a, MGM partially inhibited the degradation of EGF, resulting in an accumulation of undegraded protein of 41% of the initial input after an 8 hours incubation, versus 18% for the untreated cells. By contrast, ammonium chloride, a drug that raises the intralysosomal pH, almost totally prevented EGF degradation. The partial effect of MGM on EGF degradation left still unresolved the question of whether MGM inhibits the delivery of endocytosed membrane receptors to lysosomes or just inhibits intralysosomal degradation. To further discriminate between both possibilities, a cell fractionation experiment on Percoll gradients was performed from NRK cells incubated for 6 hours with EGF in the presence or the absence of 50 μM MGM. Both untreated and MGM-treated cells were incubated with 10 mM ammonium chloride to prevent degradation of the internalized protein once it reached lysosomes. Densitometry measurements of the results shown in Fig. 7b indicated that whereas 31% of total EGF-receptor was found in dense fractions that corresponded to lysosomes, only 9% was found in lysosomes in the presence of MGM. These results suggest that MGM partially inhibits the translocation of internalized EGF-receptor to lysosomes. However, MGM does also cause the accumulation of membrane proteins in lysosomes (Figs 1 and 2), suggesting that in addition to inhibiting transfer of endocytosed proteins to lysosomes, MGM must also inhibit their degradation. Therefore, the observed accumulation of membrane proteins in lysosomes must be a result of an accumulation of undegraded proteins despite the partial inhibition of delivery also caused by the drug.

**Lysosomal V-H⁺ATPase inhibition by MGM**

The inhibition of intra-lysosomal degradation by MGM could result from alteration of the luminal milieu, such as an acidic pH, necessary for the activity of lysosomal hydrolases. Other macrolide antibiotics, including bafilomycins and concanamycin B, have been shown to be specific inhibitors of vacuolar ATPases (Benaroch et al., 1995; Nelson and Taiz, 1989), identified in organelles belonging to the central vacuolar system such as coated vesicles (Xie and Stone, 1986; Arai et al., 1987), lysosomes (Moriyama and Nelson, 1989a), and the Golgi complex (Moriyama and Nelson, 1989b). Those ATPases are likely to be responsible for the generation of acidity in those organelles. To determine whether MGM interferes with the acidification of lysosomes, these organelles were labeled with FITC-dextran, then ATP-dependent quenching of fluorescence was measured as an index of acidification. As shown in Fig. 8, a rapid decrease of fluorescence signal intensity was measured in the control sample upon ATP addition, indicative of acidification of those organelles. This acidification was reversed by the addition of nigericin, a proton ionophore. MGM addition prior to ATP prevented lysosomal acidification by 75-85% at a concentration of 0.1 μM, 200- to 500-fold lower than the one required to produce lysosomal swelling in vivo. To test whether MGM could be disrupting the pH gradient once it had been formed, i.e. that MGM could have an H⁺ ionophore activity, 50 μM MGM was added after the pH gradient had been established. As shown in Fig. 8, MGM did
not significantly dissipate the pH gradient, suggesting that MGM does not act as an H⁺ ionophore on lysosomes and that MGM does not grossly damage lysosomal membranes. The effect on the lysosomal V-H⁺-ATPase suggests that inhibition of lysosomal acidification by MGM could be responsible for its effects on the degradation of proteins in the endocytic and secretory routes.

DISCUSSION

We have previously shown that MGM causes a profound dilation of the trans-cisternae of the Golgi apparatus and an impaired transport of newly synthesized proteins to the trans-Golgi (Bonay et al., 1996). As a result, glycoproteins are secreted in a poorly sialylated form. We now show in this paper that MGM causes a rapid swelling of lysosomes concomitant with an altered function preventing the degradation of proteins in the exocytic as well as in the endocytic routes. Interestingly in both organelles, Golgi and lysosomes, MGM caused a dilation that correlated with an impaired protein transport to the swollen organelles. Thus, we show in this paper that endocytosed proteins do not reach lysosomes in MGM-treated cells. There are other instances in the literature where the swelling of a component of the secretory route abolishes some heterotypic fusions, thus in ‘sucrosomes’ (Ferris et al., 1987), and in the large swollen vesicles stained with the PLC/late endosomes marker CI-MPR in cells treated with the PI3-kinase inhibitor wortmannin (Brown et al., 1995), the altered organelle remains rather refractory to the fusion with transport vesicles.

Other macrolide antibiotics, including bafilomycins and concanamycin B, have been shown to be specific inhibitors of the vacuolar ATPases (V-ATPases)(Bowman et al., 1988; Woo et al., 1992). Interestingly, whereas MGM did not affect the Golgi V-H⁺-ATPases (Bonay et al., 1996), it strongly inhibited the lysosomal enzyme (Fig. 8). It could be thought that V-H⁺-ATPases are the targets of MGM, and that the effects of MGM on lysosomes result from a subsequent increased lysosomal pH. Thus, the activity of lysosomal hydrolases is perturbed by a higher pH, and this effect could be responsible for the inhibition of protein degradation. On the other hand, the inhibited endosome/lysosome fusion could ultimately be due to an impaired association of coatomers to transport vesicles, a phenomenon that has been recently shown to be pH dependent (Aniento et al., 1996). Indeed bafilomycin has been shown to abrogate transport of a fluid phase marker to cathepsin D-labelled lysosomes, although it did not interfere with its transport to late endosomes (van Weert et al., 1995). However, the model of V-H⁺-ATPase inhibition by MGM as the molecular target does not explain the pleitropic effects of MGM and, thus, the effects on Golgi morphology and function would not be explained by this mechanism. In addition, one of the most prominent effects of MGM is the pronounced swelling of the trans-Golgi and lysosomes, a phenomenon not always noticed with bona fide V-H⁺-ATPase inhibitors, concanamycin B and bafilomycins (Yoshimori et al., 1991; Woo et al., 1992). Most of the acidification assays reported in the literature are in vitro assays performed with purified organelle preparations; however, in vivo measurements of acidification have shown that 100-1,000 higher concentrations of bafilomycin A₁ are required to inhibit acidification (van Weert et al., 1995; Umata et al., 1990). Consequently, while the data presented indicate that MGM can inhibit the lysosomal acidification in an in vitro assay with isolated lysosomes, they do not allow the conclusion that the inhibition of protein degradation may be totally ascribed to V-H⁺-ATPase inhibition and, indeed, an inhibition of delivery cannot be ruled out. The most likely explanation for the observed phenomena is that MGM can inhibit both lysosomal degradation of internalized and newly synthesized proteins and also can inhibit or at least delay the transfer of proteins from endosomes to lysosomes. This dual mechanism could produce simultaneously an impaired transfer of endocytosed fluid phase markers and membrane proteins to lysosomes and also an accumulation in lysosomes of proteins that slowly reach them in the presence of MGM and remain undegraded. Whether MGM could also partially inhibit the delivery of newly synthesized proteins from the Golgi to lysosomes is unknown at present.

The nature of the mechanism responsible for transport inhibition to lysosomes and to the trans-Golgi remains to be determined. As a putative mechanism for the lysosomal effects, MGM could be altering ion gradients other than proton gradients, such as monensin does for Na⁺ (reviewed by Mollenhauer et al., 1990) and promoting a net inflow of water. The dilution of the lumenal content would slow the degradation of proteins which would thus be an effect secondary to swelling. Alternatively, MGM could be inhibiting lysosomal hydrolases and in this way promoting swelling by accumulation of undegraded or partially degraded products. The mechanism of MGM inhibition on hydrolases could be direct or could be the result of an anomalous processing of the enzymes. The effects of MGM could also be explained by a block of membrane exit.
and continued vesicle incorporation arriving from other non-endosomal membranes like the Golgi apparatus although an induction of lysosome fusion is not excluded.

The inhibition of protein degradation in lysosomes by MGM could be used as a tool to enhance otherwise undetectable pathways. For instance, the degradation of newly synthesized CD3-δ complex, as well as of the other subunits of the TCR/CD3 complex, has been postulated to occur in the endoplasmic reticulum (Lippincott-Schwartz et al., 1988). This was mostly based on the fact that CD3-δ was always detectable in an endo-H sensitive form and that CD3-δ degradation was insensitive to lysosomotropic drugs. However, as shown in this paper, incubation with MGM promoted the accumulation of undergraded CD3-δ in an endo-H resistant form in lysosomes. Nevertheless, because part of CD3-δ was degraded early, even in the presence of MGM, the possibility that degradation of CD3-δ could in part take place out of a lysosomal location should not be excluded. Thus, the MGM data are mostly in agreement with those of Wileman et al. (1990b), that showed a bimodal temperature dependence for CD3-δ degradation, suggesting an ER- and a lysosome-located degradation. The glycan analysis of microsomal proteins from control and MGM-treated cells further supports that notion. The results indicate a catabolic action of enzymes rather than normal processing or trimming, consistent with the idea that, at least for CD3-δ, MGM highlights a normally occurring sorting of protein towards lysosomes, not evidenced in control cells due to the rapid degradation of the protein in the organelle. In this regard, because MGM inhibits medial- to trans-Golgi transport (Bonay et al., 1996) and causes the accumulation of endo-H resistant proteins in lysosomes (Figs 1 and 4), we have to postulate the existence of a route for the direct transport of proteins from the medial-Golgi to lysosomes. Nevertheless, a possible missorting effect of MGM on intracellular trafficking cannot be at present discarded.

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