Inhibition of glycoprotein oligosaccharide processing *in vitro* and in influenza-virus-infected cells by α -D-mannopyranosylmethyl-*p*-nitrophenyltriazene

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The effects of α -D-mannopyranosylmethyl-*p*-nitrophenyltriazene (MMNT) on mannosidases involved in asparagine-linked oligosaccharide processing were investigated. MMNT was found to inhibit the activity of rat liver Golgi α -mannosidase I in a concentration-dependent manner (50% inhibition with 0.18 mM-MMNT), whereas rat liver endoplasmic-reticulum α -mannosidase appeared to be resistant (less than 5% inhibition at 1 mM-MMNT). Jack-bean α -mannosidase was also sensitive to inhibition by MMNT (50% inhibition with 0.32 mM-MMNT). Treatment of influenza-virus-infected chick-embryo cells with 1 mM-MMNT led to a decrease in the formation of complex-type asparagine-linked oligosaccharides and an accumulation of high-mannose-type oligosaccharides with the composition $\text{Man}_8(\text{GlcNAc})_2$ and $\text{Man}_7(\text{GlcNAc})_2$ on the viral glycoproteins. The biological activities of influenza-virus haemagglutinin and neuraminidase synthesized in the presence of 1 mM-MMNT remained unchanged, but the virus was less infectious than the control.

INTRODUCTION

The complex-type and high-mannose-type oligosaccharide side chains present in mature *N*-glycosylated glycoproteins are derived from a common precursor $\text{Glc}_3\text{Man}_9(\text{GlcNAc})_2$ (Robbins *et al.*, 1977), which is assembled on a dolichol diphosphate carrier before being transferred *en bloc* to protein in the endoplasmic reticulum (ER) (Kornfeld & Kornfeld, 1985). The sequence of reactions involved in the subsequent trimming of the precursor oligosaccharide are well documented (Kornfeld & Kornfeld, 1985). The glucose residues are removed by glucosidases I and II (Grinna & Robbins, 1979; Burns & Touster, 1982) in the ER, and an ER α -mannosidase probably removes one mannose residue (Bischoff & Kornfeld, 1983) before the glycoprotein is transported to the cis-Golgi compartment, where the action of α -mannosidases IA/IB produces the high-mannose-type oligosaccharides containing five to eight mannose residues (Tabas & Kornfeld, 1979). The $\text{Man}_5(\text{GlcNAc})_2$ intermediate is the substrate for *N*-acetylglucosaminyltransferase I (Harpaz & Schachter, 1980), which is located in the medial compartment of the Golgi apparatus and adds one *N*-acetylglucosamine residue. Mannosidase II now removes two further mannose residues (Tulsiani *et al.*, 1982a), and a second *N*-acetylglucosamine residue is subsequently added by *N*-acetylglucosaminyltransferase II. Further elongation of the oligosaccharide to produce complex-type glycans

takes place in the trans-Golgi compartment (Kornfeld & Kornfeld, 1985).

Specific inhibitors of the trimming glycosidases have proved to be useful in studying the biological roles of the oligosaccharide side chains of glycoproteins as well as the functional significance of the trimming pathway (Schwarz & Datema, 1984; Datema *et al.*, 1987). Glucosidase inhibitors such as 1-deoxynojirimycin (Saunier *et al.*, 1982), *N*-methyl-1-deoxynojirimycin (Romero *et al.*, 1983) and castanospermine (Pan *et al.*, 1983) block the removal of the first glucose residue, whereas bromoconduritol (Datema *et al.*, 1982) blocks the removal of the innermost glucose residue. Swainsonine is a potent inhibitor of Golgi α -mannosidase II (Tulsiani *et al.*, 1982b), and the mannose analogue 1-deoxymannojirimycin inhibits Golgi α -mannosidase I (Fuhrmann *et al.*, 1984; Bischoff & Kornfeld, 1984). 1,4-Dideoxy-1,4-imino-D-mannitol appears to inhibit both trimming enzymes (Palamarczyk *et al.*, 1985).

Active-site-directed suicide substrates provide a novel means of irreversibly inhibiting enzyme activity, and glycosylmethyl-*p*-nitrophenyltriazenes are potential suicide substrates for glycosidases (Sinnott & Smith, 1978; Van Diggelen *et al.*, 1980). Thus it has been shown (Sinnott & Smith, 1978) that β -galactopyranosylmethyl-*p*-nitrophenyltriazene is a suicide substrate for the *Escherichia coli* lac Z β -galactosidase. Furthermore it was also shown (Van Diggelen *et al.*, 1980) that β -galactopyranosylmethyl-*p*-nitrophenyltriazene and β -

Abbreviations used: MMNT, α -D-mannopyranosylmethyl-*p*-nitrophenyltriazene; pNPMa, *p*-nitrophenyl α -D-mannopyranoside; ER, endoplasmic reticulum.

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glucopyranosylmethyl-*p*-nitrophenyltriazene could specifically inactivate lysosomal β -galactosidase and β -glucosidase respectively in cultured human fibroblasts.

On the basis of these findings the mannose derivative α -D-mannopyranosylmethyl-*p*-nitrophenyltriazene (MMNT) was synthesized and tested as an inhibitor of α -mannosidase activities present in rat liver Golgi and ER membranes and jack bean. Then the effect of MMNT on mannosidase trimming of influenza-virus glycoproteins was investigated. It is shown that MMNT inhibits Golgi α -mannosidase I and jack-bean α -mannosidase *in vitro* and oligosaccharide trimming in influenza-virus-infected chick-embryo cells, but has little effect on the ER α -mannosidase.

EXPERIMENTAL

Materials

α -D-Mannopyranosylmethylamine was prepared by a modification of the procedure of Coxon & Fletcher (1964) from α -D-mannopyranosyl cyanide, which was prepared by the method of Myers & Lee (1984). *p*-Nitrobenzenediazonium tetrafluoroborate was obtained from Kodak (Rochester, NY, U.S.A.). Jack-bean α -mannosidase was from Sigma (Munich, Germany), and *p*-nitrophenyl α -D-mannopyranoside (pNPM) and Pronase were from Serva (Heidelberg, Germany). Bio-Rad (Munich, Germany) supplied Bio-Gel P4 and Bio-Gel P6 as well as Bio-Beads SM-2. Endoglucosaminidase H was bought from Genzyme (Boston, MA, U.S.A.). Pharmacia (Freiburg, Germany) supplied concanavalin A-Sepharose 4B. Aprotinin (Trasylol) was from Bayer AG (Leverkusen, Germany). D-[2-³H]Mannose (18.3 Ci/mmol) was bought from Amersham Buchler (Braunschweig, Germany). 1-Deoxymannojirimycin and swainsonine were obtained from Boehringer Mannheim (Mannheim, Germany).

Glc₃[¹⁴C]Man₉GlcNAc and [¹⁴C]Man₉GlcNAc were prepared as described previously (Datema & Schwarz, 1981). [³H]Man₉GlcNAc was prepared from Sindbis-virus-infected BHK cells treated with 1-deoxymannojirimycin (McDowell *et al.*, 1987).

[³H]Mannose-labelled Man₉GlcNAc, Man₇GlcNAc, Man₆GlcNAc and Man₅GlcNAc were prepared from influenza-virus-infected chick-embryo cells labelled with 200 μ Ci of [³H]mannose for 3 h from 4 h post-infection. The labelled cells were extracted with chloroform/methanol (2:1, v/v) and chloroform/methanol/water (10:10:3, by vol.), the lipid-free residue was digested with Pronase, and the resulting glycopeptides were desalted by passage through a column of Bio-Gel P6. After digestion with endoglucosaminidase H, the high-mannose-type oligosaccharides were separated from the complex-type glycopeptides by gel filtration through Bio-Gel P6. The individual high-mannose-type oligosaccharides were then purified by passage through a column of Bio-Gel P4 and characterized by their elution behaviour and by mannosidase digestion.

The influenza virus strains A/PR/8/34 (H1N1) and A/FPV/Rostock/34 (H7N1, fowl-plague virus) were obtained from the Institut für Virologie collection.

Synthesis of MMNT

MMNT was synthesized by following a modified (Docherty *et al.*, 1986) procedure of Sinnott & Smith

(1978), as follows. α -D-Mannopyranosylmethylamine (11.75 mg) and NaHCO₃ (10.25 mg) were dissolved in ice-cold water (1 ml) with stirring. *p*-Nitrobenzenediazonium tetrafluoroborate (13.5 mg) was then added and stirring was continued for 30 min on ice. The mixture was then diluted with ice-cold water (2 ml) and centrifuged at 4000 *g* to pellet the solids present. The pellet was washed once with 0.2 ml of water, and the combined supernatant fluids were extracted with ice-cold water-saturated diethyl ether to remove diazonium hydroxide. Triazene was removed from the ether-washed aqueous phase by extraction into butan-1-ol (4 $\times$ 1 ml). The combined butan-1-ol extracts were evaporated to dryness under oil-pump vacuum, and the residue was dissolved in ice-cold water (0.5 ml). The MMNT concentration was determined from its absorbance in ethanol at 362 nm (Sinnott & Smith, 1978); typically solutions of 15–25 mM concentration were obtained. Triazene was usually freshly prepared for each experiment. It could be stored overnight at 4 °C, or for longer periods at –20 °C (after immediate freezing in solid CO₂), without diminishing the effect.

Preparation of rat liver Golgi membranes and assay of mannosidase I activity

Rat liver Golgi membranes were prepared by the procedure of Tabas & Kornfeld (1982) with some modifications. All sucrose solutions were in 100 mM-potassium phosphate buffer, pH 6.5, containing 5 mM-MgCl₂. All solutions were ice-cold, and centrifugation steps were carried out at 4 °C. The freshly excised rat livers, placed in 4 vol. of buffered 0.5 M-sucrose, were cut up with scissors and scalpel and homogenized in an Ultra-Turrax tissue disintegrator (Janke und Kunkel AG, Staufen i. Br., Germany) until a uniform homogenate was obtained. The homogenate was centrifuged at 600 *g* for 10 min, and the supernatant fluid was layered on top of 1.3 M-sucrose (13 ml) in Beckman SW 28 centrifuge tubes. After centrifugation at 64000 *g*_{av} for 2 h in a Beckman SW 28 rotor, the membranes banding between the 0.5 M- and 1.3 M-sucrose layers were removed and adjusted to 1.1 M-sucrose with 1.3 M-sucrose. The crude membranes (18 ml portions) were then layered on top of 1.25 M-sucrose (8 ml) in Beckman SW 28 centrifuge tubes. Next 1 M-sucrose (7 ml) was layered on top of the membranes, and then 0.5 M-sucrose (5 ml) was layered on top of the 1 M-sucrose. The discontinuous gradient was centrifuged at 83000 *g*_{av} for 90 min in a Beckman SW 28 rotor. The Golgi membranes, which band at the interface between the 0.5 M- and 1 M-sucrose layers, were removed, diluted to 0.25 M-sucrose and 0.4 M-NaCl, and centrifuged at 178000 *g*_{av} in a Beckman Ti60 rotor for 30 min. The pelleted membranes were taken up in 50 mM-Tris/HCl buffer, pH 7.5, containing 1% Triton X-100 and 5 mM-MgCl₂ (1 ml per eight rat livers) and stored at –70 °C.

The rat liver Golgi membranes were used as a source of α -mannosidase I, which was assayed as follows. Golgi membranes (10 μ l) were incubated with [³H]-Man₉GlcNAc (5000 d.p.m.), 25 mM-potassium phosphate buffer, pH 6.8, 0.05% Triton X-100 and 2.5 mM-MgCl₂ in a total volume of 30 μ l for 1 h at 37 °C. The reaction was stopped by adding pyridine (20 μ l), and released mannose was separated from oligosaccharide by paper chromatography on Whatman 3MM paper with butan-1-ol/pyridine/water (6:4:3, by vol.) as the irri-

gant. Radioactivity in 1 cm strips was measured by liquid-scintillation spectrometry. One unit of enzyme activity catalysed the release of 1 % of [^3H]mannose from [^3H]Man $_6$ GlcNAc/min at 37 °C.

Preparation and assay of ER α -mannosidase

ER membranes free of Golgi membranes were prepared exactly as described by Kreibach *et al.* (1978) as modified by Bischoff & Kornfeld (1983) with Aprotinin (0.17 trypsin-inhibitor unit/ml) in the sucrose and buffer solutions. The ER α -mannosidase was solubilized with sodium deoxycholate and partially purified by affinity chromatography through concanavalin A-Sepharose 4B as described by Bischoff & Kornfeld (1983). The enzyme preparation was active in the presence of swainsonine (pNPMan substrate) or 1-deoxymannojirimycin (Man $_6$ GlcNAc substrate), as should be the case for the ER enzyme (Bischoff & Kornfeld, 1983, 1984). The preparation appeared to be free of contaminating α -mannosidases sensitive to these inhibitors.

ER α -mannosidase activity was assayed with 4 mM-pNPMan as substrate in 50 mM-potassium phosphate buffer, pH 6.5, and 10 μM -swainsonine (to inhibit any contaminating Golgi mannosidase II activity) in a total volume of 100 μl . The assay mixture was incubated at 37 °C for 60 min. The reaction was stopped by the addition of 0.2 M- Na_2CO_3 (1 ml). Release of *p*-nitrophenol was determined by measuring the absorbance at 405 nm. ER α -mannosidase activity was also measured with [^3H]Man $_6$ GlcNAc (5000 c.p.m.) as substrate. Incubation time was for 3 h and mannose release was measured as described for the Golgi α -mannosidase I assay. However, in this case 10 μM -1-deoxymannojirimycin was included in the assay mixture as an inhibitor of any contaminating mannosidases because swainsonine inhibits ER α -mannosidase at subsaturating substrate concentrations (Bischoff & Kornfeld, 1983), and with the oligosaccharide as substrate it is not possible to saturate the enzyme. In addition, the main potential interfering enzyme is Golgi mannosidase I, which is insensitive to swainsonine but sensitive to 1-deoxymannojirimycin (Bischoff & Kornfeld, 1984).

Jack-bean α -mannosidase assay

The commercially available enzyme was diluted 50-fold with 200 mM-sodium acetate buffer, pH 4.2, containing 40 μM - ZnCl_2 . Diluted enzyme (1 μg of protein) was incubated with 100 mM-acetate buffer, pH 4.2, containing 20 μM - ZnCl_2 and 5 mM-pNPMan for 5 min at 37 °C. The reaction was stopped by the addition of 0.2 M- Na_2CO_3 , and the absorbance at 405 nm was measured to determine the release of *p*-nitrophenol. One unit of enzyme activity catalyses the formation of 1 μmol of *p*-nitrophenol/min at 37 °C.

Other procedures

Columns (1 cm $\times$ 100 cm) of Bio-Gel P6 (200–400 mesh) were used for the separation of glycopeptides generated by Pronase digestion of labelled glycoproteins. Elution was with 0.1 M-pyridinium acetate; 0.5 ml fractions were collected.

Labelled oligosaccharides were separated on columns (1 cm $\times$ 150 cm) of Bio-Gel P4 (–400 mesh) with 0.02 % NaN_3 as eluent; 0.4 ml fractions were collected.

Fig. 1. Effect of MMNT on α -mannosidases from jack bean, rat liver Golgi membranes and rat liver ER membranes

The enzymes were assayed as described in the Experimental section and were preincubated with MMNT for 5 min before the addition of substrate to initiate the reaction. When the substrate was pNPMan, separate blanks for each MMNT concentration were prepared to correct for any absorbance by the triazene. ○, Jack-bean α -mannosidase; ●, rat liver Golgi α -mannosidase I; ■, rat liver ER α -mannosidase with pNPMan substrate; □, ER α -mannosidase with [^3H]Man $_6$ GlcNAc substrate. Initial activities (100 %) were 7.8 nmol of *p*-nitrophenol formed/min (jack-bean α -mannosidase), 0.32 % [^3H]mannose released/min (rat liver Golgi α -mannosidase), 0.14 nmol of *p*-nitrophenol formed/min (rat liver ER α -mannosidase, pNPMan substrate) and 0.19 % [^3H]mannose released/min (rat liver ER α -mannosidase, [^3H]Man $_6$ GlcNAc substrate).

Digestions with endoglucosaminidase H, Pronase and α -mannosidase were performed as previously described (Datema & Schwarz, 1981).

Infection of monolayers of chick-embryo cells with influenza virus was done as described previously (Datema & Schwarz, 1981). The cells were maintained in Dulbecco's medium containing 10 mM-glucose. Quantification of virus infectivity by plaque assay was performed as described by Klenk *et al.* (1975), and the determination of haemagglutination titre was done by the procedure of Davenport *et al.* (1960). Neuraminidase activity was assayed by the method of Drzenick *et al.* (1966).

RESULTS AND DISCUSSION

Inhibition of some α -mannosidases by MMNT

The effectiveness of MMNT as an inhibitor of α -mannosidase activity was tested with a commercially available jack-bean α -mannosidase preparation and purified rat liver Golgi membranes containing α -mannosidase I activity. In addition, purified rat liver ER membranes were used as a source of ER α -mannosidase activity.

Incubation of MMNT with both jack-bean α -mannosidase and rat liver Golgi α -mannosidase I resulted in a concentration-dependent decrease in mannosidase activity towards their respective substrates (Fig. 1). In both cases high concentrations of MMNT were required for inhibition. The MMNT concentrations giving 50 %

Fig. 2. Time course of [^3H]mannose release from [^3H]Man₆GlcNAc by Golgi α -mannosidase I in the absence and in the presence of 0.5 mM-MMNT

Rat liver Golgi α -mannosidase I was incubated with [^3H]Man₆GlcNAc as described in the Experimental section in the absence (●) and in the presence (○) of 0.5 mM-MMNT. The percentage of mannose released was determined after separation of product and substrate by paper chromatography (see the Experimental section).

inhibition of activity were determined by extrapolation to be 0.18 mM and 0.32 mM for the Golgi and jack-bean enzymes respectively. These values are high when compared with 1-deoxymannojirimycin, which inhibits Golgi α -mannosidase I with a K_i of 2 μM (Bischoff & Kornfeld, 1984). However, since MMNT is a suicide substrate, one molecule will be required to inactivate each molecule of enzyme. Also, alkylaryltriazenes are known to be labile in solution at pH values below 7 and at elevated temperatures (Sinnott & Smith, 1978), and this may be partly responsible for the relatively high concentrations of MMNT needed to inactivate the jack-bean and rat liver Golgi α -mannosidases.

Incubation of MMNT with a partially purified ER α -mannosidase preparation did not inhibit enzyme activity towards pNPMAN or [^3H]Man₆GlcNAc (Fig. 1). It was noted by Bischoff & Kornfeld (1983) that rat liver ER α -mannosidase was not inhibited by 1-deoxymannojirimycin, whereas swainsonine only inhibited the enzyme when the substrate was present at subsaturating concentrations. Thus MMNT appears to have effects similar to those of 1-deoxymannojirimycin.

Effect of MMNT on mannosidase trimming *in vitro*

The time course of mannose release from Man₆GlcNAc substrate catalysed by rat liver Golgi α -mannosidase I was measured over a 4 h incubation period (Fig. 2). The inclusion of 0.5 mM-MMNT in the incubation mixture resulted in a significant decrease in mannose release over the total incubation period (Fig. 2). Analysis of the products formed during a 4 h incubation by gel filtration showed the accumulation of Man₆GlcNAc and Man₅GlcNAc and the disappearance of Man₇GlcNAc in the uninhibited reaction [results of shorter incubation times not shown; Fig. 3(a) depicts the products after 4 h incubation]. In the presence of 0.5 mM-MMNT, however, Man₆GlcNAc was the predominant oligosaccharide present, and only after 3 h incubation were significant amounts of Man₆GlcNAc and

Fig. 3. Effect of increasing MMNT concentration on the digestion of Man₆GlcNAc by Golgi α -mannosidase I

Rat liver Golgi α -mannosidase was incubated with [^3H]Man₆GlcNAc for 4 h in the absence (a) and in the presence (b-e) of MMNT. The reactions were stopped by heating in a boiling-water bath for 5 min. Denatured protein was removed by centrifugation, and the samples were analysed on a column of Bio-Gel P4 calibrated with Man₆GlcNAc (9), Man₅GlcNAc (8), Man₇GlcNAc (7), Man₆GlcNAc (6) and Man₅GlcNAc (5). (b) 0.1 mM-MMNT; (c) 0.2 mM-MMNT; (d) 0.5 mM-MMNT; (e) 1 mM-MMNT.

Man₇GlcNAc oligosaccharides produced [results of shorter incubation times not shown; Fig 3(d) depicts the products after 4 h incubation].

To assess the effects of different concentrations of MMNT on the digestion of Man₆GlcNAc, the products formed after 4 h incubation in the presence of 0 mM-, 0.1 mM-, 0.2 mM-, 0.5 mM- and 1.0 mM-MMNT were

examined by gel filtration on Bio-Gel P4 (Fig. 3). In the absence of inhibitor $\text{Man}_6\text{GlcNAc}$ and $\text{Man}_5\text{GlcNAc}$ are the only products of hydrolysis (Fig. 3a). Increasing the MMNT concentration leads to a decrease in the amount of trimming taking place and to an increase in the size of the oligosaccharides present (Figs. 3b–3e). In all incubations containing MMNT (Figs. 3b–3e) $\text{Man}_6\text{GlcNAc}$ is still present whereas it is absent from the control incubation (Fig. 3a).

These findings indicate that MMNT is an effective inhibitor of Golgi α -mannosidase I activity in rat liver Golgi membranes and may be useful as an inhibitor of mannosidase trimming *in vivo*.

Effect of MMNT on mannosidase trimming in influenza-virus-infected chick-embryo cells

To determine whether MMNT is an inhibitor of mannosidase trimming *in vivo* chick-embryo cells infected with influenza virus A/PR/8/34 were treated with the drug. The glycoproteins of this virus strain are glycosylated with a high proportion of complex-type oligosaccharides (Datema *et al.*, 1982) in chick-embryo cells. Influenza-virus-PR8-infected chick-embryo cells were treated with 1 mM-MMNT from 1 h post-infection. The cells were labelled with [^3H]mannose for 2 h from 4 h post-infection and then extracted with chloroform/methanol (2:1, v/v) and chloroform/methanol/water (10:10:3, by vol.). The lipid-free residue containing the influenza-virus glycoproteins was digested with Pronase. The resulting glycopeptides were desalted by passage through a column of Bio-Gel P6 and digested with endoglucosaminidase H to liberate high-mannose-type oligosaccharides. The endoglucosaminidase H digest was analysed by passage through a column of Bio-Gel P4 (Fig. 4). Analysis of the untreated sample showed that the viral glycoproteins are predominantly glycosylated with complex-type oligosaccharides (Fig. 4a). Complex-type glycopeptides, which were eluted between the void volume and the $\text{Glc}_3\text{Man}_6\text{GlcNAc}$ marker, constituted 54% of the eluted radioactive label. A series of high-mannose-type oligosaccharides with the elution characteristics of $\text{Man}_6\text{GlcNAc}$, $\text{Man}_5\text{GlcNAc}$, $\text{Man}_4\text{GlcNAc}$ and $\text{Man}_3\text{GlcNAc}$ were eluted after the marker (Fig. 4a) and constituted 46% of the eluted radioactive label. In contrast, in the treated sample the radioactive label associated with the complex-type glycopeptides was 25% and that associated with the high-mannose-type oligosaccharides was 75%, indicating that the formation of complex-type oligosaccharides had been inhibited by treatment with MMNT. Analysis of the treated sample showed that the viral glycoproteins are glycosylated predominantly with high-mannose-type oligosaccharides with the elution characteristics of $\text{Man}_6\text{GlcNAc}$ and $\text{Man}_5\text{GlcNAc}$ (Fig. 4b). In the untreated sample $\text{Man}_6\text{GlcNAc}$, $\text{Man}_5\text{GlcNAc}$ and $\text{Man}_4\text{GlcNAc}$ represented 22%, 22% and 20% of the high-mannose-type oligosaccharides respectively, whereas in the MMNT-treated sample the proportions were 8%, 28% and 26% respectively. This shows that in the presence of the mannosidase inhibitor the Man_7 and Man_8 intermediates have increased at the expense of the Man_6 intermediate. Increasing the MMNT concentration to 2 mM did not increase the extent of inhibition of complex-type oligosaccharide formation or the accumulation of $\text{Man}_6\text{GlcNAc}$ (results not shown).

The presence of both $\text{Man}_8(\text{GlcNAc})_2$ and

Fig. 4. Glycosylation of influenza-virus glycoproteins in the absence (a) and in the presence (b) of 1 mM-MMNT

Influenza-virus-PR8-infected chick-embryo cells were treated with 1 mM-MMNT for 3 h, then labelled with [^3H]mannose for 2 h. The labelled cells were extracted with chloroform/methanol (2:1, v/v) and chloroform/methanol/water (10:10:3, by vol.), and the lipid-free residue was digested with Pronase. The glycopeptides were desalted on a column of Bio-Gel P6 and digested with endoglucosaminidase H. The liberated high-mannose-type oligosaccharides were separated from the complex-type glycopeptides by gel filtration on Bio-Gel P4. Complex-type glycopeptides were eluted between the void volume (V_0) and the marker $\text{Glc}_3\text{Man}_6\text{GlcNAc}$ (G). The high-mannose-type oligosaccharides were eluted after this marker. The elution positions of $\text{Man}_6\text{GlcNAc}$, $\text{Man}_5\text{GlcNAc}$, $\text{Man}_4\text{GlcNAc}$ and $\text{Man}_3\text{GlcNAc}$ are represented by arrows 9, 8, 7 and 6 respectively.

$\text{Man}_7(\text{GlcNAc})_2$ on the viral glycoproteins could be due to incomplete inhibition of the cellular α -mannosidase I activity. In order to examine this possibility, the activities of the cellular α -mannosidase activities towards $\text{Man}_6\text{GlcNAc}$ at pH 6.8 and pNPMn at pH 4.2 in Triton X-100 lysates prepared from infected cells treated with up to 2 mM-MMNT were assayed (Fig. 5). Both mannosidase activities were inhibited to a maximum of about 60% after treatment of the cells with 1–2 mM-MMNT (Fig. 5), confirming the suspicion that there was incomplete inhibition of α -mannosidase I activity. These findings contrast with those obtained in a study with rat liver hepatocytes (Docherty *et al.*, 1986), in which an unspecified α -mannosidase activity was decreased to a residual level of 20% with 1 mM-MMNT. In human skin fibroblasts treated with 1 mM- β -galactopyranosylmethyl-p-nitrophenyltriazene there was less than 1% residual β -galactosidase activity (Van Diggelen *et al.*, 1980). These two results suggest that chick-embryo cells may not take up triazene as efficiently as other cell types, leading to

Fig. 5. Inhibition of α -mannosidase activities in influenza-virus-infected chick-embryo cells

Virus-infected chick-embryo cells were treated with various concentrations of MMNT for 4 h. The medium was removed, and the cell monolayers were washed three times with 170 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.6. The cells were lysed by incubation with 0.25% Triton X-100 (200 μ l) for 30 min on ice. The lysates were used immediately for the assay of α -mannosidase I (●) and acid α -mannosidase (○) as described for the rat liver and jack-bean enzymes with the exception that incubation times were 2 h and 30 min respectively. The enzyme activities were corrected for differences in protein concentration. Each point is the average for three separate experiments.

lower concentrations of triazene within the chick-embryo cell and thus a lower degree of inhibition than would have been expected from the results with hepatocytes and human skin fibroblasts. It is also possible that the residual neutral and acidic α -mannosidase activities are due respectively to cellular ER α -mannosidase and Golgi mannosidase II, which can have activity towards pNPMAN at pH 4.0 (Tulsiani *et al.*, 1982a).

The presence of $\text{Man}_8(\text{GlcNAc})_2$ and $\text{Man}_7(\text{GlcNAc})_2$ and not $\text{Man}_6(\text{GlcNAc})_2$ as the predominant high-mannose-type oligosaccharide species formed in the presence of MMNT indicates that ER α -mannosidase has probably been involved in oligosaccharide processing in influenza-virus-infected chick-embryo cells treated with MMNT. The results obtained *in vitro* with rat liver ER α -mannosidase (Fig. 1) indicated that the enzyme is not as susceptible to inhibition by MMNT as Golgi α -mannosidase I. Even allowing for the fact that the chick-embryo-cell Golgi α -mannosidase I was only 60% inhibited (Fig. 5), $\text{Man}_6\text{GlcNAc}_2$ should still have formed a significant proportion of the high-mannose-type oligosaccharides, when the data obtained *in vitro* with the rat liver enzyme (Fig. 5) are considered. In fact the proportion of $\text{Man}_6(\text{GlcNAc})_2$ was decreased in the MMNT-treated cells when compared with the proportion in untreated cells, suggesting that some trimming by the ER α -mannosidase has taken place.

Virus-infected cells have proved to be good systems for investigating the modes of action of trimming inhibitors (Schwarz & Datema, 1984; Datema *et al.*, 1987). As a consequence of these studies an essential role for glucose trimming in the establishment of a functional conformation for some viral glycoproteins has been found (Datema *et al.*, 1984; Schlesinger *et al.*, 1984,

Table 1. Biological properties of influenza virus (fowl-plague virus) synthesized in the presence of MMNT

Chick-embryo cells infected with influenza virus (A/FPV/Rostock/34) were treated with the indicated concentration of MMNT from 1 h post-infection. At 12 h post-infection the cells and medium were scraped together and subjected to three cycles of freeze-thaw treatment. Cellular debris was removed by centrifugation at 4000 g, and the resulting supernatant fluid was used immediately for measurement of haemagglutination titre and quantification of virus by plaque assay. The haemagglutination titre is the last dilution showing a positive reaction in the haemagglutination assay. Neuraminidase activity was assayed after overnight dialysis against 170 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.6, with fetuin as substrate. One neuraminidase unit is defined as the amount of enzyme that releases 1 μ mol of *N*-acetylneuraminic acid/min at 37 °C. Abbreviations: HA units, haemagglutination units; NA units, neuraminidase units; PFU, plaque-forming units.

Concn. of MMNT (mM)	Haemagglutination activity (HA units/ml)	Neuraminidase activity (NA units/ml)	Virus infectivity (PFU/ml)
0.0	4096	2.33	3.2×10^7
0.1	4096	1.42	3.5×10^7
0.2	8192	2.78	4.0×10^7
0.5	8192	2.48	5.7×10^6
1.0	16384	2.36	1.6×10^6
2.0	1024	0.97	5.0×10^4

1985; Repp *et al.*, 1985; McDowell *et al.*, 1987). In contrast, inhibition of mannose trimming has little effect with the exception of Sindbis virus, which buds from intracellular membranes in BHK cells treated with the mannosidase I inhibitor 1-deoxymannojirimycin (McDowell *et al.*, 1987). Trimming inhibitors have no effect on the formation of influenza A viruses (Romero *et al.*, 1983; Pan *et al.*, 1983; Datema *et al.*, 1984; Elbein *et al.*, 1984) except for the fowl-plague virus strain, the haemagglutinin of which is metabolically unstable when equipped with oligosaccharides of the composition $\text{Glc}_1\text{Man}_6(\text{GlcNAc})_2$ in the presence of bromoconduritol (Datema *et al.*, 1984).

The biological properties of influenza virus formed in the presence of MMNT were examined to see whether the changes in the oligosaccharide composition of the two viral membrane glycoproteins, the haemagglutinin and the neuraminidase, had altered their respective activities (Table 1). Treatment of influenza-virus-A/FPV/Rostock/34-infected chick-embryo cells with up to 1 mM-MMNT did not adversely affect either the haemagglutinin or the neuraminidase activities of the virus formed in the presence of the drug (Table 1). However, 2 mM-MMNT was inhibitory in each case (Table 1). The infectivity of the virus was not affected by low concentrations (0.1–0.2 mM) of MMNT, but at higher concentrations (0.5–2.0 mM) there was a significant decrease in the virus titre as determined by plaque assay (Table 1). Since MMNT did not affect protein synthesis in influenza-virus-infected chick-embryo cells except at a concentration of 2 mM (results not shown), it would appear that the synthesis of the influenza-virus haemagglutinin and neuraminidase are not affected by MMNT treatment, whereas the

production of infectious virus particles seems to be sensitive. This contrasts with the effects of the mannosidase I inhibitor 1-deoxymannojirimycin, which has no effect on the formation of influenza virus (Elbein *et al.*, 1984; Tlusty, 1984). It is possible that influenza-virus haemagglutinin and neuraminidase glycosylated with predominantly high-mannose-type oligosaccharides of the composition $\text{Man}_7(\text{GlcNAc})_2$ and $\text{Man}_8(\text{GlcNAc})_2$ are not incorporated efficiently into influenza-virus particles. The infectivity of influenza viruses is known to be related to the cleavability of their haemagglutinins (Webster & Rott, 1987). Thus in chick-embryo cells the haemagglutinin of fowl-plague virus is cleaved and the virus can form plaques, whereas that of influenza virus PR8 is not cleaved and plaques can only be formed if the virus is pretreated with trypsin (Klenk *et al.*, 1975). Thus there may be the possibility that MMNT treatment could have impaired the proteolytic cleavage of the fowl-plague-virus haemagglutinin, producing virus particles containing an uncleaved haemagglutinin. Such virus particles would not form plaques in the plaque assay, giving rise to diminished virus titres. However, pretreatment of virus formed in the presence of MMNT with trypsin (10 $\mu\text{g}/\text{ml}$) for 20 min at 37 °C did not increase the infectivity of the virus (results not shown). In addition, the infectivity of influenza virus PR8 was affected by MMNT treatment in a similar fashion to that of fowl-plague virus (results not shown). This would not have been the case if there was an effect on the cleavability of the haemagglutinin, since influenza virus PR8 needs to be pretreated with trypsin before determination of the virus infectivity by plaque assay in chick-embryo cells (Klenk *et al.*, 1975). Therefore an effect on the cleavage of the haemagglutinin as a cause of the decrease in infectivity of influenza virus synthesized in the presence of 1 mM-MMNT can be ruled out. Thus it would appear that the two viral glycoproteins are not being efficiently incorporated into virus particles in MMNT-treated cells.

The results presented indicate that MMNT is an inhibitor of oligosaccharide trimming catalysed by Golgi α -mannosidase I and is a useful addition to the oligosaccharide-processing inhibitors already available. Since glycosylmethyl-*p*-nitrophenyltriazines are active-site-directed irreversible inhibitors of glycosidases, they can be used to study turnover of these enzymes, as shown by Van Diggelen *et al.* (1980). Indeed the use of MMNT in a study of the degradation and biosynthesis of oligosaccharide chains on α_1 -acid glycoprotein in perfused liver and in cultured hepatocytes (Docherty *et al.*, 1986) indicates the feasibility of such an approach.

During the preparation of the manuscript a paper by Docherty & Aronson (1987) dealing with the effects of MMNT on rat liver α -mannosidases was published. Their findings were largely similar to those reported here, and in addition it was reported that Golgi mannosidase II is virtually insensitive to MMNT treatment. It was also reported that a soluble α -mannosidase, deemed to be the ER enzyme, was inhibited by 90% with 1 mM-MMNT. In contrast, we have found a more purified preparation of ER α -mannosidase to be less sensitive to MMNT. The ER α -mannosidase and soluble α -mannosidase of rat liver have essentially the same properties and have been shown to be immunologically related (Bischoff & Kornfeld, 1986). The contradiction between our findings and those of Docherty & Aronson

(1987) may be due to the fact that the free cytosolic enzyme is more sensitive to MMNT than is the membrane-bound enzyme. This could be brought about by the two enzymes adopting different conformations. The cytosolic enzyme may be less constrained than the membrane-bound enzyme, the conformation of which may be subject to interactions from the other protein components of the ER membrane.

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