Oligomannosides or oligosaccharide-lipids as potential substrates for rat liver cytosolic α-D-mannosidase

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We have previously reported the substrate specificity of the cytosolic α-D-mannosidase purified from rat liver using $Man_9GlcNAc$, i.e. Manα1-2Manα1-3(Manα1-2Manα1-6)Man α1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc, as substrate [Grard, Saint-Pol, Haeuw, Alonso, Wieruszeski, Strecker and Michalski (1994) Eur. J. Biochem. **223**, 99–106]. $Man_9GlcNAc$ is hydrolysed giving $Man_5GlcNAc$, i.e. Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc, possessing the same structure as the oligosaccharide of the dolichol pathway formed in the cytosolic compartment during the biosynthesis of *N*-glycosylprotein glycans. We study here the activity of the purified cytosolic α-D-mannosidase towards the oligosaccharide-diphosphodolichol intermediates formed during the biosynthesis of *N*-glycans, and also towards soluble oligosaccharides released from the endoplasmic reticulum which are glucosylated or not and possessing at their reducing end either a single *N*-acetylglucosamine residue or a di-*N*-acetylchitobiose sequence. We demonstrate that (1) dolichol pyrophosphate oligosaccharide substrates are poorly hydrolysed by the cytosolic α-D-mannosidase; (2) oligosaccharides with a terminal reducing di-*N*-acetylchitobiose sequence are not hydrolysed at all; (3) soluble oligosaccharides bearing a single reducing *N*-acetylglucosamine are the real substrates for the enzyme. These results suggest a role for α-D-mannosidase in the catabolism of glycans released from the endoplasmic reticulum rather than in the regulation of the biosynthesis of asparagine-linked oligosaccharides.

INTRODUCTION

We have previously purified cytosolic α-D-mannosidase activity [1]. This enzyme differs completely from other cellular mannosidases in its physicochemical and kinetic properties and its specificity towards natural substrates [2]. *In vitro* substrate-specificity studies reveal that the cytosolic α-D-mannosidase is able to cleave the $Man_9GlcNAc$ substrate, i.e. Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6(Manα1-2Manα1-2Manα1-3)-Manβ1-4GlcNAc, giving a single final product consisting of $Man_5GlcNAc$, i.e. Manα1-6(Manα1-2Manα1-2Manα1-3)-Man β1-4GlcNAc [1,3–5]. In this report, we investigate further the activity of the enzyme towards potential cellular endogenous substrates. Free oligomannosides released during N-glycosylation [6–13], and processed by the action of endoplasmic reticulum-specific glucosidases [14–16] and mannosidase [17,18], could be the physiological substrates of the enzyme. These neutral oligosaccharides, released from the endoplasmic reticulum into the cytosol by a transport process [19], possess a di-*N*-acetylchitobiose sequence (OS-$GlcNAc_2$) or a single *N*-acetylglucosamine residue (OS-$GlcNAc_1$) at their reducing end. These oligomannosides can be glucosylated as observed in the case of oligosaccharides prepared from permeabilized cells in the presence of the glucosidase inhibitor, castanospermine [12,13].

A second group of potential substrates is the oligosaccharide-lipid intermediates. Biosynthesis of precursors of protein N-glycosylation is initiated by the formation of $GlcNAc_2$-P-P-Dol. Then five mannoses are added yielding $Man_5GlcNAc_2$-P-P-Dol, i.e. Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-P-P-dolichol. Thereafter, four mannosyl and three glucosyl residues are added to form $Glc_3Man_9GlcNAc_2$-P-P-Dol which is the glycosyl donor for nascent proteins in the lumen of the rough endoplasmic reticulum [20]. Studies on the topography of the previous reactions have shown that the biosynthesis of these dolichol derivatives occurs at both sides of the endoplasmic reticulum membrane [21]. $Man_5GlcNAc_2$-P-P-Dol has been detected on the cytosolic side of the endoplasmic reticulum membrane [22] whereas the subsequent dolichol intermediates, with additional mannosyl and glucosyl residues, have been found at the lumenal side [22,23].

We hypothesized that $Man_9GlcNAc_2$-P-P-Dol biosynthesis could occur on both sides of the endoplasmic reticulum membrane. Thus cytosolic $Man_5GlcNAc_2$-P-P-Dol could be generated by both a biosynthetic pathway, using a step by step addition of mannosyl residues from GDP-Man, and a catabolic pathway from $Man_9GlcNAc_2$-P-P-Dol by the action of the cytosolic enzyme α-D-mannosidase.

In order to determine precisely the exact role of cytosolic α-D-mannosidase in either the catabolism of free oligosaccharides originating from the endoplasmic reticulum or the processing of the oligosaccharide-lipids as a possible alternative route for the

Abbreviations used: P-P-Dol, diphosphodolichol; $Man_9GlcNAc_2$-P-P-Dol, Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-P-P-Dol; GDP-Man, guanosyl diphosphomannose; Dol-P-Man, mannosylphosphoryldolichol; OS-$GlcNAc_2$, soluble oligosaccharides possessing a di-*N*-acetylchitobiose sequence at their reducing end; OS-$GlcNAc_1$, soluble oligosaccharides possessing a single *N*-acetylglucosamine residue at their reducing end; PNGase F, peptide *N*-glycosidase F (EC 3.5.1.52); Endo H, endo-*N*-acetyl-β-D-glucosaminidase H (EC 3.2.1.96).

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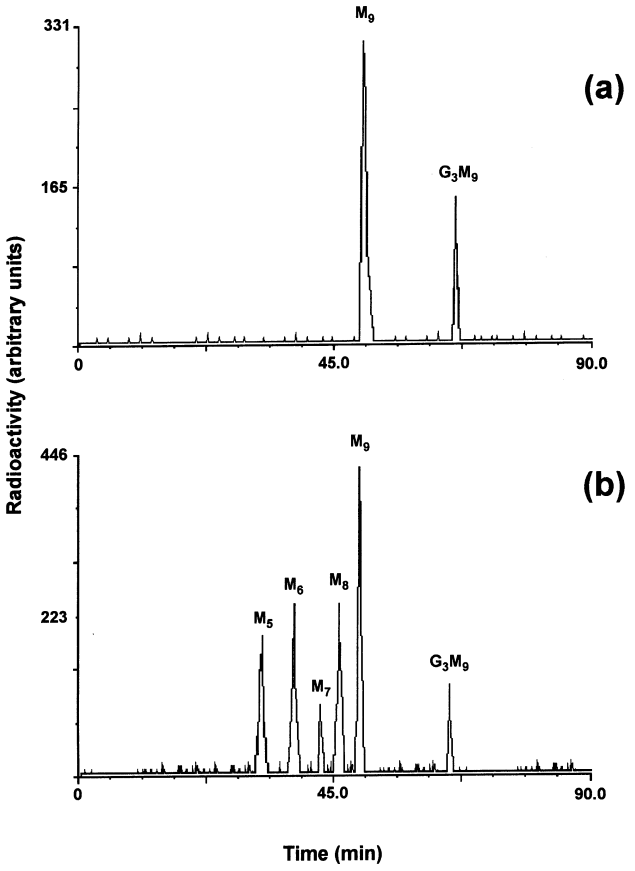


Figure 1 Degradation of lipid-intermediates by cytosolic α-D-mannosidase

Oligosaccharides-P-P-Dol were extracted after metabolic labelling of CHO cells with [2-^{3}H]mannose. Oligosaccharide-lipids were then submitted to mild acid hydrolysis before (**a**) or after (**b**) a 16 h incubation with purified cytosolic α-D-mannosidase. The glycan moieties were analysed by HPLC as described in the Materials and methods section. M_n, oligomannosides with n mannose residues; G_3M_9, oligomannosides with nine mannose residues and three glucose residues.

biosynthesis of *N*-glycosylproteins, we here investigate the specificity of the enzyme towards these different types of endogenous natural substrates.

MATERIALS AND METHODS

Cell preparation and culture

The CHO cell line was the Pro-auxotrophic clone Pro^{-5} [24]. These cells were used to prepare oligosaccharides derived from the glycan moiety linked to protein and oligosaccharide-lipids. Cells were routinely cultured in monolayers in α-minimal essential medium with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, U.S.A.) at 34 °C in 10 cm culture dishes under 5% CO_2.

Metabolic labelling and extraction

Before reaching confluence (5×10^6–7×10^6 cells/dish), cells were labelled with [2-^{3}H]mannose (429 GBq/mmol) from Amersham International (Amersham, Bucks., U.K.) (50 μCi/ml in α-minimal essential medium) with 0.5 mM glucose as described previously [25]. When used, castanospermine, an inhibitor of glucosidase I was added 30 min before labelling in order to avoid deglucosylation and was present throughout the incubation period at a final concentration of 50 μg/ml. At the end of the labelling period, the medium was removed and the cell layer washed rapidly three times with ice-cold PBS. Sequential lipid extraction was achieved as previously described [7,11]. Briefly, the interphase obtained after centrifugation of a mixture of chloroform/methanol/cell suspension (3:2:1, by vol.) was washed five times with the theoretical upper phase and extracted three times with chloroform/methanol/water (10:10:3, by vol.). This extract contained the oligosaccharide-lipids (mainly $Man_9GlcNAc_2$-P-P-Dol) [26], and the remaining pellet contained the newly glycosylated proteins.

The protein pellet was digested overnight at room temperature with 0.2 mg of Tos-Phe-CH_2Cl-treated trypsin (Sigma, St. Louis, MO, U.S.A.) in 0.1 M NH_4HCO_3, pH 7.9, in a final volume of 0.2 ml.

Substrates

Two types of oligosaccharide were prepared from the glycan moiety linked to glycopeptides as described previously [27]. The first consisted of oligosaccharide containing a single *N*-acetylglucosamine residue at the reducing end and was prepared by using the endo-*N*-acetyl-β-D-glucosaminidase H (Endo H) from Boehringer, Mannheim, Germany. The second, consisting of oligosaccharides with two GlcNAc residues at the reducing end, was prepared by cleavage using peptide *N*-glycosidase F (PNGase F) from Boehringer. The trypsin-treated protein obtained previously was boiled for 10 min to destroy trypsin activity and the tryptic peptides were dried and dissolved in either 50 mM sodium phosphate, pH 5.5, and incubated overnight with 10 m-units of Endo H or 20 mM sodium phosphate (pH 7.5) containing 50 mM EDTA, 50% glycerol and 0.02% NaN_3 and incubated overnight with 0.5 unit of PNGase F. After purification on Bio-Gel P2, oligosaccharides were separated by HPLC.

Purification of cytosolic α-D-mannosidase

Cytosolic α-D-mannosidase (EC 3.2.1.24) was purified as previously described [1]. Briefly, after 40%-satd. $(NH_4)_2SO_4$ precipitation of the cytosol, the enzyme was purified using a combination of concanavalin A–Sepharose, cobalt chelating Sepharose and anion-exchange chromatography and gel filtration.

Enzymic digestion and separation of the hydrolysis products

Enzymic digestions of oligosaccharide-P-P-Dol substrates were performed in 50 mM sodium cacodylate buffer, pH 6.2, in the presence of 0.1% Triton X-100 (to solubilize the oligosaccharide-P-P-Dol substrates) and 1 mM $CoCl_2$. Approx. 500000 c.p.m. of substrate (30 nM) were incubated with approx. 10 μg of purified cytosolic α-D-mannosidase (2400 units/mg) for 4 or 16 h. In order to determine precisely the level of hydrolysis of the glycan moiety, the oligosaccharide-lipids were then submitted to mild acid treatment (0.1 M HCl in tetrahydrofuran at 50 °C for 2 h) [28], and the released oligosaccharides were purified on a Bio-Gel P-2 column (1 cm × 50 cm; 200–400 mesh) in 0.1 M acetic acid, before HPLC analysis.

Soluble oligosaccharides (approx. 30 nM) were hydrolysed at 37 °C in 50 mM sodium cacodylate buffer, pH 6.2, and 1 mM $CoCl_2$. Incubation was carried out for 4 h as described for $Man_9GlcNAc$ substrate [1].

HPLC analysis

Soluble oligosaccharides were analysed on an amino-derivatized column (NH_2P-50 Asahipak column; Asahi Chemical Industry Co.). The column was equilibrated in a solvent system consisting of acetonitrile/water (70:30, v/v). A linear gradient up to acetonitrile/water (50:50, v/v) was then applied, at a flow rate of 1 ml/min for 90 min. Separation of the labelled oligosaccharides was followed by continuous-flow detection of the radioactivity with a Flo-one β-detector (Flotec) using Luma flow II (Lumac) as scintillation fluid.

Oligosaccharides obtained by enzymic hydrolysis were identified by co-chromatography with standard oligosaccharides obtained from urine of patients with mannosidosis (results not shown).

RESULTS

Hydrolysis of $Man_9GlcNAc_2$-P-P-Dol by cytosolic α-D-mannosidase

After 16 h incubation, $Man_9GlcNAc_2$-P-P-Dol (Figure 1a) was hydrolysed by purified cytosolic α-D-mannosidase (Figure 1b) to give the following products Man_8, Man_7, Man_6 and $Man_5GlcNAc_2$-P-P-Dol. It should be noted that a 16 h incubation time was required for the formation of 11.17% $Man_5GlcNAc_2$-P-P-Dol. No degradation products were detected after 4 h. It has been verified that Triton X-100 in the range 0–0.5% (v/v) which is used in the experiments at 0.1% (v/v), did not affect the catalytic properties of the cytosolic α-D-mannosidase (results not shown).

Hydrolysis of soluble oligosaccharides ($OS\text{-}GlcNAc_2$ or $OS\text{-}GlcNAc_1$) synthesized in the absence of castanospermine

In the absence of castanospermine, the glycan moieties of the newly synthesized glycoproteins were mainly composed of Glc_1Man_9, Man_9 and Man_8 species. These oligosaccharides may be obtained as either $OS\text{-}GlcNAc_2$ after treatment with PNGase F (Figure 2a) or $OS\text{-}GlcNAc_1$ after treatment with Endo H (Figure 2c). After 4 h of incubation, these two substrates were exposed to purified cytosolic α-D-mannosidase. $OS\text{-}GlcNAc_2$ were not hydrolysed by the enzyme (Figure 2b), whereas, under the same conditions, $OS\text{-}GlcNAc_1$ were completely hydrolysed (Figure 2d), giving $Glc_1Man_5GlcNAc_1$, $Man_5GlcNAc_1$ and $Man_4GlcNAc_1$.

It should be mentioned that a Man_4 isomer was partially formed during our experiments. This isomer (Figure 2d) may originate from the Man_8 isomer that was initially present in the substrate preparation (Figure 2c). The Man_8 isomer corresponds to the A isomer missing the mannose D_1 as a result of vesicular endo-α-D-mannosidase hydrolysis [29]. The Man_4 isomer (Figure 3d) may also be formed by hydrolysis of the Man_5 isomer after a long period of incubation *in vitro*.

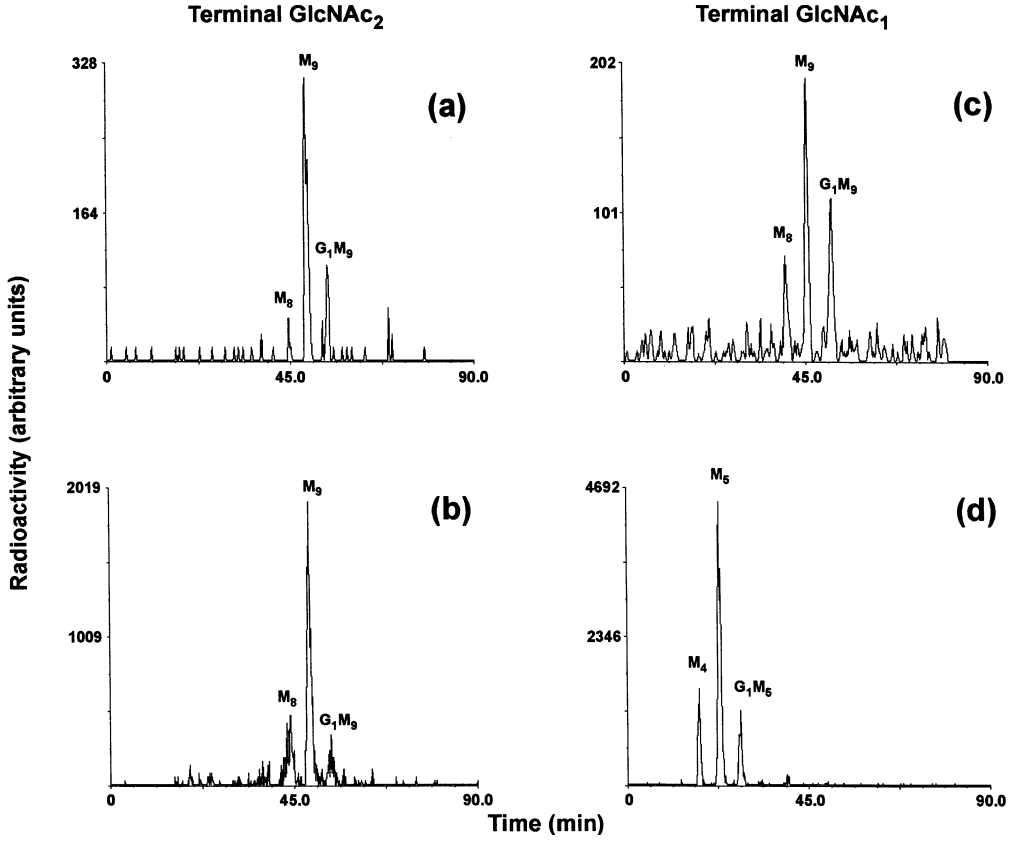


Figure 2 HPLC analysis of the products of hydrolysis of the soluble oligosaccharides ($OS\text{-}GlcNAc_2$ or $OS\text{-}GlcNAc_1$) synthesized in the absence of castanospermine

Glycan moieties of newly synthesized glycoproteins were prepared after metabolic labelling of CHO cells with [2-^{3}H]mannose in the absence of castanospermine. Oligosaccharides were released from the glycoprotein fraction by treatment with either PNGase F [as $OS\text{-}GlcNAc_2$ (**a** and **b**)] or Endo H [as $OS\text{-}GlcNAc_1$ (**c** and **d**)]. HPLC analysis was performed before (**a** and **c**) or after (**b** and **d**) a 4 h incubation with purified cytosolic α-D-mannosidase. M_n, oligomannosides with *n* mannose residues; G_1M_n, oligomannosides with *n* mannose residues and one glucose residue.

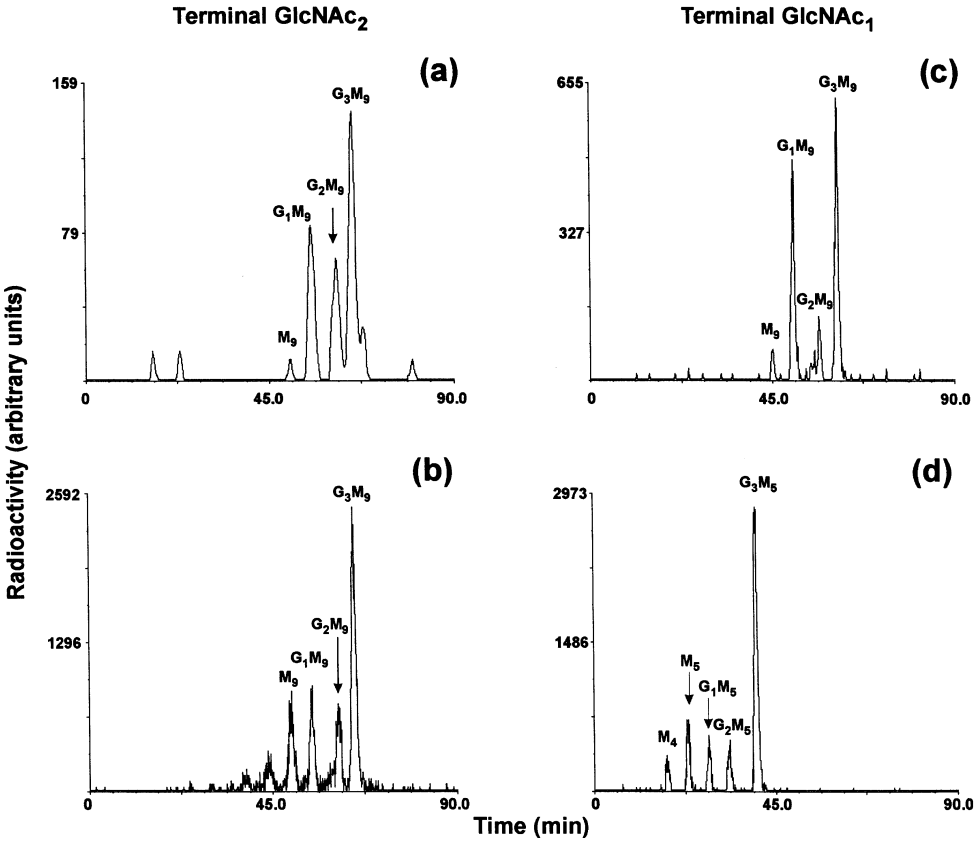


Figure 3 HPLC analysis of the hydrolysis of the soluble oligosaccharides (OS-GlcNAc$_2$ or OS-GlcNAc$_1$) synthesized in the presence of castanospermine

Glycan moieties of newly synthesized glycoproteins were prepared after metabolic labelling of CHO cells with [2-^{3}H]mannose in the presence of castanospermine. Oligosaccharides were released from the glycoprotein fraction by treatment with either PNGase F [as OS-GlcNAc$_2$ (**a** and **b**)] or Endo H [as OS-GlcNAc$_1$ (**c** and **d**)]. HPLC analysis was performed before (**a** and **c**) or after (**b** and **d**) a 4 h incubation with purified cytosolic α-D-mannosidase. M_n, oligomannosides with n mannose residues; $G_{n'}M_n$, oligomannosides with n mannose residues and n' glucose residues.

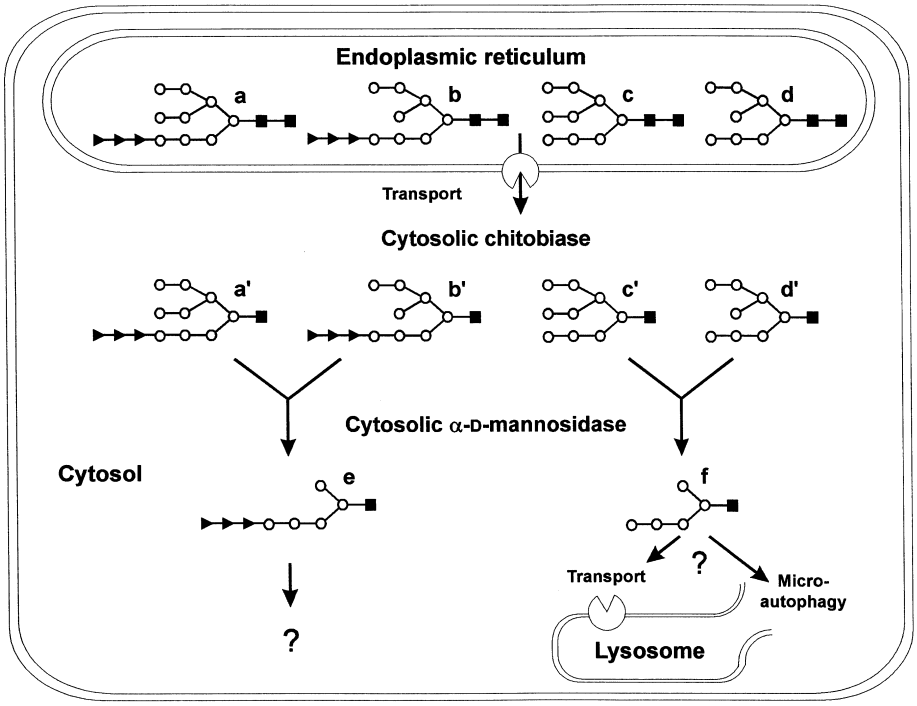
Hydrolysis of the soluble oligosaccharides (OS-GlcNAc$_2$ or OS-GlcNAc$_1$) synthesized in the presence of castanospermine

When synthesized in the presence of castanospermine, the glycan moieties of the newly synthesized glycoproteins were mainly composed of Glc_3Man_9, Glc_2Man_9, Glc_1Man_9 and Man_9 species. As previously mentioned, OS-GlcNAc$_2$ and OS-GlcNAc$_1$ may be obtained from these glycans by treatment with PNGase F (Figure 3a) or Endo H (Figure 3c). These substrates were exposed to purified cytosolic α-D-mannosidase. Again OS-GlcNAc$_2$ were not hydrolysed (Figure 3b), whereas OS-GlcNAc$_1$ were completely hydrolysed (Figure 3d) giving $Glc_3Man_5GlcNAc_1$, $Glc_2Man_5GlcNAc_1$, $Glc_1Man_5GlcNAc_1$, $Man_5GlcNAc_1$ and $Man_4GlcNAc_1$.

DISCUSSION

When exposed *in vitro* to the cytosolic form of α-D-mannosidase, $Man_9GlcNAc_2$-P-P-Dol was partially converted into Man_5-$GlcNAc_2$-P-P-Dol. The long incubation period (16 h) required for partial hydrolysis of this substrate by the cytosolic enzyme (in comparison with soluble oligosaccharides) may be interpreted in terms of poor efficiency of the enzyme towards such lipidic substrates. This result argues against the hypothesis that oligosaccharide-lipid intermediates may occur as isomers higher than $Man_5GlcNAc_2$-P-P-Dol on the cytosolic face of the endoplasmic reticulum membrane and further converted by cytosolic α-D-mannosidase into $Man_5GlcNAc_2$-P-P-Dol which is the sole compound able to be translocated in the lumen [22].

In contrast with what is observed with oligosaccharide-lipids, cytosolic α-D-mannosidase is more active with soluble oligosaccharides. In recent papers, it was demonstrated that oligomannoside-type glycans are released from the endoplasmic reticulum into the cytosol [12,13,19,25]. This material originates from both the hydrolytic capacity of the oligosaccharyltransferase [10] and presumably the action of a PNGase activity such as the one recently described in mammalian cells [30]. The soluble oligosaccharides released from the endoplasmic reticulum into the cytosol possess a di-*N*-acetylchitobiose sequence (OS-GlcNAc$_2$) at their reducing end [13]. We have demonstrated here that cytosolic α-D-mannosidase is able to hydrolyse soluble oligosaccharides terminating with a single *N*-acetylglucosamine residue (OS-GlcNAc$_1$), in contrast with soluble oligosaccharides terminating with a di-*N*-acetylchitobiose sequence (OS-GlcNAc$_2$) which are poorly hydrolysed under the same conditions. The present results are in agreement with previous studies on the substrate specificity of the neutral α-D-mannosidase purified from Japanese quail oviduct [31]. We may assume that the conversion of OS-GlcNAc$_2$ into OS-GlcNAc$_1$ is mediated by cytosolic Endo H [32]; nevertheless the absence of this enzyme from bovine and ovine tissues [33] does not support a key role for this enzyme in the catabolism of these soluble oligosaccharides. A chitobiase activity located in the cytosol of Madin-Darby



Scheme 1 Potential role for cytosolic α-D-mannosidase in the trimming of soluble oligosaccharides released from the endoplasmic reticulum

Oligomannosides possessing a di-*N*-acetylchitobiose sequence (OS-GlcNAc$_2$) at their reducing end are produced during the N-glycosylation process, and $Glc_3Man_9GlcNAc_2$ (**a**), $Glc_3Man_8GlcNAc_2$ (**b**), $Man_9GlcNAc_2$ (**c**) and $Man_8GlcNAc_2$ (**d**) have been detected in the lumen of the rough endoplasmic reticulum after the action or not of the specific endoplasmic reticulum mannosidase or glucosidases. A transport mechanism has been postulated to be responsible for the transport of OS-GlcNAc$_2$ into the cytosol where they are exposed to the action of a specific chitobiase, giving the corresponding OS-GlcNAc$_1$ (**a**′,**b**′,**c**′,**d**′). Whatever their glucosylation state, OS-GlcNAc$_1$ can be degraded by cytosolic α-D-mannosidase giving either $Glc_3Man_5GlcNAc_1$ (**e**), the fate of which is not known, or the final product $Man_5GlcNAc_1$ (**f**) which accumulates in the cytosol before its internalization in the lysosomes. ▶, glucose; ■, GlNAc; ○, mannose.

bovine kidney cells has recently been described [34]. This enzyme could be responsible for the formation of OS-GlcNAc$_1$ from OS-GlcNAc$_2$.

The substrate specificity of cytosolic α-D-mannosidase appears to be dependent on the presence of a single GlcNAc residue at the reducing end, since the presence of either a dichitobiosyl residue or an additional P-P-Dol moiety inhibits the enzyme activity. In contrast, the substrate specificity is the same for glucosylated and non-glucosylated oligomannosidic glycans, since the presence of one to three glucoses linked to the mannose D_1 does not interfere with cytosolic α-D-mannosidase activity. Glucosylated species, particularly of the $Glc_3Man_9GlcNAc_1$ species, have, however, only been detected in permeabilized cells or cells incubated with castanospermine [12,13]. In these cases, degradation of $Glc_{0-3}Man_9GlcNAc_1$ species leads to the formation of the single $Glc_{(0-3)}Man_5GlcNAc_1$ product.

These results obtained *in vitro* are in agreement with previous studies showing that, during metabolic labelling, $Man_5GlcNAc_1$ accumulates in the cytosol [12,13,35] before its possible internalization in the lysosomes [12,36]. Therefore cytosolic α-D-mannosidase may play a key role in the catabolism of oligomannosidic glycans released from the endoplasmic reticulum, as summarized in Scheme 1.

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