Demonstration of a peptide:N-glycosidase in the endoplasmic reticulum of rat liver

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Prompted by previous observations that polymannose oligosaccharides are released from newly synthesized glycoproteins [Anumula and Spiro (1983) J. Biol. Chem. **258**, 15274–15282], we examined rat liver endoplasmic reticulum (ER) for the presence of endoglycosidases that could be involved in an event presumed to be a function of the protein quality control machinery. Our investigations indicated that a peptide:N-glycanase (PNGase) is present in ER membranes that has the capacity to release from radiolabelled glycopeptides glucosylated as well as non-glucosylated polymannose oligosaccharides terminating at their reducing end in a di-*N*-acetylchitobiose sequence (OS-$GlcNAc_2$). This enzyme, which was found to be luminal in orientation, was most active in the pH range 5.5–7.0 and although it had no exogenous bivalent-cation requirements it was inhibited by EDTA. Detailed studies with $Man_9GlcNAc_2$-peptides demonstrated that in addition to the free oligosaccharide ($Man_9GlcNAc_2$) an additional neutral product characterized as $Man_9GlcNAc_2$ linked to an as yet unidentified aglycone was released in a manner that suggests its role as an intermediate. Our observation that ER, in contrast with cytosol, had no endo-β-*N*-acetylglucosaminidase activity would indicate that oligosaccharides terminating in a single GlcNAc residue (OS-$GlcNAc_1$), which have been noted to appear in the extravesicular compartment shortly after N-glycosylation [Moore and Spiro (1994) J. Biol. Chem. **269**, 12715–12721] are released from the protein as OS-$GlcNAc_2$ and undergo an ER-to-cytosol translocation in that form before undergoing cleavage of their chitobiose core.

INTRODUCTION

In recent years, studies from our laboratory [1–3], as well as those of other investigators [4–6], have shown that there is a release of free polymannose oligosaccharides concurrently or shortly after N-glycosylation takes place in the endoplasmic reticulum (ER). These components have been shown to consist of two families, which differ from each other in having either a di-*N*-acetylchiotobiose sequence (OS-$GlcNAc_2$) or a single N-acetylglucosamine residue (OS-$GlcNAc_1$) at their reducing ends [1,3]. Although neither group of oligosaccharides is secreted by the cell [7], components belonging to the latter category have attracted particular attention since studies with permeabilized HepG2 cells indicated that after their generation from newly synthesized glycoproteins [2], they rapidly appear in the cytosol [3] where an α-mannosidase closely related to ER mannosidase II [8] processes them to the $Man_5GlcNAc_1$ stage [8,9]; subsequently this oligosaccharide becomes internalized into a compartment that is believed to be the lysosomes, where enzymes are located that can perform its further degradation [9].

On the basis of these observations it has been postulated [3] that the release of these oligosaccharides might be a function of the ER-situated quality-control machinery, which is believed to degrade misfolded or improperly assembled proteins [10]. An ER–cytosolic translocation of released oligosaccharides would prevent their accumulation in the channels of the secretory system, where they would have the potential of interfering with the lectin-like chaperone interactions that have been shown to involve N-linked oligosaccharides [11,12] or to compete with intravesicular processing enzymes or saccharide-specific receptors [13].

Although previous studies have indicated that OS-$GlcNAc_2$ components can be generated by the hydrolytic capacity of oligosaccharyltransferase [2], the possibility that the release of oligosaccharides in the ER can be brought about by the action of endoglycosidases, such as peptide:N-glycosidase (PNGase) or endo-β-*N*-acetylglucosaminidase, which are known to be widely distributed in Nature [14,15], has not been explored. Therefore we have in the present study undertaken a search for such hydrolytic enzymes in rat liver ER. Our observations indicate that there is a PNGase in this subcellular compartment that *in vitro* can effectively release polymannose oligosaccharides of the OS-$GlcNAc_2$ type from exogenous glycopeptides. Furthermore our finding that, in contrast with the cytosol, the ER does not have detectable endo-β-*N*-acetylglucosaminidase activity supports the concept that OS-$GlcNAc_2$ components are translocated into the cytosol after their formation in the ER and are then converted into OS-$GlcNAc_1$ saccharide species.

EXPERIMENTAL

Preparation of rat liver ER

Rough ER was prepared from the livers of fasted male rats (175–200 g, CD strain; Taconic) by the procedure of DePierre and Dallner [16], as previously employed [8,17,18]. The purity of the ER prepared in this manner has previously been evaluated by marker enzyme assays [17], which indicated on the basis of UDP-

Abbreviations used: ER, endoplasmic reticulum; OS-$GlcNAc_1$ and OS-$GlcNAc_2$, polymannose oligosaccharides terminating at their reducing end with an *N*-acetylglucosamine or a di-*N*-acetylchitobiose sequence respectively; PNGase, peptide:N-glycosidase; KIF, kifunensine; DIM, 1,4-dideoxy-1,4-imino-D-mannitol; CST, castanospermine; DMJ, 1-deoxymannojirimycin; endo H, endo-β-*N*-acetylglucosaminidase H.

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Gal:*N*-acetylglucosamine galactosyltransferase determinations that there is less than 2% Golgi contamination.

Preparation of radiolabelled glycopeptides

Mixed ^{14}C-labelled polymannose-GlcNAc$_2$ glycopeptides

For the preparation of glycopeptides, calf thyroid slices were incubated with [U-^{14}C]glucose (325 mCi/mmol; DuPont–New England Nuclear) for 3 h at 37 °C as previously described [19]. After delipidation of the postnuclear pellet by successive chloroform/methanol/water (3:2:1, by vol.) and chloroform/methanol/water (10:10:3, by vol.) extractions, it was submitted to Pronase (Calbiochem) digestion to prepare a mixture of complex and polymannose N-linked glycopeptides as previously reported [20]. To obtain the polymannose-containing peptides, the total glycopeptide fraction (500000 d.p.m.) was applied to a concanavalin A–Sepharose column (0.7 cm × 4 cm) (Pharmacia Biotech) in a manner previously described [21], from which they were eluted, after an extensive buffer wash, with 500 mM methyl-α-D-mannoside. The glycopeptides, which contained N-linked oligosaccharides ranging in size from $Man_5GlcNAc_2$ to $Glc_1Man_9GlcNAc_2$ [20], were then desalted and separated from the methylglycoside by filtration on Bio-Gel P-2 column (1.5 cm × 32 cm) equilibrated with a 0.1 M pyridine acetate (pH 5.0) buffer which was subsequently removed by freeze-drying.

^{14}C-labelled $Man_6GlcNAc_2$-glycopeptides

For the preparation of ^{14}C-labelled $Man_9GlcNAc_2$-peptides, the thyroid slices were incubated for 3 h with [^{14}C]glucose in the presence of 0.1 mM kifunensine (KIF) (Toronto Research Chemicals) and 2 mM 1,4-dideoxy-1,4-imino-D-mannitol (DIM) (Oxford GlycoSystems) to inhibit ER and Golgi mannosidases I as well as ER mannosidase II [8,18] respectively. After Pronase digestion and concanavalin A–Sepharose chromatography, a glycopeptide fraction was obtained in this manner that contained primarily $Man_9GlcNAc_2$ oligosaccharides (approx. 85%), with the remainder occurring as $Man_8GlcNAc_2$.

[^{3}H]GlcNAc-labelled $Man_9GlcNAc_2$ glycopeptides

In order to radiolabel the GlcNAc residues selectively in the polymannose glycopeptides, BW5147.3 mouse lymphoma cells (2×10^8), obtained from ATCC (Rockville, MD, U.S.A.) and grown as previously described [18], were pulsed in suspension for 45 min with 500 μCi of [6-^{3}H]glucosamine (33 mCi/μmol; DuPont–New England Nuclear) in 2 ml of glucose-free Dulbecco's modified Eagle's medium containing 10 mM sodium pyruvate and 0.1 mM KIF as well as 2 mM DIM. This was followed by a 1 h chase in the presence of 2 mM mannose as well as 4.5 mg/ml glucose. A 15-min incubation at 37 °C with the mannosidase inhibitors preceded the addition of the radioactive substrate, and moreover these agents were present during the chase. After the incubation the cells were separated from the medium, delipidated and digested with Pronase as described for the thyroid glycoproteins.

$Glc_3Man_9GlcNAc_2$ glycopeptides

The mouse BW5147.3 lymphoma cells were also employed to prepare glycopeptides in which $Glc_3Man_9GlcNAc_2$ oligosaccharides predominated. For this purpose incubations (20 min pulse followed by 2 h chase) were performed with 300 μCi of [2-^{3}H]mannose (28 mCi/mol; DuPont–New England Nuclear) in the presence of 0.2 mM 6-*O*-butanoyl castanospermine (CST) (a gift from Dr M. Kang, Merrell Dow Research Institute, Cincinnati, OH, U.S.A.) and 0.2 mM KIF. Parallel incubations were performed in the absence of the glucosidase inhibitor to yield glycopeptides containing primarily N-linked $Man_9GlcNAc_2$ oligosaccharides. Purification of these glycopeptides was achieved by concanavalin A chromatography as described above.

Radiolabelled peptide-linked $Glc_3Man_9GlcNAc_2$ was also prepared through the action of rat liver oligosaccharyltransferase. In this approach ^{14}C-labelled oligosaccharide-lipid (200000 d.p.m.) prepared in thyroid slices as previously described [22] was incubated with the liver ER membranes (1.0 mg of protein) for 30 min at 27 °C in 100 μl of 50 μM Tris/acetate, pH 7.4, buffer containing 0.2% Triton X-100, 2 mM manganese acetate, 2 mM 2-mercaptoethanol, 5 μM KIF, 2 mM DIM and 2 mM CST. The $Glc_3Man_9GlcNAc_2$-peptides were obtained after Pronase digestion of the delipidated protein pellet as described above; the $Glc_3Man_9GlcNAc_2$ nature of the oligosaccharide unit was confirmed by TLC after digestion with endo-β-*N*-acetylglucosaminidase H (endo H).

All polymannose glycopeptide substrates were digested with endo H to determine their oligosaccharide composition and the amount of radiolabel in each component.

Assay of PNGase

In the standard assay a specified amount of rat liver ER (25–300 μg of protein) was incubated with the ^{14}C-labelled $Man_9GlcNAc_2$-glycopeptides [containing $(5–20) \times 10^3$ d.p.m. in the oligosaccharide] for various times (4–40 h) at 37 °C in 50 μl of 0.1 M NaMes buffer, pH 6.5, containing 5 μM KIF, 2 mM 1-deoxymannojirimycin (DMJ) (Genzyme) and 0.2% (v/v) Triton X-100. When glycopeptides containing glucosylated polymannose oligosaccharides were assayed, CST (2 mM) was also included in the incubations; all assays were preceded by a 40 min incubation at 2 °C without substrate to maximize the effect of the glycosidase inhibitors. The incubations were terminated by heating at 100 °C for 3 min after the addition of 0.5 ml of water. The samples were then passed through columns containing 1 ml of Dowex 1-X2, 200–400 mesh (acetate form), overlaid by 1 ml of Dowex 50-X2, 200–400 mesh (H^+ form) to yield in the water wash the released neutral oligosaccharides, which were then subjected to TLC on silica-gel-coated plates in solvent system A [propan-1-ol/acetic acid/water (3:3:2, by vol.)]. The saccharide components were detected by fluorography and quantified by scintillation counting after elution from the chromatograms.

Latency examination of PNGase in the ER

For these studies, the ER vesicles obtained from the sucrose gradient were washed twice by centrifugation (100000 ***g*** for 60 min) with 0.25 M sucrose in 5 mM Tris/acetate, pH 7.0. They were then suspended in this buffer and kept at 4 °C for 30 min with or without 0.2% (v/v) Triton X-100 in the presence of 5 μM KIF and 2 mM DMJ before the addition of the ^{14}C-labelled $Man_9GlcNAc_2$-glycopeptide substrate (25000 d.p.m.). The assay was performed in a 100 μl volume for 4 h at 37 °C; after its termination by heating at 100 °C for 3 min, the samples were loaded on Bio-Gel P-2 columns (1.5 cm × 32 cm) equilibrated with 0.1 M pyridine acetate, pH 5.0, to remove the sucrose. The radioactive fractions were pooled, freeze-dried to remove the buffer and then passed through Dowex 50 (H^+ form)/Dowex 1 (acetate form) columns to obtain the released oligosaccharides, which were then subjected to TLC, fluorography and scintillation counting. As a positive control mannose-6-phosphatase activity was determined as previously described [8].

Assay of endo β-*N*-acetylglucosaminidase activity

Determination of the activity of this enzyme in rat liver ER and dialysed cytosol was performed with 4000 d.p.m. of ^{14}C-labelled $Glc_3Man_9GlcNAc_2$, prepared by mild acid hydrolysis of metabolically labelled thyroid oligosaccharide-lipids [1], in 50 μl of 0.1 M NaMes buffer, pH 6.5, containing 2 mM CST, 5 μM KIF and 2 mM DMJ as well as 0.2% (v/v) Triton X-100 for 90 min at 37 °C. After desalting of the samples by passage through Dowex 50 (H^+ form)/Dowex 1 (acetate form) columns, the products of the incubations were identified by TLC.

Structural and analytical procedures

Reduction of oligosaccharides was performed with $NaBH_4$ as previously described [17] and the desalted oligosaccharide products, after removal of the borate with methanol, were examined by TLC. Digestions with recombinant *Streptomyces plicatus* endo H (2 m-units; Genzyme) were performed as previously described [20] and the desalted samples were examined by TLC. Protein was determined by the procedure of Peterson [23], with BSA as a standard.

TLC

Oligosaccharides were resolved on plastic sheets precoated with silica gel 60 (0.2 mm thickness; Merck) in solvent system A (propan-1-ol/acetic acid/water; 3:3:2, by vol.), whereas monosaccharides were separated on plastic sheets precoated with cellulose (0.1 mm thickness; Merck) in pyridine/ethyl acetate/water/acetic acid (5:5:3:1, by vol.) (solvent system B). The chromatography in both systems was performed with a wick of Whatman 3 MM paper clamped to the top of the thin-layer plates; the radioactive components were located by fluorography and quantified by scintillation counting after elution with water as previously described [7]. For preparative purposes, oligosaccharide eluates were extracted with peroxide-free diethyl ether to remove scintillants and passed through small columns of Dowex 50 (H^+ form)/Dowex 1 (acetate form).

Measurements of radioactivity

Liquid-scintillation counting was performed in Ultrafluor with a Beckman LS 7500 instrument. Radioactive components on thin-layer plates were detected with X-Omatic AR film (Eastman Kodak) after spraying with a scintillation mixture containing 2-methylnaphthalene [24].

RESULTS

Release of free oligosaccharides from polymannose-glycopeptides by rat liver ER

Incubation of ER membranes with a mixture of radiolabelled polymannose-containing peptides resulted in the release of a series of oligosaccharides that migrated on TLC as OS-$GlcNAc_2$ components ($Man_9GlcNAc_2$ to $Man_5GlcNAc_2$) and accordingly were converted into their respective OS-$GlcNAc_1$ forms on subsequent digestion with endo H (Figure, 1, left panel). These observations suggested that rat liver ER manifests PNGase activity and that this enzyme acts on the entire complement of N-linked polymannose oligosaccharides. Indeed on the basis of the oligosaccharide species released by direct treatment of this glycopeptide mixture with endo H, it could be calculated that the ER enzyme had released approx. 50% (range 45–58%) of the $Man_{5-9}GlcNAc_2$ components. Moreover, we observed that the enzyme effectively released $Glc_3Man_9GlcNAc_2$ from peptides containing the glucosylated oligosaccharide, irrespective of whether they were prepared in thyroid slices or by oligosaccharyltransferase action in a cell-free system. In contrast, dolichylpyrophosphate-linked $Glc_3Man_9GlcNAc_2$ was not cleaved by the enzyme; the absence of bivalent cations in our PNGase assay prevented this substrate from being transferred to

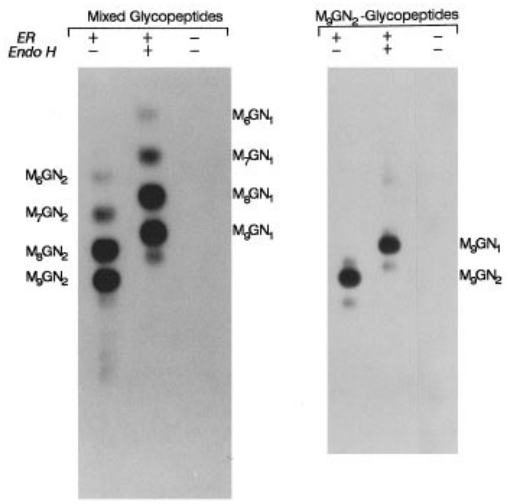

Figure 1 Thin-layer chromatographic examination of oligosaccharides released by rat liver ER from radiolabelled polymannose glycopeptides and evaluation of the effect of endo H treatment on these components

Mixed ^{14}C-labelled polymannose glycopeptides (9300 d.p.m., left panel), and $Man_9GlcNAc_2$-glycopeptides (9600 d.p.m., right panel) were incubated with (+) or without (−) ER membranes (200 and 100 μg of protein respectively) for 16 h at 37 °C in the presence of mannosidase inhibitors under the conditions for the PNGase assay described in the Experimental section. The released, desalted oligosaccharides were incubated without (−) or with (+) endo H (2 m-units; Genzyme) for 4 h at 37 °C before further desalting on Dowex 50 (H^+ form)/Dowex 1 (acetate form) columns and application to silica-gel-coated plates. Chromatography was performed in solvent system A for 24 h and the components were detected by fluorography. The oligosaccharides are identified by GN_1 if they contained a reducing GlcNAc residue and GN_2 if they terminated in a di-*N*-acetylchitobiose sequence; the suffix to the M indicates the length of the polymannose portion. Although not evident in this photograph, small amounts of M_5GN_2 and M_5GN_1 were revealed in the left and middle lanes respectively.

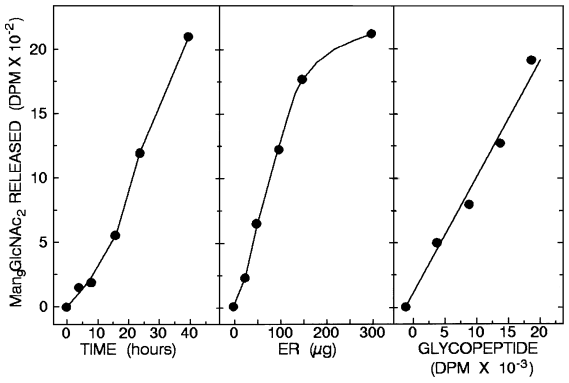

Figure 2 Effect of incubation time as well as enzyme and substrate concentrations on PNGase activity of rat liver ER

ER membranes at various concentrations (middle panel) or as 100 μg of protein (left and right panels) were incubated with various amounts of ^{14}C-labelled $Man_9GlcNAc_2$-glycopeptides (right panel) or 9600 d.p.m. of this substrate (left and middle panels) for 20 h (middle and right panels) or other periods (left panel) at 37 °C as described in the Experimental section. The released $Man_9GlcNAc_2$ oligosaccharide was quantified after TLC.

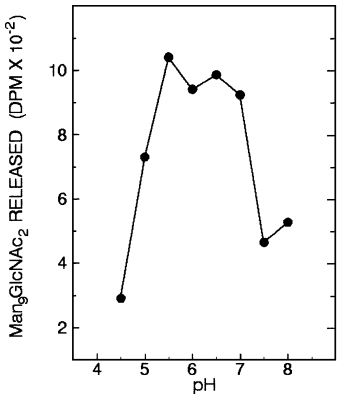

Figure 3 Effect of pH on PNGase activity from rat liver ER

ER membranes (100 μg of protein) were incubated at the indicated pH values with ^{14}C-labelled $Man_9GlcNAc_2$-glycopeptide (9600 d.p.m.) for 20 h at 37 °C as described in the Experimental section. The released $Man_9GlcNAc_2$ was quantified after TLC. The buffers (0.1 M) employed in this study were: pH 4.5–5.5, acetate; pH 6.0–7.0, Mes; and pH 7.5–8.5, phosphate.

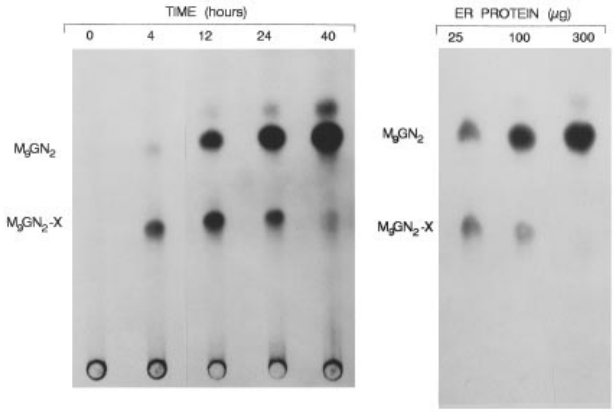

Figure 4 TLC examination of saccharide components released by rat liver ER from ^{14}C-labelled $Man_9GlcNAc_2$-glycopeptides as a function of time and protein concentration

ER membranes (100 μg of protein, left panel) or at various protein concentrations (right panel) were incubated for various times (left panel) or 20 h (right panel) with ^{14}C-labelled $Man_9GlcNAc_2$-glycopeptide (9600 d.p.m.) at 37 °C as described in the Experimental section. The released, desalted neutral components were chromatographed on silica-gel-coated plates in solvent system A for 24 h and detection was achieved by fluorography. In addition to the $Man_9GlcNAc_2$ (M_9GN_2), another more slowly migrating radiolabelled component was observed, which has been designated $Man_9GlcNAc_2$-X (M_9GN_2-X) on the basis of the subsequent characterization studies. A small amount of $Man_8GlcNAc_2$, migrating ahead of $Man_9GlcNAc_2$, can be observed at the longer incubation times.

endogenous protein by ER oligosaccharyltransferase, which is known to function only in the presence of these cofactors [22].

For the standard assay we chose to employ as substrate glycopeptides containing N-linked $Man_9GlcNAc_2$ from which the ER released an oligosaccharide that was converted into $Man_9GlcNAc_1$ after treatment with endo H (Figure 1, right panel). A more slowly migrating component, the intensity of which varied with the length of incubation, the amount of enzyme and the length of enzyme storage at −20 °C, was also observed on TLC; the nature of this product will be evaluated in a subsequent section of this paper.

Properties of PNGase in rat liver ER

Assays with the $Man_9GlcNAc_2$-peptides (Figure 2) revealed that the amount of free oligosaccharide released by the ER enzyme increased with time over a 40 h period and demonstrated a linear response to ER protein (25–150 μg) as well as glycopeptide substrate (5000–20000 d.p.m.). The pH optimum of the enzyme was evident over the pH range 5.5–7.0 (Figure 3).

Although the enzyme assay was performed in the absence of exogenous bivalent cations, the addition of EDTA almost completely inhibited activity with 23% and 13% of control values being observed at 5 and 10 mM respectively of this chelator. The addition of 10 mM concentrations of a variety of metal ions, including Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+} and Zn^{2+}, to incubations containing 5 mM EDTA failed to reverse this inhibition. Furthermore, additions of these ions (2 mM) in the absence of EDTA did not enhance activity and indeed Co^{2+}, Zn^{2+} and Mn^{2+} were inhibitory, decreasing enzyme activity to 28%, 51% and 33% of control incubations.

Latency of PNGase in ER

Determination of the enzyme activity of intact ER vesicles with and without 0.2% Triton X-100, as described in the Experimental section, indicated that the catalytic site of the PNGase was luminal in orientation, as cleavage of the glycopeptide after a 4 h incubation in the absence of the permeabilizing detergent was only 11% of that noted in its presence (50 d.p.m. compared with 463 d.p.m.). ER mannose-6-phosphatase activity, which served as a control, reflected a similar response to that of PNGase to the detergent as previously reported [8].

Characterization of the more slowly migrating component (OS-$GlcNAc_2$-X) released by rat liver ER enzyme

TLC examination of the products of $Man_9GlcNAc_2$-peptide cleavage by ER enzyme revealed, in addition to the free oligosaccharide, the presence of another radiolabelled component that migrated at only 0.63 times the rate of $Man_9GlcNAc_2$. Although the intensity of the slower component, which we termed $Man_9GlcNAc_2$-X, varied between ER preparations, depending on the length of frozen storage of the membranes, its appearance was in general related to the length of incubation (Figure 4, left panel) and amount of enzyme (Figure 4, right panel) in such a manner as to suggest that it might be an intermediate in the release of the free oligosaccharide. Such an interrelationship was also supported by a parallel response of the two components to EDTA, pH and Triton X-100 (results not shown). The possibility of a product–precursor relationship was furthermore indicated by the conversion of the purified slower component into $Man_9GlcNAc_2$ on digestions with ER membranes (Figure 5).

The observation that digestion of the slow oligosaccharide with endo H resulted in the formation of $Man_9GlcNAc_1$ in a manner comparable with such treatment of $Man_9GlcNAc_2$ (Figure 6, left panel) suggested that $Man_9GlcNAc_2$-X differed from $Man_9GlcNAc_2$ in having its internal GlcNAc residue either O-substituted or in glycosidic linkage to an additional constituent, which in either case should be excised together with the terminal GlcNAc by endo H. The latter situation seemed to exist because the chromatographic migration of $Man_9GlcNAc_2$-X was

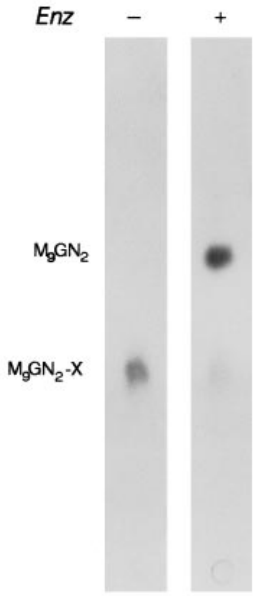

Figure 5 Effect of incubation of purified ^{14}C-labelled $Man_9GlcNAc_2$-X with rat liver ER

Radiolabelled $Man_9GlcNAc_2$-X (4000 d.p.m.) purified by preparative TLC from digests of ^{14}C-labelled $Man_9GlcNAc_2$-glycopeptides (see Figure 4) was incubated with (+) or without (−) ER membranes (100 μg of protein) for 16 h at 37 °C in the presence of mannosidase inhibitors under the conditions for the PNGase assay described in the Experimental section. The desalted digests were chromatographed in solvent system A for 24 h and detection was achieved by fluorography. The positions of migration of $Man_9GlcNAc_2$-X (M_9GN_2-X) and $Man_9GlcNAc_2$ (M_9GN_2) are indicated.

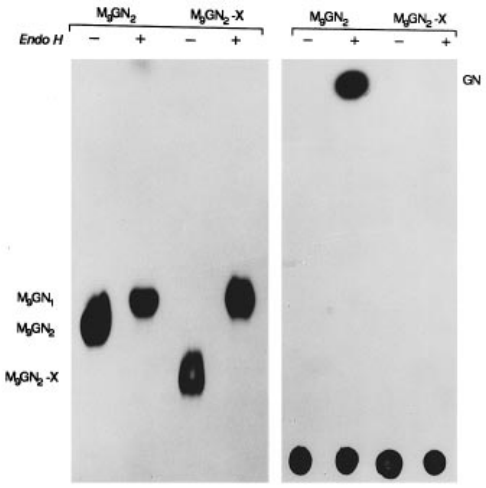

Figure 6 Comparison of the effect of digestion with endo H on the [^{3}H]GlcNAc-labelled $Man_9GlcNAc_2$ and $Man_9GlcNAc_2$-X components obtained from ER enzyme action on $Man_9GlcNAc_2$-glycopeptides

$Man_9GlcNAc_2$ (M_9GN_2) and $Man_9GlcNAc_2$-X (M_9GN_2-X) (50000 d.p.m.) that had been isolated by preparative TLC from an ER digest of [^{3}H]GlcNAc-labelled $Man_9GlcNAc_2$-glycopeptides were incubated without (−) or with (+) endo H (2 m-units; Genzyme) for 4 h at 37 °C. After passage through Dowex 50 (H^+ form)/Dowex 1 (acetate form) columns, the samples were chromatographed on a cellulose-coated plate in solvent system B for 17 h (right panel) and after elution the oligosaccharides remaining at the origins were resolved on a silica-gel-coated plate in solvent system A for 24 h (left panel). The components were revealed by fluorography. The positions of migration of M_9GN_1, M_9GN_2, M_9GN_2-X (see legend to Figure 1 for explanation) and free *N*-acetylglucosamine (GN) are indicated alongside the chromatographs. When chromatography in solvent system B (right panel) was performed for a short time (4 h), which prevented the solvent from running off the plate, no additional components were found.

not altered by reduction with $NaBH_4$, in contrast with that of the $Man_9GlcNAc_2$ oligosaccharide itself, which demonstrated the expected decrease in mobility (Figure 7).

In an attempt to characterize the nature of the aglycone, we chromatographically surveyed the cleavage products from endo H action on [^{3}H]GlcNAc-labelled $Man_9GlcNAc_2$-X alongside those obtained from $Man_9GlcNAc_2$ radiolabelled in the same manner. Although GlcNAc was released from the latter component (Figure 6, right panel), which was consistent with its increase in chromatographic mobility (Figure 6, left panel), no released product could be detected from endo H action on $Man_9GlcNAc_2$-X (Figure 6, right panel) despite clear evidence that cleavage by the enzyme had taken place with its conversion into the $Man_9GlcNAc_1$ oligosaccharide (Figure 6, left panel). Indeed the expected released radiolabelled GlcNAc-X could not be detected even when the solvent front was not permitted to move off the chromatographic plate. Although $Man_9GlcNAc_2$-X is believed to be a neutral component because it is not bound to either the Dowex 50 or Dowex 1 employed in the PNGase assay, the possibility that the excised *N*-acetylglucosaminide (GlcNAc-X) might nevertheless bear a charge was made unlikely by our observation that when the endo H digest was passed through these exchangers no radioactivity could be recovered by their elution with 1.5 M NH_4OH (Dowex 50) or 3 M formic acid (Dowex 1).

Incubation of ER enzyme with radiolabelled $Glc_3Man_9GlcNAc_2$-glycopeptides indicated that N-linked oligosaccharides in the glucosylated form are released as OS-$GlcNAc_2$ and OS-$GlcNAc_2$-X in a manner comparable with that of the non-glucosylated species (Figure 8). Moreover, when the slower component ($Glc_3Man_9GlcNAc_2$-X) from the $Glc_3Man_9GlcNAc_2$-

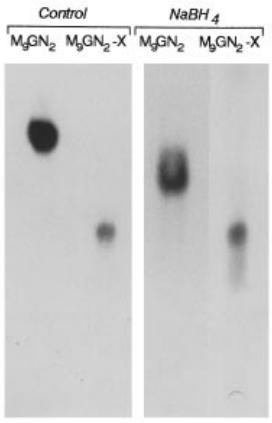

Figure 7 Effect of reduction with $NaBH_4$ on $Man_9GlcNAc_2$ and $Man_9GlcNAc_2$-X released by ER enzyme from ^{14}C-labelled $Man_9GlcNAc_2$-glycopeptides

TLC was performed on a silica gel-coated plate for 24 h in solvent system A on chromatographically purified $Man_9GlcNAc_2$ (M_9GN_2) and $Man_9GlcNAc_2$-X (M_9GN_2-X) before (Control) and after ($NaBH_4$) reduction. The components were revealed by fluorography.

peptides was treated with endo H, it was also converted into the $Glc_3Man_9GlcNAc_1$ oligosaccharide (results not shown).

Evaluation of ER endo-β-*N*-acetylglucosaminidase activity

Because our laboratory has previously reported that OS-$GlcNAc_1$ as well OS-$GlcNAc_2$ components are released shortly after N-

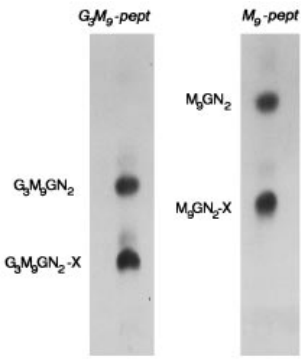

Figure 8 Comparison of the action of PNGase in rat liver ER on glucosylated and non-glucosylated polymannose glycopeptides

ER membranes (200 μg of protein) were incubated for 8 h at 37 °C with [^{3}H]Man-labelled $Glc_3Man_9GlcNAc_2$-glycopeptides (25 200 d.p.m., left panel) or similarly labelled $Man_9GlcNAc_2$-glycopeptides (48 600 d.p.m., right panel) as described in the Experimental section. TLC of the released neutral components was performed as in Figure 4. Each substrate yielded, in addition to the di-*N*-acetylchitobiose-terminating oligosaccharide ($G_3M_9GN_2$ and M_9GN_2 respectively), other more slowly migrating components, which have been designated $G_3M_9GN_2$-X and M_9GN_2-X.

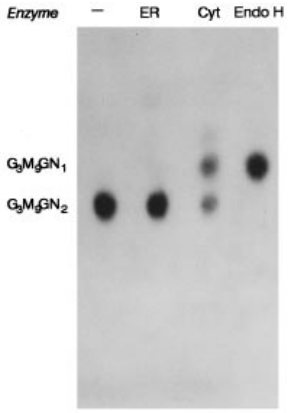

Figure 9 Evaluation of endo-β-*N*-acetylglucosaminidase activity in rat liver ER and cytosol

Incubations were performed with 200 μg of protein from ER membranes (ER) and cytosol (Cyt) with the ^{14}C-labelled $Glc_3Man_9GlcNAc_2$ oligosaccharide (4000 d.p.m.) in 50 μl of 0.1 M NaMes buffer, pH 6.5, containing CST (2 mM), KIF (5 μM) and DMJ (2 mM) and 0.2% (v/v) Triton X-100, for 90 min at 37 °C. For comparison, recombinant endo H (endo H) from *Streptomyces plicatus* (2 m-units; Genzyme) was incubated with the oligosaccharide under similar conditions. After passage through Dowex 50 (H^+ form)/Dowex 1 (acetate form) columns the desalted samples were applied to a silica-gel-coated plate and chromatographed in solvent system A for 24 h; the components were revealed by fluorography. The abbreviations are: $G_3M_9GN_1$, $Glc_3Man_9GlcNAc_1$; $G_3M_9GN_2$, $Glc_3Man_9GlcNAc_2$. The incubation without enzyme is denoted by (—).

glycosylation takes place, we were prompted to determine whether an endo-β-*N*-acetylglucosaminidase resides in the ER that could act on the OS-$GlcNAc_2$ components released by the PNGase in this compartment and convert them into OS-$GlcNAc_1$ species. This possibility was, however, made unlikely by our observation that conversion of $Glc_3Man_9GlcNAc_2$ into $Glc_3Man_9GlcNAc_1$ was not effected by ER membranes (Figure 9); in contrast, as expected from a previous report [25], incubations with cytosolic protein demonstrated endo-β-*N*-acetylglucosaminidase activity, which yielded an OS-$GlcNAc_1$ product comparable with that formed by endo H (Figure 9).

DISCUSSION

The results obtained in the present study indicate that the ER contains a luminally oriented enzyme that can generate OS-$GlcNAc_2$ components from a wide spectrum of N-linked polymannose oligosaccharides, including those that are glucosylated. Release of these oligosaccharides was accompanied by the appearance of an additional component (OS-$GlcNAc_2$-X) that, on the basis of time and ER membrane concentration studies as well as parallel responses to pH, Triton X-100 and EDTA, seems to be related to the free oligosaccharide as an intermediate. The possibility of a product–precursor relationship was further supported by the finding that this slowly migrating component purified from $Man_9GlcNAc_2$-glycopeptide digests ($Man_9GlcNAc_2$-X) was converted into $Man_9GlcNAc_2$ on treatment with ER membranes.

Although endo H acted effectively, as expected from its broad substrate specificity for polymannose-di-*N*-acetylchitobiose oligosaccharides whether free, linked to peptide or a variety of aglycones [14,26], on both the OS-$GlcNAc_2$ and OS-$GlcNAc_2$-X components, we were unable to recover the anticipated cleaved GlcNAc-X product; until this aglycone can be identified its complete structure still remains uncertain. The possibility that the OS-$GlcNAc_2$-X is a 1-amino-oligosaccharide seems to be reasonable in view of reports that PNGase from various sources [14,27] as well as the β-aspartyl-*N*-acetylglucosamine hydrolases [28,29] are in fact amidases that function to release the free oligosaccharide in a two-step process. In both groups the 1-amino intermediate is unstable below pH 8.0 and is believed to be undergoing rapid non-enzymic hydrolysis to the reducing saccharide with the release of ammonia at more acidic pH values [30,31]. Our observations did not seem to conform to such a model because the relative amount of $Man_9GlcNAc_2$-X obtained did not increase at higher pH values; indeed, the ratio of $Man_9GlcNAc_2$ to $Man_9GlcNAc_2$-X after a 20 h incubation at pH 6.0 was observed to be 1.67, whereas that at pH 8.0 was 2.4. We must therefore entertain the possibility that two enzymes (or one enzyme with a two-step action) are involved in generating the OS-$GlcNAc_2$ components.

Although the absence of exogenous metal requirement for the action of the ER enzyme conforms with observations made on PNGases from other sources, the pronounced effect of EDTA in abolishing its activity seems to be quite distinctive [14,15].

Although the substrate specificity of the ER PNGase in regard to the length of the peptide and the complete spectrum of N-linked oligosaccharides remains to be determined after the isolation of the enzyme, we focused our attention in the present study on the polymannose carbohydrate units as it is these that are physiologically present in the ER compartment and subsequently appear in the cytosol. Our observation that Pronase-generated glycopeptides were such favourable substrates for the enzyme might indicate that degradative mechanisms, whether proteolytic or glycosidic, which have conventionally been assigned to the lysosomes, might also exist in a proximal compartment such as the ER where they participate in molecular editing.

The absence of endo-β-*N*-acetylglucosaminidase activity from the ER, in contrast with the cytosol, where it has previously been localized in rat liver [25], is consistent with an ER-to-cytosol translocation of OS-$GlcNAc_2$ followed by cleavage of the chitobiose core; indeed, a recent report has suggested that an energy-requiring transport mechanism for this movement might occur in the HepG2 cells [32].

The migration of oligosaccharides from the ER to the lysosomes via the cytosol might seem a circuitous route but it has the clear advantage of bypassing the secretory channels of the cells where specific and defining interactions of oligosaccharides N-linked to proteins are known to occur [11–13].

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