Occurrence of a cytosolic neutral chitobiase activity involved in oligomannoside degradation: a study with Madin–Darby bovine kidney (MDBK) cells

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Neutral oligomannosides possessing one GlcNAc (OS-Gn1) and two GlcNAc (Os-Gn2) at the reducing end have been reported to be released during the N-glycosylation process in various biological models. To investigate which enzyme is responsible for OS-Gn1 formation, we used the Madin–Darby bovine kidney (MDBK) cell line which exhibits neither lysosomal chitobiase nor endoglucosaminidase activities. However, these cells produced OS-Gn1 and we showed that a neutral chitobiase is responsible for the transformation of OS-Gn2 into OS-Gn1. Using streptolysin O-permeabilized MDBK cells, we demonstrated that this neutral chitobiase activity is located in the cytosolic compartment and is active on oligomannoside species released during the N-glycosylation process.

INTRODUCTION

The production and the degradation of oligomannosides in mammalian cells are now well documented. Two major pathways have been reported to be involved in the release and degradation of mannose-containing oligosaccharides [1]. The first one is the lysosomal catabolism of asparagine-linked glycoproteins leading to the liberation of the glycan moieties which are further degraded by acidic glycosidases, the specificity of which is now well understood [1,2]. In contrast, the degradation of free oligomannoside material liberated during the biosynthetic N-glycosylation process at the rough endoplasmic reticulum (RER) level remains to be clarified. This latter pathway involved both the release of free oligosaccharides during the 'quality control' mechanism for glycoprotein maturation [3,4], and the release of phosphorylated and neutral oligosaccharides as regulatory steps for the control of the dolichol cycle [5,6]. Three subcellular compartments involving the action of specific α-mannosidases have been shown to take part in the catabolism of RER-derived oligosaccharide material: i.e. RER itself, cytosol and lysosome [1,6,7]. According to their subcellular location, the natural substrates for α-mannosidases are oligomannosides terminating in one or two GlcNAc residues at their reducing end: the presence of two GlcNAc residues does not affect the ordered pathway for the removal of mannose residues by the major lysosomal α-mannosidase [8]. In contrast, the neutral cytosolic mannosidase has been suggested to be involved in the degradation pathway of lipid-intermediate-derived oligosaccharides [1] and requires oligomannosides terminating in a single GlcNAc residue (Os-Gn1) for efficient activity [9].

Up to now, two enzymes have been described in mammals as being responsible for producing oligosaccharide structures possessing one GlcNAc residue at the reducing end. The first one is the chitobiase which is involved in a key step of the catabolism of Asn-linked glycoprotein, the second one corresponds to the endo-*N*-acetyl-β-D-glucosaminidase activity. Chitobiase has been predominantly located in lysosomes, with a maximum of activity at pH 3.5, and is active only towards non-conjugated oligosaccharides possessing a chitobiosyl residue at the reducing end [10]. It has been purified from rat liver [11] and the full-length clones for human placenta and rat liver have been described recently [12]. Endoglucosaminidase has been located in the cytosolic fraction of rat liver and human kidney [13,14], with a maximum activity at pH 6.5, and the enzyme is active both towards non-conjugated oligosaccharides and towards glycopeptides of oligomannosidic [13–15] and asialo-*N*-acetyl-lactosaminic types [14].

The distribution of lysosomal chitobiase among mammalian species has been widely studied. Chitobiase levels were not measurable in the kidney of sheep, cattle and pig [16], and only residual amounts of chitobiase enzyme activity were detected in bovine liver and brain [12]. In these animals, a deficiency in lysosomal mannosidase, or treatment with swainsonine, results in the accumulation of an intact di-*N*-acetylchitobiose unit at the reducing end of oligosaccharides fragments, although some oligomannoside isomers possessing a single terminal GlcNAc have been detected [17]. As these latter isomers resemble the dolichol-linked oligosaccharides, it has been postulated that they originate from the degradation of either newly synthesized glycoproteins or lipid intermediates by the dual action of neutral mannosidase and cytosolic endoglucosaminidase.

Using a cell line derived from bovine kidney (Madin–Darby bovine kidney; MDBK), we demonstrated in the present work that these cells, which did not exhibit either lysosomal chitobiase or endoglucosaminidase activities, possessed a cytosolic neutral chitobiase activity which was responsible for the liberation, during the N-glycosylation process, of oligomannosides possessing one GlcNAc residue at the reducing end.

MATERIALS AND METHODS

Cells and cell culture

The Chinese hamster ovary (CHO) cell line used in these experiments was the proline-auxotrophic clone Pro^{-5}. The CHO cells were routinely cultured in monolayers in alpha minimal

Abbreviations used: endo H, endo-*N*-acetyl-β-D-glucosaminidase H (EC 3.2.1.96); CHO, Chinese hamster ovary; MDBK, Madin–Darby bovine kidney; RER, rough endoplasmic reticulum; $GlcNAc_2$, $(GlcNAc)_2$.

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essential medium with 10% (v/v) fetal-calf serum (Gibco Laboratories, Grand Island, NY, U.S.A.). The MDBK cells were cultured in Eagle's minimal essential medium with 10% (v/v) horse serum (Gibco Laboratories, Grand Island, NY, U.S.A.). Both cell lines were grown at 34 °C in 10-cm-diam. Petri dishes, under a 5% CO_2 atmosphere.

Metabolic labelling

In the standard conditions, cells were labelled for 1 h with [2-^{3}H]mannose (429 GBq/mmol; from Amersham International, Amersham, Bucks., U.K.), at a level of 50 μCi/ml in the culture medium with 0.5 mM glucose and 10% (v/v) dialysed calf serum. At the end of the labelling period, the medium was removed by aspiration and the cell layer was rapidly washed three times with ice-cold PBS. The reaction was terminated, and the sequential lipid extractions were achieved as previously reported [5].

Preparation and analysis of free oligomannosides released during the labelling period

The oligosaccharide material contained in the upper phase of the sequential lipid extraction, was purified on a Biogel P2 column. The neutral oligosaccharide were separated from phosphorylated material on a QAE-Sephadex A-25 column (Pharmacia LKB, Sweden) as already described [5]. The analysis of the neutral oligosaccharide material was achieved by HPLC on an amino-derivatized column, Asahipak NH2-P-50 (5 μm; Asahi, Kawasaki-hsu, Japan), with the following gradient solvent system: acetonitrile/water from (70:30, v/v) to (50:50, v/v), at a flow rate of 1 ml/min for 80 min. The separation of the labelled oligosaccharides was followed by continuous-flow detection of the radioactivity with a Flo-One beta detector (Flotec, France) using Luma Flow II (Lumac, The Netherlands) as scintillation fluid. Authentic oligomannosides with one GlcNAc at the reducing end, used as internal markers, were purified from urine of patients with mannosidosis and were a generous gift from Dr. G. Strecker, Villeneuve d'Ascq, France. Oligomannosides with a chitobiosyl unit at the reducing end were prepared from mild acid hydrolysis of radiolabelled lipid intermediates as described in [5].

Hydrolysis of oligomannoside by endo-*N*-acetyl-β-D-glucosaminidase H (endo H)

Neutral 2-[^{3}H]mannoside material was dissolved in 50 mM sodium phosphate, pH 5.5, and incubated overnight with 10 m-units of endo-H (Boehringer Mannheim, Mannheim, Germany). Neutral oligomannosides were purified on Biogel P2 and the size analysis was achieved by HPLC.

Determination of the chitobiase activity

The radioactive oligomannoside [^{14}C]$Man_8GlcNAc_2$ was prepared after extensive Pronase digestion of bovine lactotransferrin according to [18], followed by hydrazinolysis and N-reacetylation with [1-^{14}C]acetic anhydride (1.1 GBq/mmol; Amersham International, Bucks., U.K.), as described in [19]. The specific radioactivity was around 10^5 d.p.m./mg of oligosaccharide.

Cell homogenate was obtained by scraping the cells with a rubber policeman in a buffer containing 10 mM Hepes, 15 mM KCl, 1% Triton X-100, pH 7.2. Cell homogenate was kept at −80 °C until required for use.

Assays were performed by incubating 10^4 d.p.m. (0.1 mg) of the above radioactive substrate in 10 μl of distilled water with 10 μl of the appropriate buffer and 25 μl of homogenate in a final volume of 50 μl for 6 h at 37 °C. Lysosomal mannosidases were inhibited by the use of 0.5 μg of swainsonine. The reaction was stopped by addition of 50 μl of cold ethanol. Separation of the released [^{14}C]GlcNAc was achieved by using descending paper chromatography for 16 h in solvent system A (pyridine/ethyl acetate/acetic acid/water; 5:5:1:3, by vol.). Control incubations without homogenate have been made to take into account the GlcNAc liberated by chemical hydrolysis of the substrate (less than 10% of the released GlcNAc). The radioactivity was determined along the chromatographic path by counting 1 cm × 5 cm bands. Specific enzyme activity was expressed as a percentage of [^{14}C]GlcNAc released per 6 h and per mg of protein.

Determination of the endo-*N*-acetyl-β-D-glucosaminidase activity

The glycoasparagine, $Man_8GlcNAc_2Asn$, prepared after extensive Pronase digestion of bovine lactotransferrin [18] was radioactively labelled by ^{14}C-acetylation of the asparagine residue as described in [20] using 1-[^{14}C]acetic anhydride (1.1 GBq/mmol.) from Amersham International, Bucks., U.K. The final specific radioactivity was around 2×10^6 d.p.m./mg of glycoasparagine. The cell homogenate was prepared as described in the chitobiase assay. Determination of the endo-*N*-acetyl-β-D-glucosaminidase activity was carried out by detecting the formation of GlcNAc-[^{14}C]Asn liberated from the $Man_8GlcNAc_2$-[^{14}C]Asn. The glyco-[^{14}C]asparagine (10000 d.p.m. in 20 μl of water) was incubated with 20 μl of cell homogenate and 60 μl of TKM buffer [30 mM Tris/HCl (pH 7.5), 120 mM KCl, 4 mM magnesium acetate buffer] for 2 h at 37 °C. The reaction was stopped by addition of 100 μl of cold ethanol. Separation of the GlcNAc-[^{14}C]Asn was achieved by using descending paper chromatography for 16 h in solvent system A as described in [13].

Incubation with oligomannosides possessing a chitobiosyl unit at the reducing end

A mixture of labelled oligomannosides possessing a chitobiosyl unit at the reducing end was prepared from oligosaccharide lipids extracted from metabolically labelled CHO cells with 2-[^{3}H]mannose under standard conditions. The oligosaccharide moieties of the oligosaccharide lipids were released by mild acid treatment (0.1 M HCl in tetrahydrofuran, 50 °C for 2 h). The mixture was desalted on a Biogel P2 column equilibrated and eluted with 0.1 M acetic acid. Incubations with cell lysate were performed by incubating 5×10^4 d.p.m. of the oligomannosides in 10 μl of distilled water and 50 μl of lysate (5×10^6 cells), adjusted to 100 μl with TKM buffer (pH 7.5). Lysosomal mannosidases were inhibited by adding 0.5 μg of swainsonine. After incubation, the oligosaccharide material was purified on a Biogel P2 column and analysed by HPLC.

Cell permeabilization and labelling

MDBK cells were routinely permeabilized using streptolysin O (BioMérieux, Marcy l'Étoile, France) as follows: cells were resuspended in PBS, pH 6.6, containing 13 mM dithiothreitol and incubated on ice for 30 min with streptolysin O (7.5 units/μl of cell suspension). Cells were then washed with TKM buffer and incubated at 37 °C for 15 min. For the removal of the cytosol, permeabilized cells were centrifuged (10000 ***g*** for 10 min) and the cell pellet was washed with TKM buffer.

Use of endo-H as a probe to measure the integrity of RER vesicles

Permeabilized cells were first labelled for 20 min using the standard incubation conditions [5 μCi of GDP-[^{14}C]mannose

(11.4 GBq/mmol) from Amersham International, 50 μM UDP-GlcNAc, 50 μM UDP-Glc, 5 mM AMP, 2 mM $MnCl_2$, and 5 mM $MgCl_2$]. After incubation, the cells were washed and resuspended in TKM buffer adjusted to pH 6.8. Endo-H (5 m-units for 10^6 cells) was added in the presence of various concentrations of Triton X-100 from 0 to 1%. After 1 h incubation, the extent of oligosaccharides released in the presence of endo H was monitored by treating with 10% trichloroacetic acid and by comparing the amount of acid-insoluble radioactivity to a control incubated without endo H.

RESULTS

Demonstration of a neutral chitobiase activity in MDBK cells

The results of preliminary experiments with cell homogenates indicated that CHO and MDBK fibroblasts exhibited a chitobiase activity. The liberation of the terminal GlcNAc from the $Man_8GlcNAc_2$ used as substrate was linear for 6 h and the activity was strictly dependent on the amount of protein in the cell extract. Figure 1 shows the pH dependence of the chitobiase activity of both cell lines. As already demonstrated for rodent cells [11], CHO fibroblasts exhibited two chitobiase activities; one at acidic pH, the other at neutral pH (Figure 1A). In MDBK cells the optimum pH of the enzymic activity was 7, and no activity was detected at acidic pH values, as expected for bovine cells [16]. This demonstrated that, like bovine kidney from which they originate, the MDBK cells lack lysosomal chitobiase activity.

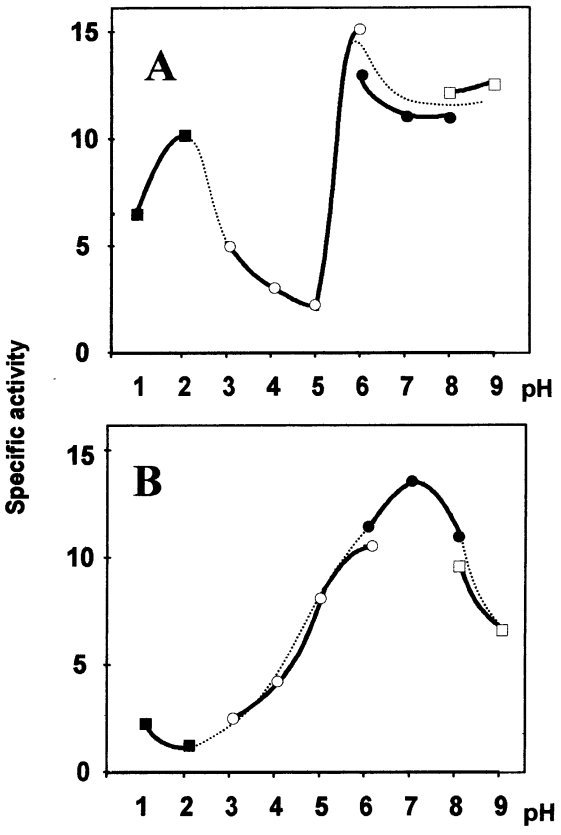

Figure 1 pH dependence of the chitobiase activity in CHO and MDBK cells

The chitobiase activity of CHO (**A**) and MDBK cells (**B**) was determined in buffers at different pH: (■) KCl/HCl buffer; (○) sodium citrate buffer; (●) sodium phosphate buffer; (□) Tris/HCl buffer. The specific activity is expressed as a percentage of [^{14}C]GlcNAc liberated over 6 h per mg of protein.

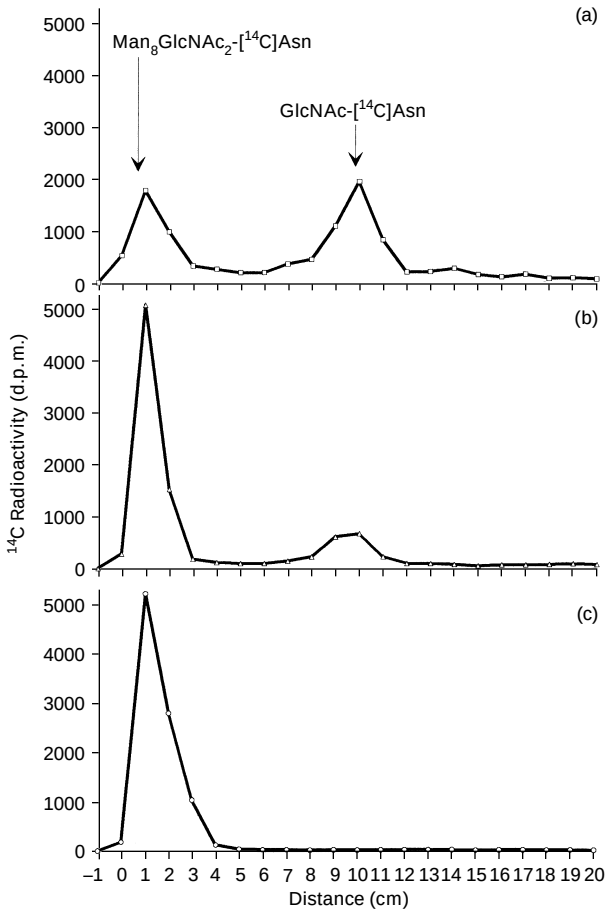

Figure 2 Determination of the endo-*N*-acetyl-β-D-glucosaminidase activity

$Man_8GlcNAc_2$-[^{14}C]Asn was incubated with endo H (**a**), CHO cells lysate (**b**) and MDBK cells lysate (**c**). After incubation at pH 7.4, at 37 °C for 2 h, separation of the hydrolysis products was achieved by descending paper chromatography in solvent system A.

To determine whether an endo-*N*-acetyl-β-D-glucosaminidase activity was present in MDBK cells, cell lysates were incubated at pH 7.5 with $Man_8GlcNAc_2$-[^{14}C]Asn. The liberation of GlcNAc-[^{14}C]Asn, which co-migrates with authentic GlcNAc-[^{14}C]Asn obtained after incubation of the substrate with endoH (Figure 2a), was demonstrated with CHO (Figure 2b) cells but not with MDBK cells (Figure 2c). This demonstrates that the chitobiase activity exhibited at neutral pH by MDBK cells is not due to a broad specificity of an endo-*N*-acetyl-β-D-glucosaminidase activity, which could be active on non-conjugated oligosaccharides.

Release of oligomannosides with a single GlcNAc at their reducing end during the N-glycosylation process of MDBK cells

As previously reported for CHO fibroblasts [4–6], the glycoprotein biosynthesis is accompanied by the release of soluble oligomannosides which are heterogeneous, possessing either a chitobiosyl unit or a single GlcNAc residue at their reducing end. MDBK cells were pulse-labelled for 1 h with [2-^{3}H]mannose and the released oligomannosides were purified from the aqueous

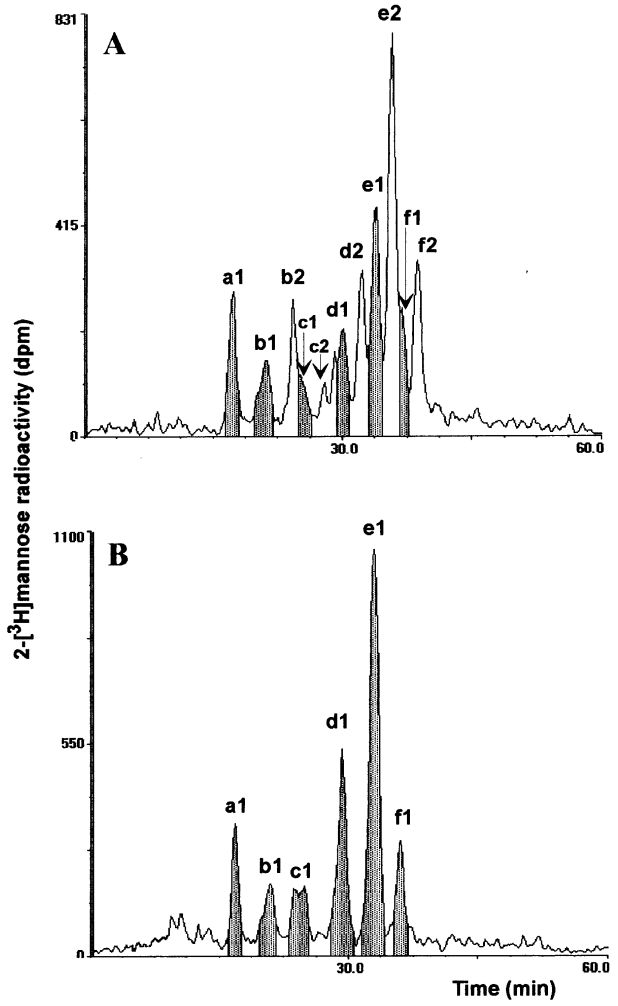

Figure 3 HPLC analysis of free oligomannosides liberated during [2-^{3}H]mannose labelling of MDBK cells

Free oligomannosides liberated during [2-^{3}H]mannose labelling of MDBK cells were analysed by HPLC before (**A**) and after (**B**) endo-H treatment. Oligomannosides possessing only one terminal GlcNAc residue are represented as shaded areas: a1, $Man_5GlcNAc_1$; b1, $Man_6GlcNAc_1$; c1, $Man_7GlcNAc_1$; d1, $Man_8GlcNAc_1$; e1, $Man_9GlcNAc_1$; f1, $Glc_1Man_9GlcNAc_1$. Oligomannosides possessing two terminal GlcNAc residues are b2, $Man_6GlcNAc_2$; c2, $Man_7GlcNAc_2$; d2, $Man_8GlcNAc_2$; e2, $Man_9GlcNAc_2$; f2, $Glc_1Man_9GlcNAc_2$.

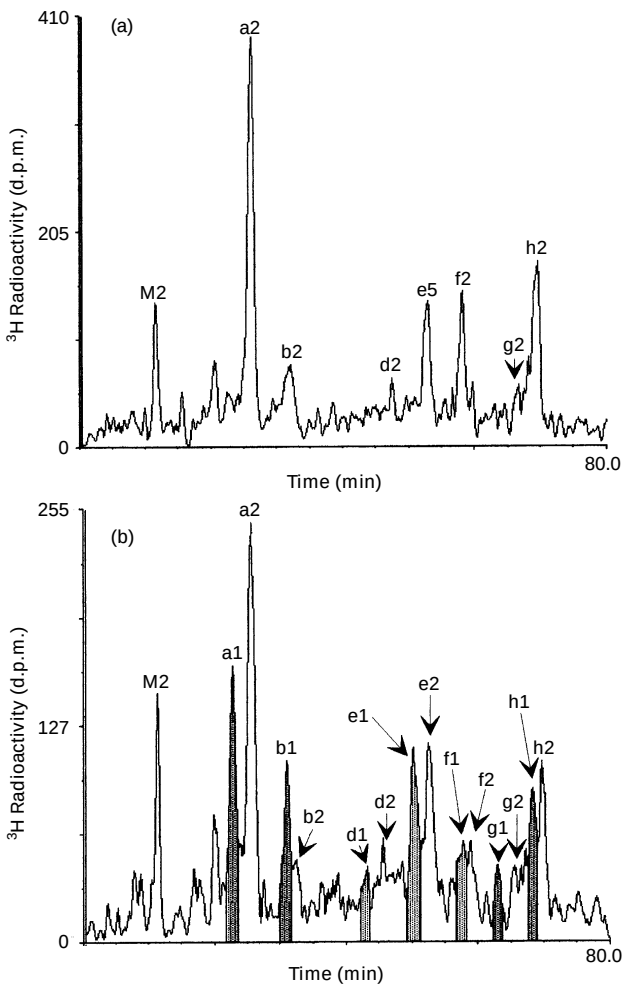

Figure 4 Incubation of a mixture of oligosaccharides possessing two GlcNAc residues at their reducing end with MDBK cells homogenate

A mixture of labelled oligomannosides was prepared by mild acid hydrolysis of oligosaccharide-lipid extraction from [2-^{3}H]mannose-labelled CHO cells in the standard conditions. The mixture was analysed by HPLC before (**a**) and after (**b**) incubation with MDBK cells lysate. Peaks: a1, $Man_5GlcNAc_1$; b1, $Man_6GlcNAc_1$; c1, $Man_7GlcNAc_1$; d1, $Man_8GlcNAc_1$; e1, $Man_9GlcNAc_1$; f1, $Glc_1Man_9GlcNAc_1$; g1, $Glc_2Man_9GlcNAc_1$; h1, $Glc_3Man_9GlcNAc_1$. a2, $Man_5GlcNAc_2$; b2, $Man_6GlcNAc_2$; c2, $Man_7GlcNAc_2$; d2, $Man_8GlcNAc_2$; e2, $Man_9GlcNAc_2$; f2, $Glc_1Man_9GlcNAc_2$; g2, $Glc_2Man_9GlcNAc_2$; h2, $Glc_3Man_9GlcNAc_2$. M2 represents $Man_2GlcNAc_2$ used as internal standard.

phase of the sequential lipid extraction. Figure 3(A) shows an HPLC analysis of this oligosaccharide fraction. Peaks a1, b1, c1, d1, e1 and f1 co-migrated with authentic oligomannoside standards isolated from urine of a patient with mannosidosis i.e. $Man_5GlcNAc$, $Man_6GlcNAc$, $Man_7GlcNAc$, $Man_8GlcNAc$, $Man_9GlcNAc$ and $Glc_1Man_9GlcNAc$ respectively. Peaks b2, c2, d2, e2 and f2 co-migrated with oligomannosides possessing a chitobiosyl unit at their reducing end, i.e. $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, $Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$ respectively. They were converted into oligomannosides possessing a single GlcNAc residue by incubation with endo-H (Figure 3B). This demonstrates that the release of both oligomannoside populations possessing one and two terminal GlcNAc residues occurs during glycoprotein biosynthesis in MDBK cells. This free oligosaccharide material has been shown to originate from the degradation of dolichol-derived oligosaccharides [5,6] and from the degradation of newly synthesized glycoproteins [3–5]. We further examined whether the neutral chitobiase activity were involved in the degradation of this free oligosaccharide material.

Specificity of the neutral chitobiase activity towards oligomannoside substrates

As shown in Figure 3, a wide variety of oligomannosides (from Glc_1Man_9- to Man_5- species) were released during glycoprotein biosynthesis. Except for the Man_5 oligomannoside, which was only of the $GlcNAc_1$ species, the other free oligosaccharides released belonged to the $GlcNAc_1$ and $GlcNAc_2$ species. Figure 4 shows the result of an incubation at pH 7.5 of a mixture of radioactive oligomannosides of the $GlcNAc_2$ family, from $Glc_3Man_9GlcNAc_2$ to the $Man_5GlcNAc_2$ with a lysate from

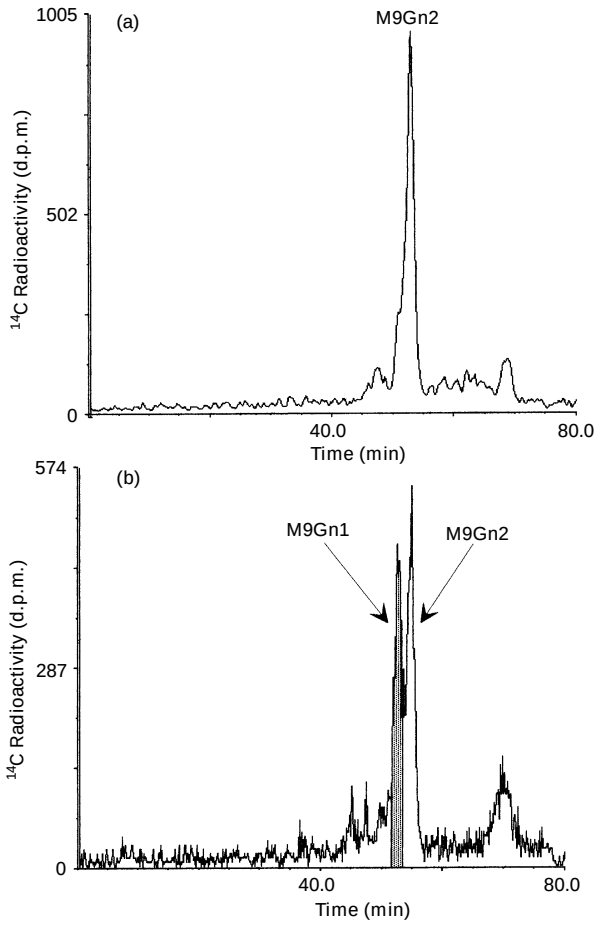

Figure 5 Incubation of [^{14}C]$Man_9GlcNAc_2$ with permeabilized MDBK cells

After streptolysin O permeabilization, MDBK cells were submitted (**a**) or not submitted (**b**) to centrifugation and washings to eliminate the cytosol. Permeabilized cells were then submitted to lysis and incubated with labelled [^{14}C]$Man_9GlcNAc_2$ for 2 h at 37 °C in TKM buffer, pH 7.5. After incubation, labelled material was purified on a Biogel P2 column and analysed by HPLC. Abbreviations: M9Gn1, $Man_9GlcNAc_1$; M9Gn2, $Man_9GlcNAc_2$.

MDBK cells. All the oligomannosides were cleaved by the neutral chitobiase activity into the corresponding $GlcNAc_1$ family. This indicates that the neutral chitobiase is active towards the oligomannosides released during the N-glycosylation process. Such an hydrolysis could be a prerequisite for their further hydrolysis by the neutral cytosolic mannosidase as suggested by Daniel et al. [1].

Cytosolic location of the chitobiase activity of MDBK cells

Streptolysin O has been demonstrated to permeabilize plasma membrane while leaving intracellular organelles intact. It has been used after metabolic labelling of HepG2 cells to show the presence of cytosolic oligomannosides [7]. Figure 5 shows the result of an incubation of radioactive $Man_9GlcNAc_2$ with a lysate of streptolysin O-permeabilized MDBK cells. When the permeabilization process was followed by the elimination of the cytosol [by low-speed centrifugations (200 ***g***, 5 min) and washings of the permeabilized cells before lysis] no transformation was observed (Figure 5a), in contrast to the result obtained when the cytosol was not removed before lysis (Figure 5b). In this latter

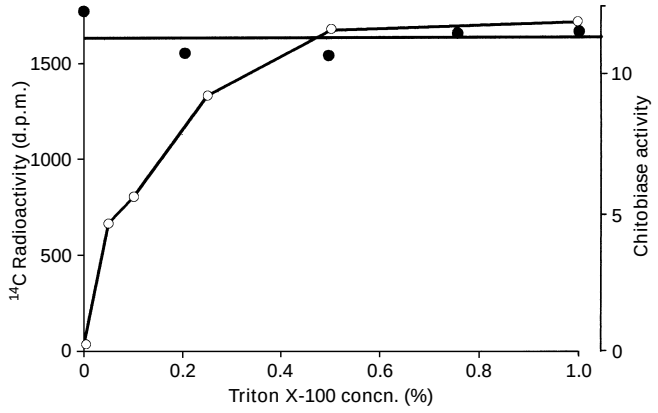

Figure 6 Effect of Triton X-100 at different concentrations on the latency of chitobiase activity and RER integrity in the permeabilized cells

MDBK cells were permeabilized with streptolysin O and labelled without previous washings with GDP-[^{14}C]Man, UDP-GlcNAc and UDP-Glc. After 20 min incubation at 37 °C, the cells were submitted to various Triton X-100 concentrations. At each concentration the chitobiase activity of the lysate was measured (●) and the quantity of radioactivity released from [^{14}C]glycoproteins by exogenous endo H (○) was determined as described in the Materials and methods section. The chitobiase activity is expressed as in Figure 1.

case, 50 % of the radioactivity was converted into $Man_9GlcNAc_1$ due to the chitobiase activity in the cytosol.

In order to unambiguously demonstrate the cytosolic location of the chitobiase activity, latency experiments were performed using permeabilized cells as the enzyme source. After the permeabilization process, the semi-intact cells were labelled with GDP-[^{14}C]Man, UDP-GlcNAc and UDP-Glc for 20 min and submitted to various Triton X-100 concentrations from 0 to 1 %. At each detergent concentration, the chitobiase activity was measured and the latency of the RER was determined by measuring the sensitivity of [^{14}C]glycans bound to glycoproteins to be released by endo H used as an exogenous probe [21] (Figure 6). When permeabilized cells were incubated with endo H without Triton X-100, only 2 % of the [^{14}C]oligosaccharides could be released from endo H-sensitive glycoproteins (latency 98 %), the endo H-sensitive glycoproteins being fully deglycosylated at a concentration of 0.4 % Triton X-100. In contrast, chitobiase activity was not affected by the detergent (Figure 6), demonstrating that the enzyme activity was not trapped in RER vesicles but was located in the extravesicular compartment.

DISCUSSION

In contrast to the catabolism of asparagine-linked glycoproteins, which is mainly restricted to lysosomes, the degradation of free oligomannoside material released during the N-glycosylation process requires several subcellular compartments: RER, cytosol and, presumably, finally the lysosomal compartment. For both pathways, the formation of an oligosaccharide possessing one GlcNAc residue at the reducing end seems to be crucial. For the lysosomal pathway, the role of the acidic di-*N*-acetylchitobiase, which has been defined as 'reducing-end exohexosaminidase' [11] is now well documented [10,22]. For the degradation of the oligosaccharide material generated in the lumen of the RER during the biosynthetic N-glycosylation process, the presence of oligosaccharides possessing one or two GlcNAc residues has

been demonstrated in the cytosol of several cell types [3,5,7] and a catabolic pathway leading to a single $Man_5GlcNAc_1$ isomer has been pointed out [6].

In this paper, we demonstrate that MDBK cells, which originate from bovine kidney and do not exhibit any acidic chitobiase activity, possess a cytosolic neutral chitobiase activity able to cleave the reducing chitobiosyl residue from oligomannosides.

The chitobiase activity found in MDBK cells has a similar optimum pH and the same specificity towards oligomannosidic-type glycans as the endo-*N*-acetyl-β-D-glucosaminidase activity described in rat [13,14] and in human tissue [23]. The endo-glucosaminidase activity described in rat and human tissues has the characteristics of a soluble enzyme present in the cytosolic fraction after subcellular fractionation, and its physiological role is not yet understood. In the case of MDBK cells, no endo-*N*-acetyl-β-D-glucosaminidase activity has been detected and the substrates of the enzyme are only free oligosaccharide material.

In fact, MDBK cells released oligomannosides possessing both two ($OS\text{-}Gn_2$) and one ($OS\text{-}Gn_1$) GlcNAc residues during the glycosylation process. We postulate that the described chitobiase activity is responsible for the conversion of $OS\text{-}Gn_2$ to $OS\text{-}Gn_1$. This conversion could occur at each step of the oligomannoside degradation, since all glycan species which are susceptible to release during the N-glycosylation process can be the substrates for such a chitobiase activity.

Using streptolysin O-permeabilized cells, we demonstrated that the chitobiase was released during the permeabilization process as a cytosolic enzyme, in the conditions where the latency of the RER vesicles was 98%. This methodology, based on a plasma membrane permeabilization technique, allowed us to avoid the liberation of the lumenal content of microsomal vesicles which can occur when using subcellular fractionation techniques.

The action of the neutral cytosolic chitobiase could be the prerequisite step for the degradation by further glycosidases, since it has been established that the cytosolic mannosidase is more active on oligomannosides terminating with a single GlcNAc residue [9]. We suggest that, in rodent or human, the hydrolysis of the chitobiose residue of oligomannosides is achieved by the cytosolic endo-*N*-acetyl-β-D-glucosaminidase which possesses the same subcellular location and is active towards free oligomannosides. As oligomannosides are released into the lumen of RER vesicles by the N-glycosylation machinery, a translocation process has to be involved for the transport of the material through intracellular membranes. Such a transport mechanism has already been suggested [6,7], but needs further investigation.

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