Release of oligomannoside-type glycans as a marker of the degradation of newly synthesized glycoproteins

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The N-glycosylation of proteins is accompanied by the release of soluble oligosaccharide material. Besides oligosaccharide phosphates originating from the cleavage of lipid intermediates, neutral free oligosaccharides represent the major part of this material and are heterogeneous depending on whether the reducing end has one or two *N*-acetylglucosamine residues. The present study focuses on the intracellular origin of neutral free oligosaccharides in a CHO cell line. Kinetic and pulse-chase experiments clearly indicate that oligosaccharides possessing a chitobiosyl unit are derived from oligosaccharide pyrophosphodolichol, whereas oligosaccharides possessing one *N*-acetylglucosamine residue are derived from newly synthesized glycoprotein. This relationship is confirmed by comparing the

glycosylation pattern of lipid donors and glycoproteins with those of neutral free oligosaccharides under various incubation conditions (inhibition of protein synthesis, presence of processing inhibitors, presence or absence of glucose). Degradation of newly synthesized glycoprotein and formation of neutral oligosaccharides with one *N*-acetylglucosamine residue are inhibited at 16 °C but not affected by lysosomotropic agents such as leupeptin or NH_4Cl . Together with the fact that the degradation of newly synthesized glycoproteins and the subsequent release of the glycan are recovered in permeabilized cells, these results suggest that this phenomenon occurs in the rough endoplasmic reticulum or in a closely related compartment.

INTRODUCTION

Glycoprotein biosynthesis results from two separate metabolic pathways which are localized in the rough endoplasmic reticulum (ER), i.e. the dolichol cycle for the lipid donor and protein synthesis for the acceptor. The fate of the glycan moieties of the lipid intermediates is not always linked to protein, as the release of soluble oligosaccharide material associated with glycoprotein synthesis has been demonstrated in various biological models, for example splenocytes [1], thyroid gland [2] and fibroblasts [3]. The latter studies support the idea that degradative mechanisms generating free oligosaccharide phosphates and neutral free oligosaccharides may be a way of adjusting the level of lipid donor to the amount of protein acceptor.

Although the origin of the oligosaccharide phosphates has been clearly elucidated [3], that of the neutral free oligosaccharide material is not yet established. In order to obtain a better insight into the relationships between the lipid donors, newly synthesized glycoproteins and neutral free oligosaccharides, we have chosen the B3F7 cell line as a biological model. This cell line is a glycosylation mutant of Chinese hamster ovary (CHO) cells which does not synthesize mannose phosphodolichol [4]. At low glucose concentration (0.5 mM), we observed that the amount of neutral free oligosaccharide released was higher than in wild-type CHO cells. Furthermore, as previously published [3], the nature of the glycan moieties of glycoproteins can be manipulated by varying the incubation conditions (with or without 0.5 mM glucose).

Recent studies have revealed that the ER is a site at which newly synthesized proteins can be degraded (for general reviews, see refs. [5] and [6]). Degradation of newly synthesized proteins in the rough ER is distinct from lysosomal degradation, as misfolded proteins or overexpressed subunits of oligomeric

complexes are in most cases degraded without being released from the rough ER.

In the present report, we demonstrate that newly synthesized glycoproteins can also be degraded at the rough ER level generating free oligosaccharides with one GlcNAc at the reducing end (OS-Gn1). In contrast, the free oligosaccharides terminating in a chitobiosyl unit at the reducing end (OS-Gn2) originate from the hydrolase activity of the oligosaccharyltransferase. Studies based on temperature-dependence and effects of lysosomotropic agents show that this degradation is not localized in the lysosomes but occurs in the rough ER or a closely related compartment.

MATERIALS AND METHODS

Cells and cell culture

Mutant cell line B3F7, which does not synthesize mannose phosphodolichol, was a gift from Dr. S. S. Krag (The Johns Hopkins University, Baltimore, MD, U.S.A.). In these cells, the nature of the glycan moiety bound to the glycoprotein can be manipulated according to the incubation conditions (absence or presence of 0.5 mM glucose and processing inhibitors [3]). Cells were routinely cultured in monolayers in α -minimum essential medium with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY, U.S.A.) at 34 °C in 10 cm culture dishes, under 5% CO_2 [4].

Metabolic labelling and extraction

Before reaching confluency (5×10^6 – 7×10^6 cells/dish), cells were labelled with $[2\text{-}^3\text{H}]\text{mannose}$ (429 GBq/mmol) from Amersham International (Amersham, Bucks., U.K.) (50 $\mu\text{Ci/ml}$ in α -minimal essential medium) with or without 0.5 mM glucose; 10% dialysed fetal calf serum was present. When used, castano-

Figure 1 Flow diagram explaining the isolation procedure for oligosaccharide fractions

Free oligosaccharide material was isolated from the aqueous phase of the sequential lipid extraction. Glycan moieties of lipid intermediates and glycoproteins are liberated by mild acid hydrolysis with 0.1 M HCl in tetrahydrofuran (THF) and peptide *N*-glycanase F (PNGase F) treatment respectively.

spermine (Cst) and 1-deoxymannojirimycin (dMM) (Boehringer-Mannheim, Mannheim, Germany) were added 30 min before labelling and were present throughout the incubation period at final concentrations of 50 µg/ml and 100 µg/ml respectively. B3F7 cells were transferred to a temperature of 39 °C 8 h before labelling and kept at this temperature during incubation [4].

For incubations in the presence of a protein-synthesis inhibitor, a 30 min preincubation was performed with 1 mM cycloheximide. For incubations in the presence of leupeptin, a 30 min preincubation was carried out with 100 µg/ml leupeptin. The effect of NH₄Cl was studied after an overnight preincubation of the cells in 20 mM NH₄Cl in the culture medium. This concentration was maintained during the incubation with radioactive mannose.

At the end of the labelling period, the medium was removed and the cell layer washed rapidly three times with ice-cold PBS. Sequential extraction was then achieved as previously described [3].

Incorporation of radioactivity into oligosaccharide lipids was measured by liquid-scintillation counting. The protein pellet was digested overnight at room temperature with 0.2 mg of Tos-Phe-CH₂Cl-treated trypsin (Sigma, St. Louis, MO, U.S.A.) in 0.1 M NH₄HCO₃, pH 7.9, in a final volume of 0.2 ml, and a sample was counted for radioactivity. The soluble oligosaccharide material contained in the upper aqueous phase was separated from GDP-mannose, mannose 1-phosphate and mannose by gel filtration on a Bio-Gel P2 column. This material was eluted a few fractions later than the void volume, as reported previously [1]. The fractions containing the soluble oligosaccharide material were pooled, and the radioactivity was determined by liquid-scintillation counting.

Protein neosynthesis was evaluated by incubation with 2.5 µM L-[³⁵S]methionine (37 TBq/mmol) from Amersham International. After incubation, cells were scraped, precipitated by 2 vol. of 10% trichloroacetic acid, filtered and washed on glass-fibre filters.

Preparation and incubation of permeabilized cells

Cells were allowed to swell for 10 min in a hypotonic buffer containing 15 mM KCl and 10 mM Hepes, pH 7.2, at 4 °C, then scraped [7]. Permeabilization was checked by the Trypan Blue exclusion test. Incubations of 5 × 10⁶ semi-intact cells were carried out in an isotonic buffer containing 90 mM KCl and 50 mM Hepes, pH 7.2, in the presence of 5 µCi of GDP-[¹⁴C]mannose (11.4 GBq/mmol) from Amersham International, 50 µM UDP-GlcNAc, 1 mM ATP, 2 mM MnCl₂ and 5 mM MgCl₂ in a final volume of 100 µl during 1 h at 37 °C. The extraction procedure was as described previously [3].

Release and h.p.l.c. analysis of glycan moieties from oligosaccharide lipids and glycoproteins

The glycan moiety of the oligosaccharide lipids was released by mild acid treatment (0.1 M HCl in tetrahydrofuran, 50 °C for 2 h). The glycan moiety linked to protein was cleaved by peptide *N*-glycanase F (EC 3.2.2.18) as follows: the trypsin-treated protein obtained previously was boiled for 10 min to destroy the trypsin activity and the tryptic peptides were dried and dissolved in 20 mM sodium phosphate (pH 7.5) containing 50 mM EDTA, 50% glycerol and 0.02% NaN₃. Peptide *N*-glycanase F (Boeh-

ringer-Mannheim) was then added (0.5 unit) for an overnight incubation at 37 °C. All the preparation and analytical steps are summarized in Figure 1.

Size analysis of the glycan moieties was by h.p.l.c. on an amino-derivatized column: Supelcosil LC-NH₂, 5 µm (Supelco, Bellefonte, PA, U.S.A.) with a solvent system of acetonitrile/water from 70:30 (v/v) to 50:50 (v/v), at a flow rate of 1 ml/min over 80 min. The 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 liquid.

Purification and h.p.l.c. analysis of free oligosaccharide material released into the aqueous phase

For further analysis, the free oligosaccharide material released into the aqueous phase of the lipid extraction after purification on a Bio-Gel P2 column was passed through a 1 ml QAE-Sephadex column equilibrated with 10 mM Tris base [8]. The neutral free oligosaccharides were eluted in the first 2 ml of 10 mM Tris base. Phosphorylated oligosaccharides were eluted with 70 mM NaCl [3]. After QAE-Sephadex chromatography, phosphorylated oligosaccharides were incubated with 5 units of alkaline phosphatase overnight at 37 °C in 100 mM Tris/HCl, pH 8.8. The neutral free oligosaccharides are heterogeneous depending on the number of GlcNAc residues at their reducing end [3]. Oligosaccharides with one GlcNAc residue are designated OS-Gn1 in this study and oligosaccharides with a chitobiosyl unit are designated OS-Gn2. The relative proportions of OS-Gn1 and OS-Gn2 were deduced from a comparison between two h.p.l.c. profiles before and after rat liver chitobias treatment of soluble material. Chitobias prepared as described previously [9] converts the neutral free oligosaccharide populations into OS-Gn1. Incubations with rat liver chitobias were in the following buffer: 200 mM citrate/phosphate (pH 3.5), 100 µl of enzyme preparation at 37 °C overnight in a final volume of 0.2 ml [3]. All the preparation and analytical steps are summarized in Figure 1. H.p.l.c. analyses were performed under the conditions described for the analysis of glycan moieties (a representative h.p.l.c. profile and an example of the calculation are presented in Figure 3).

RESULTS

Time course of the liberation of free neutral oligosaccharides in B3F7 cells

B3F7 cells were labelled with [2-³H]mannose in the presence of 0.5 mM glucose for 2 h. At various incubation times, the neutral free oligosaccharide material was analysed to discriminate between OS-Gn1 and OS-Gn2. The radioactivity incorporated into OS-Gn1 increased over 100 min after a lag period of 20 min. This was in contrast with the formation of OS-Gn2 (Figure 2). The lag period indicated that OS-Gn1 formation required previous synthesis of a sufficient amount of a precursor. The higher proportion of OS-Gn2 formed in short incubation times is illustrated in Figure 3, which shows h.p.l.c. profiles of neutral free oligosaccharides after 10 min of incubation. The only oligomannoside possessing one GlcNAc is Man₅GlcNAc₁ (9 % of the radioactivity). The major part (91 %) is composed of OS-Gn2 (mainly Glc₃Man₅GlcNAc₂, 78 %). As previously reported, phosphorylated free oligosaccharides are liberated during incubation [3]. Analysis of this material after phosphatase treatment confirmed, as demonstrated in [3], that this material (10 %

of the labelled soluble oligosaccharide material) is composed of Man₂GlcNAc₂-P (90 % of the radioactivity of the charged material) and Man₅GlcNAc₂-P (10 % of the radioactivity of the charged material). Thus this material cannot generate neutral free oligosaccharides by dephosphorylation, as they are mainly glucosylated, as shown in Figure 3. Thus neutral free oligosaccharides must originate from either a lipid donor or a glycoprotein. To investigate this, we began by examining the origin of OS-Gn2, by inhibiting glycoprotein formation with cycloheximide, a protein-synthesis inhibitor.

Effect of protein-synthesis inhibition on neutral oligosaccharide formation

Incubation of B3F7 cells with 1 mM cycloheximide caused protein-synthesis inhibition (98 % inhibition of [³⁵S]methionine incorporation into acid-precipitable material). Table 1 shows that mannose incorporation into protein was reduced in the presence of cycloheximide as expected. In contrast, the incorporation into OS-Gn2 was concomitantly increased. The total amount of radioactivity in glycoproteins plus OS-Gn2 was constant (1 822 000 d.p.m. in the control, 1 805 000 d.p.m. in the presence of cycloheximide). This indicates competition between transfer of the glycan moiety to protein and the formation of OS-Gn2, supporting the hypothesis that oligosaccharide pyrophosphodolichol is a precursor of both glycoprotein and OS-Gn2. In addition, as shown in Table 2, analysis of the neutral free oligosaccharides formed in the presence of the cycloheximide revealed that they are mainly glucosylated (Glc₃Man₅GlcNAc₂), i.e. identical with the glycan moiety bound to oligosaccharide pyrophosphodolichol from which they originate (not shown).

As previously indicated (Figure 2), the formation of OS-Gn1 requires the synthesis of a precursor. This precursor could be either OS-Gn2 or glycoprotein (Scheme 1). During protein-synthesis inhibition, the amount of radioactivity bound to OS-Gn1 was decreased 3-fold (Table 1) which is of the same order of magnitude as the observed inhibition of mannose incorporation into glycoprotein (2.7). This favours hypothesis 2. However,

Figure 2 Kinetics of formation of neutral free oligosaccharides

B3F7 cells were incubated in the presence of 0.5 mM glucose with 200 µCi of [2-³H]mannose for 10, 15, 30, 60 and 120 min. At each time, the relative proportions of OS-Gn1 (●) and OS-Gn2 (▲) were measured as described in the Materials and methods section and illustrated in Figure 3 for a 10 min incubation.

Figure 3 H.p.l.c. analysis of neutral free oligosaccharides and determination of the relative proportions of OS-Gn2 and OS-Gn1

B3F7 cells were incubated in the presence of 0.5 mM glucose with 200 μ Ci of [2-³H]mannose for 10 min. After incubation, the neutral free oligosaccharides were isolated and a fraction was treated with rat liver chitobiase as described in the Materials and methods section. H.p.l.c. profiles of the control (a) and the chitobiase-treated fraction (b) are shown. Peaks A, B, C and D of control are OS-Gn2 populations, as they are converted into A', B', C' and D' after rat liver chitobiase treatment. Peak D' of the control is not affected (OS-Gn1). The nature of the peaks and the relative percentages of OS-Gn2 and OS-Gn1 are indicated in (c). G, Glucose; M, mannose; Gn, *N*-acetylglucosamine.

cycloheximide is a non-specific protein-synthesis inhibitor which affects all cell proteins especially those with a high turnover rate. According to hypothesis 1, OS-Gn1 would be generated by degradation of OS-Gn2 by the action of a hypothetical chitobiase. The synthesis of this enzyme could be inhibited by cycloheximide leading to the observed result, i.e. accumulation of the substrate, OS-Gn2. To resolve this, the fates of OS-Gn2 and newly synthesized glycoproteins were followed in a pulse-chase experiment.

Pulse-chase experiment

Figure 4 shows a 240 min chase experiment after a 10 min pulse

Table 1 Influence of cycloheximide on mannose incorporation into glycoproteins and neutral free oligosaccharides

B3F7 cells were incubated for 1 h with 200 μ Ci of [2-³H]mannose in 4 ml of culture medium without glucose in the absence (control) or presence of 1 mM cycloheximide.

	10 ⁻³ × Mannose incorporation (d.p.m./10 ⁷ cells)	
	Control	+ Cycloheximide
Glycoproteins	1725	640
OS-Gn2	97	1165
OS-Gn1	492	150

Table 2 Influence of cycloheximide on neutral free oligosaccharide composition

B3F7 cells were incubated in the absence of glucose with 200 μ Ci of [2-³H]mannose in the absence (control) or presence of 1 mM cycloheximide as described in the Materials and methods section. Values in parentheses indicate percentages of the radioactivity associated with neutral free oligosaccharides.

	Composition (d.p.m./10 ⁷ cells)	
	Control	+ Cycloheximide
Man ₄ GlcNAc ₂	18 750 (2.7%)	17 540 (1.1%)
Man ₂ GlcNAc ₂	41 670 (6.0%)	131 580 (8.0%)
GlcMan ₅ GlcNAc ₂	22 220 (3.2%)	—
Glc ₂ Man ₅ GlcNAc ₂	13 890 (2.0%)	61 400 (3.7%)
Glc ₃ Man ₅ GlcNAc ₂	18 750 (2.7%)	1307 020 (78.2%)
Total OS-Gn2	116 670 (16.6%)	1517 540 (91.0%)
Man ₄ GlcNAc	51 390 (7.4%)	—
Man ₂ GlcNAc	316 665 (45.6%)	147 630 (9.0%)
GlcMan ₅ GlcNAc	211 111 (30.4%)	—
Glc ₂ Man ₅ GlcNAc	—	—
Glc ₃ Man ₅ GlcNAc	—	—
Total OS-Gn1	579 170 (83.4%)	147 630 (9.0%)

Scheme 1 Possible routes of synthesis of OS-Gn1

Oligosaccharide-PP-Dol, oligosaccharide pyrophosphodolichol.

with [2-³H]mannose. During the 240 min chase, the radioactivity associated with the lipid donor decreased with a $t_{1/2}$ of 15 min and was recovered in cellular and secreted glycoproteins (Figure 4a) and in soluble oligosaccharide material (Figure 4b). As previously suggested, lipid donors are precursors of OS-Gn2 as its formation strictly depends on the chase of the lipid donor. In contrast, the radioactivity associated with OS-Gn1 increased linearly throughout the chase period (Figure 4b). After a 60 min chase, the radioactivity bound to glycoprotein decreased with a $t_{1/2}$ of 150 min. This decrease in radioactivity can be explained by either the action of mannosidases at the Golgi level or degradation of

Figure 4 Pulse-chase experiment

B3F7 cells were labelled for 10 min in the presence of 0.5 mM glucose with 200 μ Ci of $[2\text{-}^3\text{H}]\text{mannose}$ and then chased for the indicated times. (a) Radioactivity associated with lipid donors ($\triangle$), intracellular glycoproteins ($\circ$) and total glycoproteins (intracellular + secreted) ($\bullet$); (b) formation of neutral free oligosaccharide OS-Gn1 ($\square$) and OS-Gn2 ($\boxtimes$).

the glycoproteins synthesized in the first 60 min chase. To check this, a chase experiment was performed in the presence of processing inhibitors Cst and dMM.

Pulse-chase experiment in the presence of processing inhibitors

The pulse-chase experiment was performed in the presence of 0.5 mM glucose, and Cst (50 μ g/ml) and dMM (100 μ g/ml) in order to inhibit glucosidase and mannosidase respectively. Using these incubation conditions, the lipid donor synthesized after the 10 min pulse was only of the $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ family (Figure 5a), and glycoproteins exhibited a pattern of mainly glucosylated populations (80 %) (Figure 5d). Analysis of the OS-Gn2 population synthesized during the pulse showed a pattern which was almost identical with that observed for the lipid donor, i.e. $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ (Figure 5b), whereas analysis of OS-Gn1 revealed a pattern similar to that of glycoproteins (i.e. 70 % of species glucosylated) (Figure 5e). After a 240 min chase in the presence of inhibitors, a decrease in radioactivity associated with glycoprotein was observed similarly to previously reported in

Figure 5 Structural pattern of glycan moieties of lipid intermediates, glycoproteins and free OS-Gn2 and OS-Gn1 during a pulse-chase experiment performed in the presence of processing inhibitors and 0.5 mM glucose

Pulse-chase of B3F7 cells was performed in the presence of 0.5 mM glucose and 50 μ g/ml Cst and 100 μ g/ml dMM. Cells were pulsed for 10 min with 200 μ Ci of $[2\text{-}^3\text{H}]\text{mannose}$ (a, b, d, e) and then chased for 240 min (c, f). $\square$, glycan moieties isolated from lipid donors (a); $\boxtimes$, glycan moieties isolated from glycoproteins (d); $\square$, neutral free oligosaccharides OS-Gn2 (b, c); $\boxtimes$, neutral free oligosaccharides OS-Gn1 (e, f). M_4 and M_5 represent oligomannosides with four and five mannose residues respectively. G_1M_5 , G_2M_5 and G_3M_5 represent oligomannosides with one, two and three glucose residues (and five mannose residues) respectively.

Figure 4(a). However, under these conditions, as glucosidases and mannosidases are inhibited, the observed loss of radioactivity can only be explained by degradation of newly synthesized glycoprotein and not by loss of radioactive mannose due to processing. Analysis of oligosaccharide material after the 240 min chase indicated that the OS-Gn2 exhibited the same pattern as observed during the pulse, i.e. mainly $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ (Figure 5c), demonstrating that this material was released into the lumen of rough ER from the lipid donor and remained glycosylated, as rough ER glucosidases were inhibited. The population pattern of OS-Gn1 after the 240 min chase (Figure 5f) was in agreement with a glycoprotein origin. These results clearly demonstrate that the radioactivity lost from glycoprotein during the chase was recovered associated with soluble free oligosaccharides of the OS-Gn1 family.

Influence of glycosylation on glycoprotein degradation in monolayer cells

When the pulse-chase experiment was performed in the absence of glucose and in the presence of processing inhibitors, the main species transferred to protein was $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$ (Figure 6a). The $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$ was recovered in the OS-Gn1 family after either a 10 min pulse (Figure 6b) or a 240 min chase (Figure 6c). These results paralleled those obtained in the presence of 0.5 mM glucose and processing inhibitors in which the glyco-

Figure 6 Structural pattern of glycan moieties of glycoproteins and free OS-Gn2 and OS-Gn1 during pulse-chase experiments performed in the presence of processing inhibitors and without glucose

Pulse-chase of B3F7 cells was performed in the absence of glucose and the presence of 50 $\mu\text{g/ml}$ Cst and 100 $\mu\text{g/ml}$ dMM. Cells were pulsed for 10 min with 200 μCi of $[2\text{-}^3\text{H}]\text{mannose}$ (a, b) and then chased for 240 min (c). $\square$, Glycan moieties isolated from glycoproteins (a); $\boxtimes$, neutral free oligosaccharides OS-Gn1 (b, c). M_4 and M_5 represent oligomannosides with four and five mannose residues respectively. G_1M_5 , G_2M_5 and G_3M_5 represent oligomannosides with one, two and three glucose residues (and five mannose residues) respectively.

proteins and OS-Gn1 were mainly glucosylated (Figures 5d, 5e and 5f). Glycoprotein degradation seems to be independent of the number of glucose residues linked to the glycan moiety.

Importance of intracellular trafficking in glycoprotein degradation

Three major sites for intracellular protein degradation are known: (i) the lysosome, where leupeptin and lysosomotropic agents such as NH_4Cl can be used as inhibitors; (ii) the cytosolic pathway, where ubiquitin is a degradation signal for the protein; (iii) a non-lysosomal degradation pathway in a poorly defined post-ER compartment described in various systems [10]. In each case, the degradation depends on the intracellular traffic. When cells were incubated at low temperature (16 °C), intracellular traffic was blocked or greatly slowed down. Figure 7 shows a comparison between two chase experiments, one performed at 39 °C, the other at 16 °C. The decrease in radioactivity associated with glycoprotein at 39 °C after 40 min can be correlated with the increase in radioactivity associated with OS-Gn1. When the chase was performed at 16 °C, the level of OS-

Figure 7 Effect of temperature on the degradation of glycoproteins and formation of OS-Gn1

B3F7 cells were incubated at 39 °C with 200 μCi of $[2\text{-}^3\text{H}]\text{mannose}$ for 10 min in the presence of 0.5 mM glucose and processing inhibitors (50 $\mu\text{g/ml}$ Cst and 100 $\mu\text{g/ml}$ dMM). The cells were then chased at either 16 °C (●) or 39 °C (▲) for various times from 0 to 240 min. The fate of labelled glycoproteins (a) or OS-Gn1 (b) was followed during the chase.

Gn1 remained constant and no degradation of glycoprotein was observed. This allows correlation of glycoprotein degradation with OS-Gn1 production. The inhibition of glycoprotein degradation at 16 °C is reminiscent of the temperature-dependence of membrane transport processes.

Properties of the degradation process

Non-lysosomal degradative mechanisms of newly synthesized proteins have recently been highlighted in different biological models: in 2B4 T-cell hybridoma, degradation is not affected by lysosomotropic agents (NH_4Cl , leupeptin) [10], whereas degradation is recovered in permeabilized cells [11], providing evidence for the ER as the site of degradation. Another degradative mechanism has been described in HT29 cells [12], in which ER oligomannoside-type glycoproteins are degraded in a lysosome-like compartment avoiding the secretory pathway via the Golgi apparatus. In the present case, neither leupeptin nor NH_4Cl inhibited glycoprotein degradation (not shown) and, as demon-

Table 3 Influence of lysosomotropic agents and cell permeabilization on the relative proportions of OS-Gn2 and OS-Gn1

B3F7 intact cells were incubated for 60 min with 200 μ Ci of [3 H]mannose. The relative percentages of OS-Gn2 and OS-Gn1 were measured as described in the Materials and methods section. Incubations were performed without (control) or with lysosomotropic agents: 100 μ g/ml leupeptin or 20 mM NH_4Cl . Permeabilized cells were incubated with 5 μ Ci of GDP-[14 C]Man and 50 μ M UDP-GlcNAc. The relative proportions of OS-Gn1 and OS-Gn2 were determined after 20 min incubation. Results are expressed as percentage of radioactivity associated with neutral free oligosaccharides.

	Amount (%)	
	OS-Gn2	OS-Gn1
Intact cells		
Control	38.5	61.5
+ Leupeptin	37.0	63.0
+ 20 mM NH_4Cl	52.3	47.7
Permeabilized cells	60.0	40.0

strated in Table 3, did not strongly affect the formation of OS-Gn1. When permeabilized B3F7 cells were incubated with GDP-[14 C]mannose and UDP-GlcNAc, release of OS-Gn1 was observed (Table 3), demonstrating that the degradative mechanism is not inhibited by the permeabilization process.

DISCUSSION

During the membrane-bound synthesis of a *N*-glycosylprotein, soluble material is produced containing free neutral and phosphorylated oligosaccharides. The origin of the phosphorylated oligosaccharides has been clearly demonstrated [3,8]. They represent a by-pass of the dolichol cycle which may control the availability and structural suitability of lipid intermediates for protein glycosylation. The present study focuses on cellular aspects of the origin of the free neutral oligosaccharides.

The relationship between protein glycosylation and release neutral oligosaccharides has already been demonstrated *in vitro* using thyroid rough ER [2]. These oligosaccharides were also found by metabolic labelling in B3F7 cells [3].

Synthesis of a glycoprotein requires the adjustment of two different metabolic pathways: the formation of a lipid donor and protein synthesis. Degradation of either one to neutral oligosaccharides could be a reflection of this adjustment. In order to test this hypothesis, we have studied conditions in which one of the metabolic pathways was abolished. Using cycloheximide to inhibit protein synthesis, we have shown that the combined mannose incorporation into glycoprotein and OS-Gn2 is constant, suggesting that they are both derived by the action of oligosaccharyltransferase. This enzyme uses the lipid donor pool at a constant rate, generating OS-Gn2 when the protein acceptor is deficient. Thus oligosaccharyltransferase could possess two enzymic activities: the well-known '*en bloc*' transferase activity and a hydrolase activity that liberates OS-Gn2. The competition between these two functions is revealed when the protein level is lowered. A decreased liberation of OS-Gn1 is also observed during cycloheximide treatment as previously described for puromycin-treated thyroid slices by Spiro and Spiro [13]. This favours a glycoprotein origin for the OS-Gn1 family, but can be questioned as cycloheximide and puromycin are non-specific protein-synthesis inhibitors and could affect the

synthesis of a hypothetical chitobiase which could cleave one GlcNAc from OS-Gn2, generating OS-Gn1.

In fact, if OS-Gn1 originates from glycoprotein, a degradative mechanism for newly synthesized glycoprotein must be demonstrated. Such a degradative mechanism has been examined *in vitro* in thyroid microsomal vesicles [2]. In B3F7 cells, a decrease in the radioactivity associated with glycoprotein was observed after 40 min of chase following a 10 min pulse. Some of the radioactivity was recovered in the medium as secreted glycoprotein but an overall loss of glycoprotein radioactivity was observed. This decrease was not affected when the chase experiment was performed in the presence of Cst and dMM, demonstrating that liberation of the glycan moiety rather than processing is responsible for this loss of radioactivity. With regard to the neutral oligosaccharide material, the time progress curve during the chase (Figure 4b) may suggest a substrate/product relationship between OS-Gn2 and OS-Gn1. Labelled lipid donors were no longer formed between 120 and 240 min of the chase. During the same period, the formation of OS-Gn2 was completely abolished, confirming that lipid donors are precursors of OS-Gn2. During this chase period, the loss of the radioactivity associated with OS-Gn2 was 200 000 d.p.m./h, whereas, during the same period, the radioactivity associated with OS-Gn1 increased by 400 000 d.p.m./h. Thus, even if some OS-Gn2 generates OS-Gn1, at least half of the OS-Gn1 originates from glycoprotein, as glycoprotein-associated radioactivity is decreasing during this period. Furthermore, it should be noted that some of the oligosaccharide material was recovered in the incubation medium after the chase. This radioactivity could explain the decrease in intracellular OS-Gn2.

Secretion of oligosaccharides by cultured cells has been described by Lubas and Spiro [14]. This secretory process is rapid as it depends on the bulk flow rate [15]. The nature of such extracellular oligosaccharide material released during the chase period is under investigation.

In order to demonstrate unambiguously that OS-Gn1 originates from degradation of newly synthesized glycoprotein, we analysed oligosaccharide populations from neutral free oligosaccharides and from glycoproteins under various incubation conditions. To avoid any processing during the chase period, experiments were performed in the presence of processing inhibitors (Cst and dMM). In previous work [3], we have demonstrated that, whatever the glucose concentration, the neutral free oligosaccharides and glycan moieties of glycoprotein are similar and mostly glucosylated. During these chase experiments, whatever the incubation conditions (presence or absence of glucose), whereas OS-Gn2 remains identical with the lipid donor, OS-Gn1 has a similar structural pattern to glycan moieties of glycoprotein. This reinforces the hypothesis that glycoprotein degradation leads to release of OS-Gn1 and that the number of glucose residues linked to the glycoprotein moieties does not affect this degradative mechanism.

Glucosyl residues attached to the glycan of glycoproteins have been implicated in the retention of these molecules in the rough ER. This glycosylation is achieved via a glucosyltransferase/glucosidase shuttle [13] or via a specific UDP-glucose glucosyltransferase [16]. It has been speculated that, during protein folding or subunit assembly, glucosylation is a means of preventing incompletely folded or unassembled protein complexes from being channelled into the secretory pathway. The presence of glucosyl residues on OS-Gn1 thus indicates that their release occurs in the subcellular compartment where glycoproteins are retained.

Furthermore, the patterns of OS-Gn2 and OS-Gn1 observed after the 10 min pulse remain glucosylated during the chase

period. This indicates that the neutral oligosaccharides are released in a subcellular compartment that contains Cst-sensitive glucosidases, i.e. ER or a compartment closely related to ER.

Pre-Golgi or rough ER degradation of newly synthesized proteins has already been observed [5,6,10,11]. In these studies, two observations were reported that suggest the possibility that some transport takes place between synthesis and degradation of these newly synthesized proteins. First, degradation proceeds after a variable lag period (5–20 min) after synthesis; second, the degradation is inhibited at temperatures below 20 °C (temperatures at which membrane transport processes are slowed down). In the present case, OS-Gn1 formation exhibited a lag period of 20 min (Figure 2), and we demonstrated that its appearance was strictly temperature-dependent (Figure 7b), as well as glycoprotein degradation (Figure 7a). This allows correlation of the release of OS-Gn1 with a glycoprotein-catabolic pathway, suggesting that some transport is involved between the synthesis of glycoproteins and their degradation. Degradation of newly synthesized proteins via a lysosomal proteolytic pathway has also been described [12,17]. In these models, the use of leupeptin or acidotropic agents (NH₄Cl) prevented protein degradation. In the present case, these agents did not prevent the formation of OS-Gn1. The fact that the degradation occurs in permeabilized cells, in which a total absence of transport between the rough ER and the Golgi has been reported [11], strongly supports the idea that, in our model, ER or a closely related compartment is the site of glycoprotein degradation and OS-Gn1 formation.

Recent observations indicate that OS-Gn1 is also generated in the wild-type CHO cell line. Thus the release of oligomannoside-type oligosaccharides can be considered as a marker of degradation of newly synthesized glycoproteins. In mammalian cells, two pathways are known to generate OS-Gn1 from glycoproteins: a two-step hydrolysis by an aspartamidase followed by a chitobiase as described in the lysosome [18] and a one-step hydrolysis by endo- β ,*N*-acetylglucosaminidase [19] without pro-

tein degradation. The precise subcellular localization and nature of the enzyme(s) involved in the degradative pathway described here for newly synthesized glycoproteins remain to be determined.

This work was supported by the Centre National de la Recherche Scientifique (U.M.R. no. 111, director Professor A. Verbert), by the Ministère de l'Éducation Nationale, by the Université des Sciences et Technologies de Lille Flandres-Artois and by an EEC grant no. BIOT-CT 92-0304 (Bridge contract on 'Control of recombinant protein glycosylation under defined cultivation conditions'). We are indebted to Dr. Sharon S. Krag from the School of Hygiene and Public Health (The Johns Hopkins University, Baltimore, MD, U.S.A.) for her precious gift of B3F7 cells and to Steven G. Ball for his careful reading of the manuscript.

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