Monoglucosylated Oligomannosides Are Released during the Degradation Process of Newly Synthesized Glycoproteins*

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The Chinese hamster ovary mutant MI8–5 is known to synthesize Man9GlcNAc2-P-P-dolichol rather than the fully glucosylated lipid intermediate Glc3Man9GlcNAc2-P-P-dolichol. This nonglucosylated oligosaccharide lipid precursor is used as donor for N-glycosylation. In this paper we demonstrate that a significant part of the glycans bound to the newly synthesized glycoproteins in MI8–5 cells are monoglucosylated. The presence of monoglucosylated glycans on glycoproteins determines their binding to calnexin as part of the quality control machinery. Furthermore, we point out the presence of Glc3Man9GlcNAc2 in the cytosol of MI8–5 cells. This indicates that part of the monoglucosylated glycoproteins can be directed toward a deglucosylation process that occurs in the cytosol. Besides studies on glycoprotein degradation based on the disappearance of protein moieties, MI8–5 cells can be used as a tool to elucidate the various steps leading to glycoprotein degradation by studying the fate of the glycan moieties.

A key reaction of N-glycosylation is the transfer en bloc of a Glc3Man9GlcNAc2 oligosaccharide from a lipid intermediate to an Asn residue in the Asn-Xaa-Ser(Thr) consensus sequence of a nascent protein. This N-glycosylation process is immediately followed by sequential deglucosylation leading to Man9GlcNAc protein. It is now clearly established that oligosaccharide trimming in the ER is intimately linked to the quality control process, leading to the degradation of misfolded glycoproteins or unassembled multimeric proteins. Indeed the presence of monoglucosylated oligomannosides on glycoproteins retained in the ER is essential for their binding to the molecular chaperones calnexin and calreticulin (1, 2). The presence of a glucosyl residue is controlled by reglucosylation-deglucosylation cycles involving UDP-glucose: glycoprotein glucosyltransferase acting as a folding sensor (3) and glucosidase II, respectively. It has been demonstrated that glucose trimming and reglucosylation cycles determine the association of a glycoprotein with calnexin, as well as its folding (4, 5).

During this quality control, part of newly synthesized glycoproteins are degraded after translocation of the glycosylated polypeptide chains from the ER to the cytosol. As first demonstrated by Wiertz et al. (6), using labeled protein with an amino acid precursor, this retrotranslocation is accompanied by the removal of the glycans by a cytosolic peptide N-glycanase (PNGase), such as the one isolated by Suzuki et al. (7). The relationship between the location of the enzyme and the degradation process has been discussed by Karaivanova and Spiro (8). However, it is worth mentioning that few labs follow the fate of newly synthesized proteins by labeling with [2-3H]mannose. In this case, the N-glycosylation process is accompanied by the release of glycans from either lipid intermediates or from newly synthesized glycoproteins (9–11). The trafficking of the released oligosaccharides from both origins leads to the formation of oligosaccharides possessing one GlcNAc residue at the reducing end (OSGn1) in the cytosol (for reviews see Refs. 12–14).

A Chinese hamster ovary mutant, MI8–5, was found to synthesize Man9GlcNAc2-P-P-Dol rather than fully glucosylated lipid intermediate (15). This mutant cell line was found to be deficient in dolichol-P-Glc:Man9GlcNAc2-P-P-dolichol glucosyltransferase activity. This defect is similar to the one observed for CDGIC (congenital disorders of glycosylation) (16).

In this paper, we demonstrate that although in MI8–5 the oligosaccharide lipids are nonglucosylated, part of the glycans bound to glycoproteins are monoglucosylated. Furthermore, we observe the formation of Glc3Man9GlcNAc2 species that could only originate from a deglucosylation mechanism occurring during glycoprotein degradation. Our work demonstrates that the quality control system for newly synthesized glycoproteins is intact in MI8–5 cells. This cell line gives us a biological model to determine the nature of oligosaccharides released from protein independently of what is happening at the reducing end.

MATERIALS AND METHODS

Reagents—Mutant cell line MI8–5 was isolated as described previously (15). [2-3H]Manose (429 Gbq/mmol) and [U-14C]glucose (10 Gbq/mmol) were from Amersham Pharmacia Biotech. PNGase F and castanospermine were purchased from Glyko, (Novato, CA). α-Mannosidase from jack bean, protease inhibitors, and trypsin were obtained from Sigma. Polyclonal antibody anti-calnexin was from StressGen. Protein A-Sepharose CL-4B and concanavalin A-Sepharose were from Amersham Pharmacia Biotech.

Metabolic Labeling of Oligosaccharides and Chase Experiments—MI8–5 cells were routinely cultured in monolayers in α-minimal essential medium with 10% (v/v) fetal calf serum at 34 °C in 10-cm Petri dishes under air/CO2 (19:1). Cells were labeled with 50 μCi/ml of [2-3H]mannose in α-minimal essential medium containing 0.5 mM glucose and 10% dialyzed fetal bovine serum. After incubation, the medium...
was removed, and the cell layer was rinsed rapidly twice with ice-cold PBS. When a chase was performed, pulse-labeled cells were washed twice with PBS and incubated for different times in α-minimal essential medium containing 5 mM glucose. When used, castanospermine (Cat) was maintained throughout the incubation and the chase periods at a final concentration of 50 μg/ml. Sequential extraction and purification of free oligosaccharide material were then achieved as described previously (9).

**Analysis of Oligosaccharide Material**—The protein pellet was digested overnight at room temperature with 0.2 mg of trypsin in 0.1 M ammonium bicarbonate, pH 7.9. The glycan moiety linked to protein was cleaved by PNGase F as follows: the trypsin-treated protein was boiled for 10 min to destroy the trypsin activity, and the peptides were dried and dissolved in 20 mM sodium phosphate buffer, pH 7.5, containing 50 mM EDTA, 50% (v/v) glycerol, and 0.02% NaN3. PNGase F was then added (0.5 mM/μl) for incubation overnight at 37 °C.

Size analysis of the glycan moieties was achieved by HPLC on an amino-derivatized Asahipak NH₂-P-50 (250 mm × 4.6 mm) column (Asahi, Kawasaki-ku, Japan) 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. Under these conditions the oligomannosides can be resolved by water from 70:30 (v/v) to 50:50 (v/v) at a flow rate of 1 ml/min over 80 min. Under these conditions the oligomannosides can be resolved by their numbers of mannose, glucose, and GlcNAc residues from Man to GlcNAc₉. Oligomannosides were identified by their retention times as described previously (9); separation of the labeled oligosaccharides was monitored by continuous-flow detection of the radioactivity with a Flo-one β detector (Packard).

**Jack Bean α-Mannosidase Treatment**—For enzymatic treatment with jack bean α-mannosidase, oligosaccharides released after PNGase digestion were dissolved in 20 μl of 50 mM sodium acetate buffer, pH 5. The incubation was achieved overnight at 37 °C with 0.5 units of jack bean α-mannosidase. The incubation mixture was then analyzed by HPLC.

**[U-¹⁴C]Glucose Labeling for Glycosyl Residue Detection**—MI8–5 cells were labeled with 25 μCi/ml of [U-¹⁴C]glucose per dish in α-minimal essential medium without glucose containing 5 mM mannose, 50 μg/ml Cst, and 10% dialyzed fetal bovine serum. Pulse-labeled cells were washed twice with PBS and chased during 2 h in α-minimal essential medium containing 5 mM glucose and 50 μg/ml Cst. Sequential extraction was then achieved as described previously (9).

The protein pellet was digested overnight at room temperature with trypsin in 0.1 M ammonium bicarbonate, pH 7.9, and glycan were then removed using PNGase F treatment. Both oligosaccharides bound to glycoproteins, and free oligosaccharides were purified via concanavalin A chromatography. The lectin column (concanavalin A-Sepharose; 5 × 0.5 cm) was equilibrated in 5 mM sodium acetate buffer, pH 5.2, containing 0.1 mM NaCl, 1 mM MnCl₂, and 1 mM MgCl₂, at room temperature. The fraction resulting from PNGase F action on glycoprotein fraction and free oligosaccharides were applied to the column. The strongly retained [¹⁴C]-labeled oligomannosides were eluted with 100 mM α-mannoside. Peak A (originating from glycoproteins) and peak B (originating from free oligomannoside material) were then isolated by preparative HPLC. The isolated peaks A and B were then submitted to PNGase treatment. Although the oligosaccharide bound to lipid intermediates in K1–2 and MI8–5 cells, the oligosaccharide species bound to proteins seems to be similar. In particular, a species having a higher retention time than Man₉GlcNAc₂ can be detected bound to proteins of parental (K1–2) and MI8–5 cell lines.

**Pattern of Glycans Bound to Glycoproteins Synthesized by MI8–5 Cell Line**—MI8–5 cells transfer onto proteins only nonglycosylated oligosaccharides (15). To compare the fate of glycans bound to glycoproteins synthesized in parental (K1–2) cells and MI8–5 these cells were pulse-labeled during 60 min with [2-³H]mannose in the conditions described under “Materials and Methods.” Fig. 1, a and b represent the pattern of glycans released from glycoproteins of K1–2 and MI8–5 after PNGase treatment. Although the oligosaccharide bound to lipid intermediates in K1–2 and MI8–5 cells was different (Glc₃Man₉GlcNAc₂–PP-Dol for parental cells and Man₉GlcNAc₂–PP-Dol for mutant cells), the oligosaccharide species bound to proteins seems to be similar. In particular, a species having a higher retention time than Man₉GlcNAc₂ can be detected in MI8–5 (peak A). This raised the question of the nature and of the molecular composition of this oligosaccharide species.

Peak A has been isolated, and its sensitivity toward jack bean α-mannosidase has been compared with the Man₉GlcNAc₂ species used as control. The action of jack bean α-mannosidase on isolated Man₉GlcNAc₂ species (Fig. 2a) leads to the formation of 88% of free mannose and 12% of Man₇GlcNAc₂ species (see Fig. 2b and Table I). These results indicate that the totality of α-linked mannose residues has been removed. In contrast, the same incubation of isolated peak A (Fig. 2c) with jack bean α-mannosidase leads to the formation of 30% of free mannose and 70% of higher species migrating as hexose₉₋₈GlcNAc₂, peaks 1,
2, and 3, respectively (see Fig. 2 and Table I). The absence of a resulting Man1GlcNAc2 species suggests that only partial hydrolysis of peak A was performed by the enzyme. It has to be noted that when incubation was achieved with higher enzyme concentration, the proportion of peak 1 increases, but peaks 2 and 3 remained detectable. This suggests that peak 1 is the limit product of the hydrolysis of peak A by jack bean \( \alpha \)-mannosidase. Moreover, peak 1 comigrated with the Glc1Man5GlcNAc2 species that corresponds to the major oligomannoside species synthesized by mannosylphosphoryldolichol-deficient cell line B3F7 (not shown). Partial digestion of Glc9Man9GlcNAc2 species isolated from the glucosidase I-deficient cell line Lec23 has also been obtained leading to a mixture of three species (17). As in our case, complete digestion to a single product was not observed even after longer incubations. Furthermore, it has been clearly demonstrated on bovine rhodopsin that jack bean \( \alpha \)-mannosidase is not able to cleave an unsubstituted \( \alpha,1,6 \)-linked mannose from the core \( \beta \)-mannose (18). Our results are consistent with these observations, because the smallest species is Glc1Man5GlcNAc2. According to our results, we can postulate that peaks 1, 2, and 3 correspond to Glc1Man5GlcNAc2, Glc1Man6GlcNAc2, and Glc1Man7GlcNAc2, respectively. 

**TABLE I**

Proportion of various species obtained after jack bean \( \alpha \)-mannosidase treatment of \([2-3H]\)mannose-labeled oligomannoside species isolated from MI8–5 glycoproteins

The values are expressed as percent of the input radioactivity.

| Hydrolysis products | Treated substrates |
|---------------------|-------------------|
|                     |          | Isolated peak A | Oligosaccharides released from glycoproteins bound to calnexin |
| Free mannose        | 88  | 30  | 30 |
| Man6GlcNAc2         | 12  | 0   | 0  |
| Species \( \geq \) Glc6Man6GlcNAc2 (peaks 1 + 2 + 3) | 0   | 70  | 70 |

2, and 3, respectively (see Fig. 2d and Table I). The absence of a resulting Man1GlcNAc2 species suggests that only partial hydrolysis of peak A was performed by the enzyme. It has to be noted
Effect of Castanospermine on the Pattern of Glycans Bound onto Glycoproteins in MI8–5 Cells—MI8–5 cells were metabolically labeled with tritiated mannose in the presence of 50 μg/ml Cst (an inhibitor of rough ER glucosidases). Comparing panels a and b of Fig. 3, there was clearly an increase of the radioactivity bound to peak A in the presence of Cst. As Cst has been demonstrated to prevent deglucosylation of newly synthesized glycoproteins, this observation strongly suggests that peak A represents a glucosylated species.

Occurrence of a Glucose Residue on Glycoproteins Synthesized by MI8–5 Cells—MI8–5 cells were pulse-labeled with [U-14C]glucose during 1 h in the presence of 50 μg/ml of Cst as described under "Materials and Methods." After a PNGase treatment of the protein pellet, oligomannoside-type glycans were purified by concanavalin A chromatography. [14C]-Labeled peak A (see Fig. 1b) was then isolated by HPLC and submitted to acid hydrolysis to release monosaccharides. Fig. 4b shows chromatography of the [14C]-labeled hydrolysate of peak A with [2-3H]mannose as an internal standard. The HPLC profile showed the presence of both [14C]mannose and glucose residues. All the data from HPLC, α-mannosidase treatment, and composition analysis demonstrate that the species migrating as peak A is glucosylated and possesses the 

\[ \text{Glc}_1\text{Man}_7\text{GlcNAc}_2 \]

respectively.

Release of Monoglucosylated Glycans during Glycoprotein Degradation

Fig. 3. Effect of castanospermine on the pattern of glycans bound to glycoproteins and free oligomannosides synthesized by MI8–5 cells. MI8–5 cells were pulse labeled with [2-3H]mannose for 1 h in the absence (panels a and c) or in the presence (panels b and d) of 50 μg/ml Cst. After sequential extraction, the free oligosaccharide material (panels c and d) and the glycans released from glycoproteins with PNGase (panels a and b) were analyzed by HPLC as described under "Materials and Methods." M9, M8, M5, M4, and M3 indicate oligomannosides containing nine, eight, five, four, and three mannose residues, respectively. Open peaks represent oligomannosides possessing two N-acetyl glucosamine residues at the reducing end, and hatched peaks represent oligomannosides possessing one N-acetyl glucosamine residue at the reducing end.
following molar composition: Glc₃Man₉GlcNAc₂.

Glucosylation of Newly Synthesized Glycoproteins in MI8–5 Allows Binding to Calnexin—Because oligomannosides synthesized in MI8–5 are transferred onto nascent proteins as non-glucosylated species, it can be assumed that the presence of Glc₃Man₉GlcNAc₂ bound to glycoproteins originated from a subsequent glucosylation process of the Man₉GlcNAc₂ species.

At the present time it has been well demonstrated that the presence of the glucosyl residue is controlled by reglucosylation-deglucosylation cycles involving the UDP-glucose : glycoprotein glucosyltransferase and glucosidase II, respectively (3). Recently it has been demonstrated that the trimming and readdition of glucose to N-linked oligosaccharides determine the association of a glycoprotein with calnexin, as well as its folding (4, 5). We next determined whether the reglucosylation of oligomannosides onto glycoproteins seen in MI8–5 cells resulted in an association with calnexin. MI8–5 cells were pulse-labeled for 1 h with [2-3H]mannose, and the radioactivity was chased for 1 h. To preserve glucosylated oligomannosides, Cst was maintained in the media during the entire experiment. After immunoprecipitation of the cell lysate with anti-calnexin antibodies, immunoprecipitated glycoproteins were trypsin-treated and subjected to a PNGase treatment before being analyzed by HPLC. Fig. 5 reveals the presence of two peaks, the first one migrating as Glc₃Man₉GlcNAc₂ and the second one as Glc₃Man₈GlcNAc₂. The hydrolysis of each peak by jack bean a-mannosidase leads to the same pattern as the one obtained after hydrolysis of Glc₃Man₉GlcNAc₂ (peak A) isolated from glycoproteins (Table I). These results indicate that the reglucosylated oligomannoside glycans of MI8–5 could associate with calnexin. Therefore, the quality control system for newly synthesized glycoproteins appeared intact in MI8–5 cells.

Monoglucosylated Free Oligomannosides Are Produced by MI-5 Cells—It has been shown that the N-glycosylation process is accompanied by the release of free oligosaccharide material originating from both oligosaccharide-lipid hydrolysis and degradation of glycoproteins (12–14). It has also been demonstrated in mutant cell lines lacking mannosylphosphoryldolichol that the free oligosaccharides released from oligosaccharide-lipid or glycoprotein are degraded into an oligomannoside isomer, Man₅GlcNAc₁ (9). Because MI8–5 cells do not glucosylate the glycans bound to oligosaccharide-PP-Dol but do glucosylate the glycans on proteins, this offers a biological model to discriminate oligosaccharides originating from glycoproteins.
The more interesting aspect of this work is the demonstration that the Glc$_3$Man$_5$GlcNAc$_2$ species is a component of the soluble free oligomannoside material. These glycosylated oligosaccharides originated from glycoproteins, because in MI8–5 cells glucosyl residues are recovered only on glycoproteins. Endoplasmic reticulum to cytosol transport of oligomannosides possessing two GlcNAc residues at the reducing end (OSGn$_2$) is known to be specific for nonglucosylated species (19, 20). Moreover, it is now well established that OSGn$_2$ are converted to OSGn$_1$ species by a cytosolic chitobiase (21). Thus the finding of glycosylated OSGn$_1$ in MI8–5 cells indicates that the deglycosylation process of glycoproteins occurred in the cytosol prior to their degradation.

When looking at the reducing end of the released oligomannosides, a discrepancy appears between the previous demonstration of the PNGase action (6) releasing OSGn$_2$ and the monoglucosylated OSGn$_1$ observed in this study. Two alternatives can be proposed, either there was successive action of PNGase and chitobiase, or there was cleavage by a cytosolic β-endoglucosaminidase (22, 23).

The study of oligomannosides produced by MI8–5 cells suggests the following pathway for the formation of Glc$_3$Man$_5$GlcNAc$_2$; after retrotranslocation of polypeptides bearing monoglucosylated glycan (Glc$_3$Man$_5$GlcNAc$_2$ and Glc$_3$Man$_5$GlcNAc$_2$), deglycosylation occurs in cytosol liberating Glc$_3$Man$_5$GlcNAc$_2$ and Glc$_3$Man$_5$GlcNAc$_2$. These OSGn$_1$ are substrates for the cytosolic a-mannosidase (24) generating the observed Glc$_3$Man$_5$GlcNAc$_2$. The fate of this oligosaccharide is still in question, because up to now, no α-glucosidase activity has been detected in the cytosol. Although its transport into lysosomes has not yet been studied (25), our results suggest a lysosomal degradation into Man$_5$GlcNAc$_2$ and lower species. The question remains whether glycoproteins bearing monoglucosylated glycans and being directed toward degradation must be linked to calnexin, as demonstrated by Liu et al. (26) for the degradation of misfolded α1-antitrypsin.

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