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BREAKTHROUGHS AND VIEWS

Free and N-Linked Oligomannosides as Markers of the Quality Control of Newly Synthesized Glycoproteins

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It appears increasingly evident that the oligomannoside type N-glycans play important roles in the fate of newly synthesized glycoproteins in the rough endoplasmic reticulum. The variety of protein-bound oligomannoside isomers are involved in the quality control of glycoprotein, in their transport into the Golgi and probably as a degradation signal. A prerequisite of the degradation in the cytosol by the proteasome pathway is the release of the glycans as free oligomannosides. These oligomannosides are further processed in the cytosol into a peculiar isomer of $Man_5GlcNAc_1$ which enters into the lysosome to be further degraded into monosaccharides. In this review, we will illustrate how the different species of N-linked and free oligomannosides either are involved or are markers of the quality control and fate of newly synthesized glycoproteins.

Key Words: **N-glycosylation; endoplasmic reticulum; cytosol; oligomannoside trafficking; quality control; protein degradation.**

There are more and more evidences that oligosaccharide moieties of glycoconjugates play a role as recognition signal in many biological events; (for review see 1). Most of these recognition phenomena which in fact occur at the cell surface or in extracellular fluids involve complexe type N-glycans. It appears that the terminal sugars such as such sialic acids, galactose and fucose are mainly implicated. In contrast, it is striking to note that, intracellularly, sugar recognition phenomena concern mostly oligomannoside type glycans. This is for example the case for the interaction of calnexin or calreticulin with newly synthesized glycoproteins, for mannose binding protein ERGIC 53 (MR 60) and for nuclear lectins. The mannose-6 phosphate receptors could also be included in this list. Such diverse roles require structural diversity of N-linked oligomannosides. Restricting ourselves to events occurring in the rough endoplasmic reticulum, we will examine how the different N-linked oligomannoside species are synthesized, how they are involved in the quality control and the degradation mechanisms of newly synthesized glycoproteins.

I. SYNTHESIS AND PROCESSING OF N-LINKED OLIGOMANNOSIDE IN ROUGH ER

N-glycosylation of proteins in eukaryotic cells involves two separate metabolic pathways which are localized in the rough endoplasmic reticulum (ER) i.e., the dolichol cycle for the lipid donors and the protein synthesis for the acceptor.

The key reaction is the transfer en bloc of a tetradecasaccharide from a lipid intermediate (oligosaccharide pyrophospho dolichol:oligosaccharide-PP-Dol) to an Asn residue in the Asn-X-Ser(Thr) consensus sequence of a nascent protein. The tetradecasaccharide is preassembled on a specific polyisoprenol (the dolichol) via a pyrophosphate bond to form the $Glc_3Man_9GlcNAc_2$-PP-Dol. This compound is assembled by sequential addition of monosaccharides first at the cytosolic face of the ER up to $Man_5GlcNAc_2$-PP-Dol, then, after translocation to the lumenal face of the ER by a still unknown mechanism, this precursor is elongated up to the final $Glc_3Man_9GlcNAc_2$-PP-Dol. This compound is the substrate for the oligosaccharyltransferase which transfers the oligosaccharide onto the acceptor asparagine residue. Nascent glycoproteins are processed in the ER by the action of specific glucosidases (glucosidases I and II) and of the ER mannosidase I or II, leading to specific $Man_8GlcNAc_2$-protein which is the key structure to leave the rough ER for the Golgi ap-

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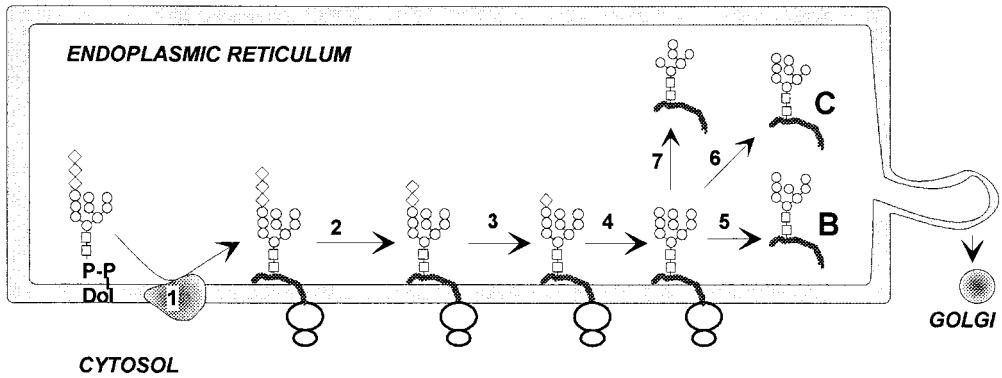

FIG. 1. Processing of N-linked oligomannosides in rough endoplasmic reticulum. The mature $Glc_3Man_9GlcNAc_2PPDol$ is a substrate of oligosaccharyl transferase (1). The $Glc_3Man_9GlcNAc_2$ bound to the nascent glycoprotein is submitted to the action of glucosidase I (2), glucosidase II (3, 4). The N-linked $Man_9GlcNAc_2$ is substrate of the ER mannosidase I (5) or ER mannosidase II (6) or Man_9 mannosidase (7). The Man_8 isomers are identified as having a mannose residue missing in middle (isomer B) and α 1,6linked chain (isomer C) of the oligomannoside respectively. Symbols: ◇, glucose; ○, mannose; □, N-acetylglucosamine.

paratus. This oligomannoside type glycoprotein can be further processed in the different Golgi stacks being trimmed by several mannosidases and elongated by different glycosyltransferases to give a variety of complex or hybrid type glycans.

Thus, ER located glycoproteins can possess a variety of oligomannoside structures (for review; see 2):

—$Glc_3Man_9GlcNAc_2$ species are present just after the transfer "en bloc" of the glucosylated oligomannoside from the lipid intermediate precursor to the protein acceptor site (Fig. 1, step 1).

—This species is rapidly (half time less than 5 minutes) deglucosylated by the glucosidase I to give $Glc_2Man_9GlcNAc_2$ (Fig. 1, step 2).

—The deglucosylation process is further achieved by the action of glucosidase II leading to $Glc_1Man_9GlcNAc_2$ and $Man_9GlcNAc_2$ (Fig. 1, steps 3 and 4).

—Two isomers of $Man_8GlcNAc_2$ species can be obtained by the action of ER mannosidases I and II (Fig. 1, step 5 for isomer B and step 6 for isomer C, respectively). In some instances, these Man_8 isomers can still possess a glucose residue.

—The $Man_9GlcNAc_2$ species can also be processed to a peculiar $Man_6GlcNAc_2$ isomer by a different mannosidase, the Man_9 mannosidase described by Bause *et al.* (3), (Fig. 1, step 7). The biological significance of these different $Glc_1Man_8GlcNAc_2$, $Man_8GlcNAc_2$, $Man_7GlcNAc_2$ or $Man_6GlcNAc_2$ isomers is not yet understood.

II. N-LINKED OLIGOMANNOSIDES IN RELATION WITH QUALITY CONTROL

Calnexin, a membrane bound ER protein, was suggested to have chaperon-like function in glycoprotein maturation. It recognises monoglucosylated glycan moieties of glycoproteins and retained them in the rough ER, allowing their folding by interaction with the polypeptide chain. However calnexin does not seem to be able to sense the folding state of the glycoproteins (4, 5). Thus, when released from calnexin binding, the monoglucosylated glycoproteins are substrate for glucosidase II. If correctly folded, the deglucosylated Man_9 (or Man_8) oligomannoside type glycoprotein will follow the route for Golgi. If uncorrectly folded, it will be recognized by a specific UDP-Glc:glycoprotein glucosyltransferase which interacts with the hydrophobic amino-acid sequence exposed in misfolded conformation (6). Thus, this glucosyltransferase acts as a "folding sensor" and re-offer the substrate for calnexin binding (7).

Thus, immatured glycoproteins shuttle between monoglucosylated and unglucosylated structures until they are either properly folded or rejected by the quality control towards a degradation process. A similar "glyco-deglyco"cycle was observed with a soluble ER protein, calreticulin, which is structurally and functionally related to calnexin (8) (see Fig. 2, step A).

III. N-GLYCOSYLATION STATUS AND FOLDING OF PROTEINS

The role of N-glycans in the folding process of glycoproteins is not yet understood and appears so far to be different for one glycoprotein to the other, as had already suggested by early experiments using tunicamycin a glycosylation inhibitor.

We have recently observed that truncated glycosylation could influence proper folding and secretion of soluble alkaline phosphatase (9). In brief, soluble alkaline phosphatase was used as a tool for screening the temperature dependent secretion in mutagenized CHO cell line. One clone (MadIA214) displaying such a phenotype revealed that the polypeptide possessed a truncated oligomannoside core ($Man_5GlcNAc_2$). A similar

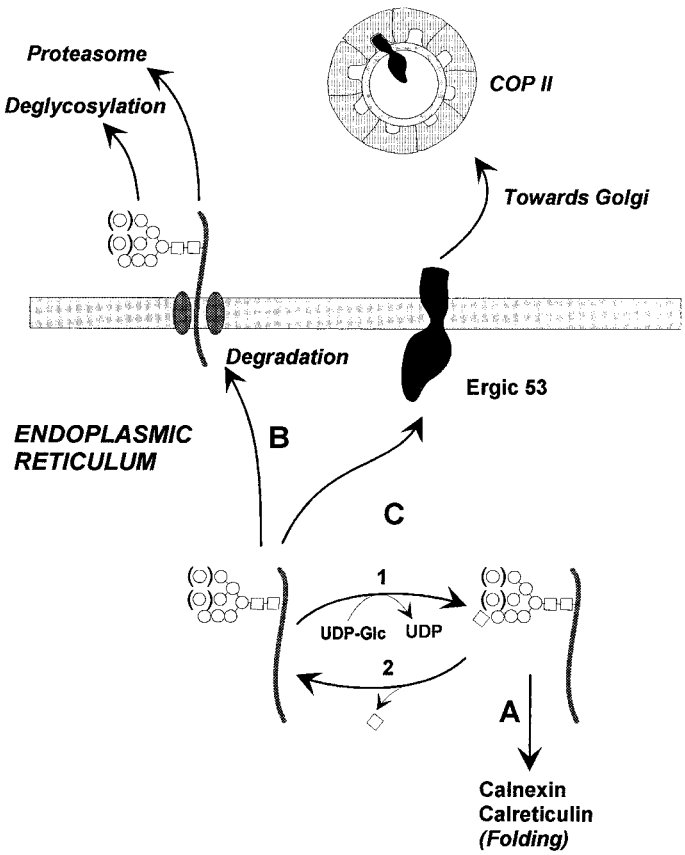

FIG. 2. N-linked oligomannosides and the fate of glycoproteins in rough endoplasmic reticulum. The $Man_9GlcNAc_2$ or $Man_8GlcNAc_2$ species bound to glycoproteins are signal for: (A) The folding mechanism by binding to calnexin or calreticulin via the glucosyltransferase (1)-glucosidase II (2) shuttle. (B) The degradation by the proteasome pathway. (C) The export via the binding to ERGIC 53. Symbols: ◇, glucose; ○, mannose; □, N-acetylglucosamine.

observation had been made with hemagglutinin of influenza virus (10, 11). This fact is renforced by the fact that Man-P-Dol deficient cells which synthesized only $Man_5GlcNAc_2$-N-linked glycans, exhibits an increased ER retention of glycoproteins indicating that the folding process is disturbed (12).

An other role of N-linked oligomannoside core has been recently pointed out with in *S. cerevisiae* by Jakob *et al.* (13), showing that $Man_8GlcNAc_2$ oligomannoside could be a signal for the degradation process of newly synthesized glycoproteins. They suggested the presence of a $Man_8GlcNAc_2$ binding lectin involved in targeting misfolded glycoproteins to degradation. The role of the ER mannosidase I in directing the selection of a misfolded glycoprotein for degradation by the proteasome has been recently demonstrated by Liu *et al.* (14) (see Fig. 2, step B).

Finally, N-linked oligomannoside plays a role in the exit of glycoprotein toward the Golgi vesicles. ERGIC-53, a transmembrane protein of both ER and ERGIC, possesses a lumenal lectin like domain that binds to high mannose glycans (15) and a short cytosolic tail interacting with the coat proteins COP I and COP II. Recent studies (16, 17) suggested that ERGIC-53 could serve as an adaptator between the glycoproteins to be transported to Golgi and the coat components responsible for ER to ERGIC and Golgi transport via coat vesicles (Fig. 2, step C).

IV. DEGRADATION OF NEWLY SYNTHESIZED GLYCOPROTEINS

Alternatively when newly synthesized glycoproteins are not correctly folded, the question is how ER-retained misfolded proteins are finally degraded. It is important to note that this is not a lysosomal mechanism but it occurs both inside the ER and in the cytosol by a proteasome mediated pathway.

It has been recently reported that degradation of class-I heavy chains by cytomegalovirus occurs by transport from ER to cytosol during which the glycans are removed by a cytosolic PNGase, prior to proteolysis by proteasomes and a deglycosylated form of the glycoprotein has been isolated (18). This process involves the Sec61 complex, which appears to be a reversal of the reaction by which it translocates nascent chains into the endoplasmic reticulum (19). Bonifacino (20) proposed a generalization of this model for both soluble and membrane bound glycoproteins. The cytosolic degradation of CFTR (21), α1-antitrypsin (22) and class I heavy chains of the major histocompatibility complex (23) has also been demonstrated, but here again no correlation has been made between the degradation of these glycoproteins and release of their glycans.

V. RELEASE OF OLIGOMANNOSIDE AS MARKERS OF GLYCOPROTEIN DEGRADATION

For the past fifteen years, few labs including ours (for review, see 24) have observed that the N-glycosylation process was accompanied by the release of oligomannoside type oligosaccharides. This material is constituted of oligosaccharide-phosphates and of neutral oligosaccharides possessing one GlcNAc (OS-Gn1) or two GlcNAc (OS-Gn2) at the reducing end.

We have demonstrated that oligosaccharide-phosphates originated from the cleavage by a specific pyrophosphatase, of non-glucosylated cytosolic faced oligosaccharide-PP-Dol and chiefly the $Man_5GlcNAc_2$-PP-Dol. The $Man_5GlcNAc_2$-P, as the main product, is recovered in the cytosolic compartment and is further degraded to $Man_5GlcNAc_1$.

In contrast, OS-Gn2 produced from hydrolysis of oligosaccharide-PP-Dol (presumably as a transfer reaction onto water) when the amount of protein acceptor is limiting, are generated into the lumen of rough endoplasmic reticulum (ER). They are further submitted

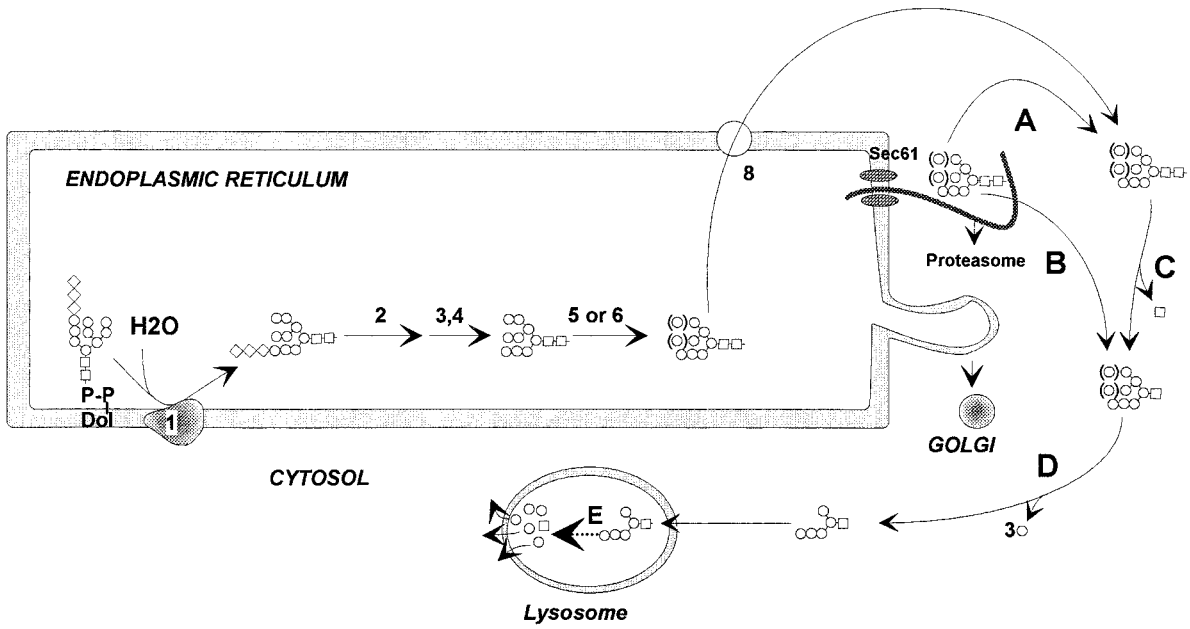

FIG. 3. Oligomannosides trafficking and glycoproteins deglycosylation. Lumenal oligomannosides released by the hydrolytic activity of the oligosaccharyl transferase (1) are submitted to ER glycoproteins processing enzymes:glucosidase I (2), glucosidase II (3, 4) and ER mannosidases I and II (5 or 6). The $Man_8GlcNAc_2$ species are then transported into the cytosol (8). The deglycosylation of newly synthesized glycoproteins which occurs before the degradation in the proteasome is achieved either by the sequential action of a cytosolic peptide N-glycanase (A) and a chitobiase (C) or by a β-endoglucosaminidase (B). The oligomannoside being then degraded by the cytosolic mannosidase (D). The $Man_5GlcNAc_1$ enters into the lysosome to be further degraded in mannose and N-acetyl glucosamine (E). Symbols: ◇, glucose; ○, mannose; □, N-acetylglucosamine.

to processing α-glucosidases and rough ER mannosidases and are (mainly as $Man_8GlcNAc_2$) exported into the cytosolic compartment (Fig. 3, step 8). This material is further degraded into a single component, the $Man_5GlcNAc_1$ (25, 26): Man $\alpha 1 \rightarrow 2$ Man $\alpha 1 \rightarrow 2$ Man $\alpha 1 \rightarrow 3$(Man$\alpha 1 \rightarrow 6$) Man$\beta 1 \rightarrow 4$ GlcNAc by the sequential action of a cytosolic neutral chitobiase (27) followed by cytosolic mannosidase (28) (Fig. 3, steps C and D).

It has been demonstrated that OSGn1 could originate from OSGn2 due to the action of the cytosolic chitobiase as described above. However, this process cannot be considered as exclusive. First, in thyroid microsomes OS-Gn1 formation was not affected by EDTA which inhibited the formation of OS-Gn2 (29). Second, quantitation of the pulse-chase experiment indicated that the amount of OS-Gn1 is far higher that the amount of OS-Gn2 formed at the beginning of the chase (30). This indicates that OS-Gn1 does not originate exclusively from OS-Gn2 and another origin has to be envisaged. Our recent experiments with protein synthesis inhibitor, anisomycin (12) demonstrated that OS-Gn1 are released in the cytosol and originated from the cleavage of newly synthesized glycoproteins. This raises the question of which enzyme could be responsible for such a cleavage of the glycan moieties of glycoproteins.

As early as 1979, Pierce *et al.* (31, 32) discovered in the cytosol of rat liver an enzyme able to cleave the chitobiose unit of glycans from oligomannosidic and non-sialylated complex type glycopeptides. The properties of this enzyme have been described to be entirely different from those of the lysosomal chitobiase activity on free glycans (33,34). This enzyme activity would explain the presence of cytosolic OS-Gn1 but implies that the protein has to be retrotranslocated still bearing the glycan moieties. The question is to know whether the steric hindrance of the whole glycosylated protein is compatible with the size of the "translocon channel". In 1983, Anumula and Spiro (29) demonstrated the presence of an endo-β-D-N-acetylglucosaminidase activity in the thyroid microsomal fraction. This enzyme could, in one step, generate OS-Gn1 (Fig. 3, step B) from ER located glycoproteins. To our knowledge, this enzyme has not yet been isolated, neither from thyroid nor from another animal tissue.

Another possibility would be the sequential activity of a PNGase such as the one described by Suzuki *et al.* (35) followed by the action of a cytosolic chitobiase (see Fig. 3, steps A and C). We have reported the occurrence of such an activity in MDBK cells (27) and it has also recently been isolated from hen oviduct by Kato *et al.* (36). Again the subcellular location of the PNGase is of importance. It could be located in the ER lumen as the

one described by Weng and Spiro in rat liver (37), to generate OS-Gn2 a species further transported in the cytosol, but this remains to be documented. Alternatively, as proposed by Suzuki (38) its location is cytosolic. Recent studies of Suzuki *et al.* (39) reporting a soluble peptide N-glycanase in yeast reinforced this hypothesis.

CONCLUSION

Thus, it appears that the different species of N-linked oligomannosides are involved at many steps of the processing and quality control of glycoproteins in the rough ER.

N-linked oligomannosides appears as substrates to specific enzymes and ligands for lectins involved in the retention of glycoproteins in the ER up to their proper folding or degradation. In this latter case, the first step (may be its prerequisite) would be an "en bloc deglycosylation" releasing oligomannosides. Their relative abundance would be a marker of the importance of the degradation process of newly synthesized glycoproteins in different cell types or different physiological states of a given cell.

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