PLANT CELLS SYNTHESIZE GLUCOSE-CONTAINING LIPID-LINKED OLIGOSACCHARIDES SIMILAR TO THOSE FOUND IN ANIMALS AND YEAST

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1. Introduction

From a series of studies it has become apparent that a glucose-containing lipid-bound oligosaccharide with the composition $Glc_3Man_9GlcNAc_2$ [1] seems to be the immediate donor in the glycosylation of asparagine residues of membrane-bound as well as secretory proteins. This Glc-oligosaccharide is transferred en bloc from the lipid to nascent polypeptides and subsequently processed giving rise to both polymannose and complex oligosaccharide chains. There is evidence that glucose could have a signal function in the oligosaccharide transfer [2,3]. Studies delineating the 'dolichol pathway' thus far, both in vivo and in vitro, have used mainly animal [4–8] and yeast cells [9–10].

Here I show that plant tissues also are able to catalyze the formation of lipid-bound Glc-oligosaccharide with the composition $Glc_3Man_9GlcNAc_2$. The data provide initial evidence that *N*-glycosylation of glycoproteins in all eucaryotic cells is very similar, if not identical.

2. Materials and methods

Preparation of membrane fraction:

1. *Nicotiana tabacum* membranes were isolated from cells grown in liquid suspension culture in LS-medium [11] supplemented with 2,4-D (2 mg/l) and kinetin (0.03 mg/l). Cells from the logarithmic phase were digested with 1% cellulase Onozuka-SS, 1% cellulysin and 0.2% macerase in LS-medium containing 0.4 M sorbitol (5 ml/g cells). After 2 h cells were centrifuged, washed with the same medium without enzymes and lysed with a potter homogenizer in 20 mM Tris–HCl (pH 7.4) containing 3 mM $MgCl_2$, 160 mM NaCl and 2 mM mercaptoethanol (lysis buffer). The homogenate was centrifuged for 45 min at 50 000 × *g*. The pellet was resuspended in lysis buffer and used as enzyme source.
2. Crude spinach membranes were prepared as follows: Leaves were separated from the middle rib and homogenated for 2 s with a Warring blendor in a medium, containing 50 mM MES buffer (pH 6.5), 0.33 M sorbitol, 2 mM Na-EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 20 mM NaCl. The homogenate was filtered through cheese cloth and centrifuged at 2500 × *g* for 70 s. The resulting supernatant was centrifuged at 50 000 × *g* for another 30 min and the pellet was suspended in lysis buffer to give ~25 mg protein/ml.

Transfer of mannose from GDP-Man and glucose from UDP-Glc into glycosyl lipids, oligosaccharide–lipid and polymer was assayed as for yeast [9]. Mild acid hydrolysis, endo-*N*-acetylglucosaminidase digestion and gel-filtration of oligosaccharides were done as in [9].

Thin-layer chromatography was performed on Merck Silica Gel G using the following solvents (A) chloroform/methanol/water (65/25/4, by vol.) or (B) *n*-propanol/acetic acid/water (3/3/2, by vol.).

3. Results

A membrane fraction isolated from tobacco suspension cells catalyzes the incorporation of mannose or glucose from GDP-[^{14}C]Man and UDP-[^{14}C]Glc,

Abbreviations: Dol-P, dolichyl monophosphate; Dol-PP, dolichyl diphosphate; Man, mannose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; GDP-Man, guanosine diphosphate mannose; UDP-Glc, uridine diphosphate glucose

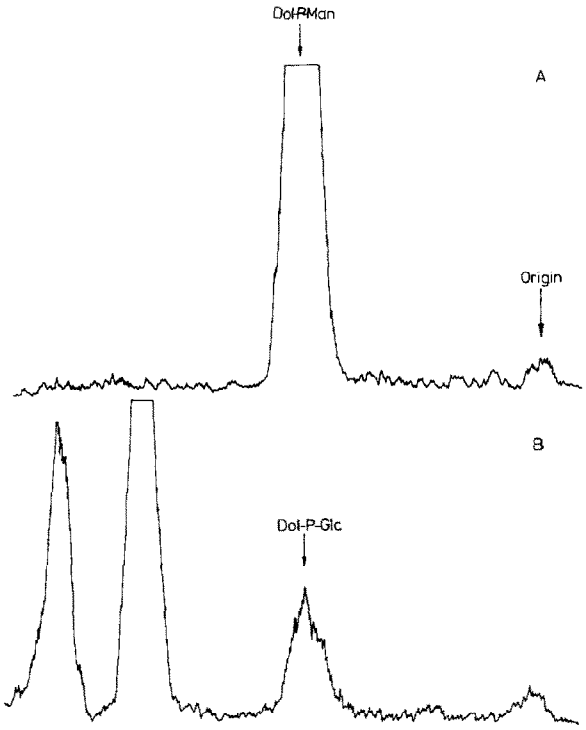

Fig.1. Thin layer chromatography of chloroform/methanol (3/2, v/v) soluble glycolipids obtained by incubation with GDP-Man (A) or UDP-Glc (B). The arrows indicate the positions of authentic Dol-P-Man (A) or Dol-P-Glc (B).

respectively, into various lipid-linked saccharides and polymer fraction, probably a mixture of glycoprotein and polysaccharide. The main constituent (~90%) of the chloroform/methanol extract upon incubation with GDP-Man has chromatographic properties of Dol-P-Man (fig.1). With UDP-Glc as glycosyl donor, however, 3 compounds are formed. Most of the radioactivity partitions on thin-layer chromatography as a sterylglucoside and its acylated derivative, as for *Phaseolus aureus* [12]. Only a minor amount of radioactivity has a R_F value characteristic of Dol-P-Glc. The observation that addition of exogenous Dol-P stimulates glycosyl transfer to the compounds migrating like Dol-P-Man and Dol-P-Glc, respectively, provides an additional evidence for the dolichol nature.

Lipid material not soluble in chloroform/methanol, but extractable into chloroform/methanol/water, representing larger lipid-linked oligosaccharide species, chromatographs on a DEAE-cellulose column like Dol-PP-oligosaccharides from yeast. The radioactivity is rendered water soluble by mild acid hydrolysis. Fig.2 shows the gel filtration profile of this material synthe-

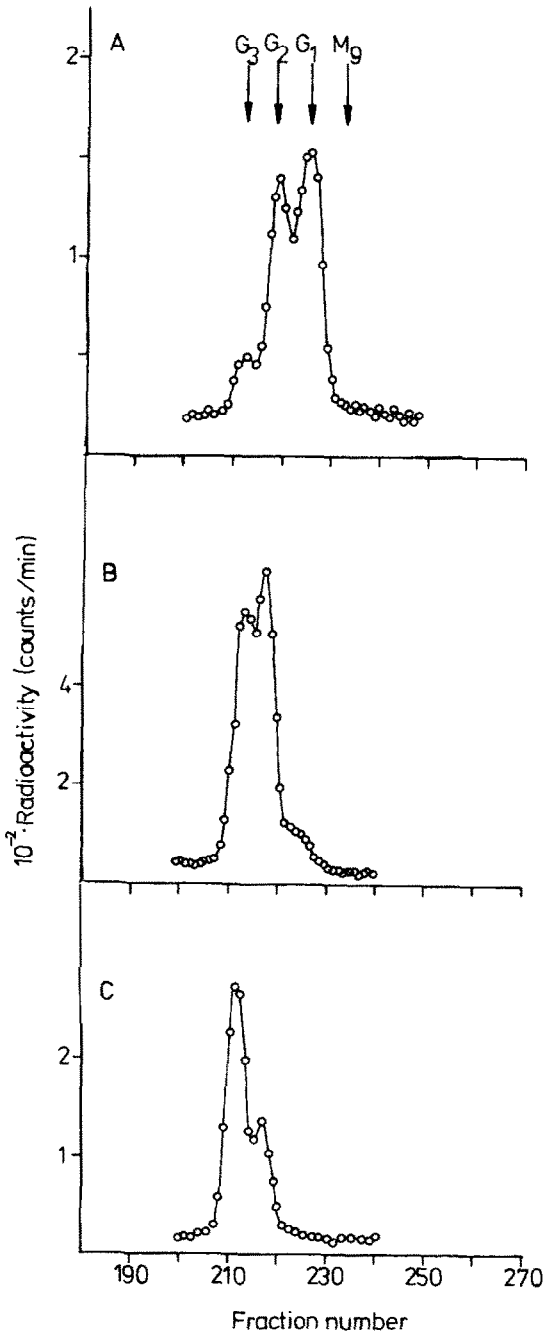

Fig.2. Gel filtration profile of [*Glc*-^{14}C]oligosaccharides from tobacco. Lipid-linked oligosaccharides were synthesized with UDP-Glc. The oligosaccharides were released from the chloroform/methanol/water (10/10/3, by vol.) extract by mild acid, treated with endo-*N*-acetylglucosaminidase and fractionated on Bio-Gel P4. (A) The profile of tobacco cells, (B) of yeast and (C) of liver synthesized according [13]. The arrows indicate the position of marker oligosaccharides M_9, $Man_9GlcNAc_1$; G_1, G_2, G_3, $Glc_{1/2/3}Man_9GlcNAc_1$.

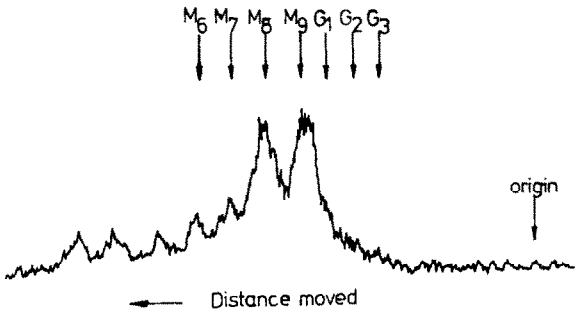

Fig.3. Chromatography of lipid-linked [*Man*-^{14}C]oligosaccharide fraction from tobacco. Membranes were incubated with GDP-Man and mild acid hydrolysis products were separated in solvent (B). Plates were developed twice for 24 h. Whatman paper was clamped to the top of the plate to absorb the solvent. Arrows indicate marker position M_9, $Man_9GlcNAc_2$; M_8, M_7, M_6, $Man_{8/7/6}GlcNAc_2$.

sized with UDP-Glc after additional endo-*N*-acetylglucosaminidase H treatment. Three oligosaccharides are formed migrating as reference compounds of the composition $Glc_1Man_9GlcNAc_1$, $Glc_2Man_9GlcNAc_1$, $Glc_3Man_9GlcNAc_1$. The largest detectable species has the same size as the longest oligosaccharide from yeast [9] or from liver [14]. The reason for the relatively small amount of Glc_3-oligosaccharide is not clear at the moment. However, it might be due to the presence of an active glucosidase, specific for the terminal glucosidic linkage of the Glc_3-oligosaccharide [15] or due to a preferential transfer of the Glc_3-species to endogenous protein as shown for liver [14]. Moreover digestion with α-mannosidase causes a shift in mobility towards lower M_r-values. Treatment of the Glc-oligosaccharide fraction with a membrane-bound yeast glucosidase [9] led to a decrease of radioactivity in the oligosaccharide with a concomitant appearance of free glucose (not shown). The largest lipid-oligosaccharide formed with GDP-Man as donor migrates with the $Man_9GlcNAc_2$ marker (fig.3, in this case the endo H treatment was omitted) and minor amounts of radioactivity were incorporated into species of the size $Man_{8-3}GlcNc_2$, which seem to act as precursors for the larger ones. Table 1 shows that formation of glycolipids requires Mg^{2+} or Mn^{2+}, whereas polymer is synthesized only in the presence of Mn^{2+}. The presence of Triton X -100 has no pronounced effect on polymer synthesis, but strongly inhibits glycosyl transfer to endogenous lipid.

To determine whether the formation of lipid-linked Glc-oligosaccharides involved in *N*-glycosylation of

Table 1
Requirements for glycosyl transfer with tobacco membrane preparation

Additions	Radioactivity (cpm) incorporated into fraction		
	C/M	C/M/W	Polymer
(A) GDP-mannose			
$+Mg^{2+}$	14 559	720	100
$+Mn^{2+}$	16 348	1440	2767
$+Mn^{2+}$, Triton X-100	3268	414	2146
+ EDTA	3273	25	10
(B) UDP-glucose			
$+Mg^{2+}$	10 556	479	15
$+Mn^{2+}$	8790	942	2971
$+Mn^{2+}$, Triton X-100	2671	234	4100
+ EDTA	3867	15	25

Membranes were incubated for 15 min with either GDP-Man or UDP-Glc according to standard assay with 7 mM Mg^{2+} or Mn^{2+}; Na-EDTA was 10 mM and Triton X-100 was 1.5%; C/M = fraction soluble in chloroform/methanol (2:1); C/M/W = fraction soluble in chloroform/methanol/water (10:10:3)

proteins is of general occurence in plants, too, another tissue was investigated. Using a microsomal fraction from spinach similar glycolipids were synthesized with GDP-Man or UDP-Glc as in the tobacco cells. Fig.4 shows the oligosaccharide pattern, released from the

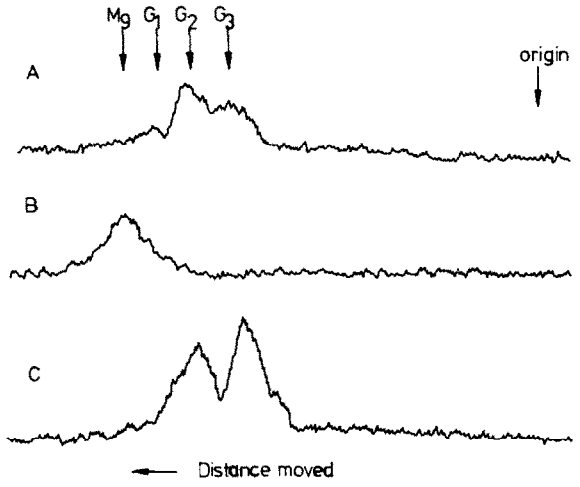

Fig.4. Chromatography of lipid-linked [*Glc*-^{14}C]oligosaccaride fraction from spinach. Microsomal fraction from spinach was incubated either with UDP-Glc (A) or GDP-Man (B) and the oligosaccharides released from lipid–oligosaccharide fraction was separated in solvent (B) as in fig.3. (C) is the Glc-oligosaccharide fraction from yeast.

glycolipid fraction by mild acid. The main compounds of the Glc-oligosaccharide fraction have the mobility of Glc_3-and Glc_2Man_9-$GlcNAc_2$. When GDP-Man was the donor, as expected, the largest oligosaccharide formed migrated with the $Man_9GlcNAc_2$ marker.

4. Discussion

Our knowledge about the structure and assembly of glycoproteins in plants is still very limited as compared to that of animal organisms. So far in plants lipid-linked saccharides containing mannose [16–18], glucose [17,19,20], *N*-acetylglucosamine [18,20] or *N*-acetylglucosamine-mannose-oligosaccharides [18, 20–22] of undefined size have been synthesized and implicated in the glycosylation of glycoproteins. In some cases the nature of the lipid was shown to be an α-saturated polyprenol (dolichol) rather than of undecaprenol of bacteria [18–20,23].

Here, for the first time in vitro, the formation of Dol-PP-$GlcNAc_2$-Man_9 and Dol-PP-$GlcNAc_2Man_9Glc_3$ has been shown. The oligosaccharides seem to be identical by several criteria to those found in animals [1,8,14,24] and yeast [9,10]. Of particular interest is the finding of the derivative with 3 glucose residues, since this compound is thought to play the key role in the glycosylation of asparagine residues. From an evolutionary point of view the observations made here further strengthen the argument that the dolichol part of the biosynthetic pathway of *N*-glycosylation of proteins seems to be the same in all eucaryotic cells.

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