EVIDENCE AGAINST THE PARTICIPATION OF LIPID INTERMEDIATES IN THE IN VITRO BIOSYNTHESIS OF SERINE(THREONINE)-*N*-ACETYL-D-GALACTOSAMINE LINKAGES IN SUBMAXILLARY MUCIN

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1. Introduction

The role of lipid-bound sugars in the biosynthesis of polysaccharides and glycoproteins is well established in bacteria, animals, plants and fungi [1]. These have been shown in eucaryotic systems to be intermediates in glycosylating reactions, transferring sugars like *N*-acetylglucosamine, mannose, and glucose. In particular, the biosynthesis of the carbohydrate–protein linkage has been shown to be mediated by transfer from lipid-linked saccharides to the respective amino acid in two cases, namely, the *N*-acetylglucosamine–asparagine and the mannose–serine/threonine bonds within certain glycoproteins. It is unlikely that lipid carriers are generally involved in the attachment of sugars to amino acid, since direct glycosyltransfer via nucleoside diphosphate sugars has been observed in numerous investigations. This study was undertaken to examine a possible participation of lipid-linked *N*-acetyl-galactosamine derivatives in the biosynthesis of mucin-type glycoproteins, where the amino-sugar is linked *O*-glycosidically to serine/threonine residues. No such involvement could be observed. Furthermore, neither the synthesis of lipid-linked *N*-acetylgalactosamine seems to occur in three other eucaryotic in vitro systems known to catlyze lipid–saccharide formation.

2. Materials and methods

Enzyme preparations: (i) Submaxillary gland microsomal preparations were prepared according to [2]. Cow glands were obtained within 15 min of killing from the local slaughter house. Glandular tissue was excised, cut into pieces and ground in a mixer at 4°C. After addition of 0.07 M KCl the crude homogenate was stirred overnight, filtered through cheesecloth, and the filtrate centrifuged at 500 × *g* for 10 min. The resulting supernatant was again centrifuged at 50 000 × *g* for 20 min. The pellet was then washed twice with 0.05 M KCl, once with 50 mM Tris–HCl (pH 7.4), containing 0.15 mM $MgCl_2$, and finally suspended in 50 mM Tris–HCl (pH 7.4), containing 3.5 mM $MgCl_2$ by a Potter homogenizer. (ii) Membrane preparations from *Saccharomyces cerevisae* (strain 66.24, Fleischman Labs.) were prepared as in [3]. (iii) Crude rat-liver microsomes were obtained according to [4]. (iv) Castor-bean microsomes were produced as in [5].

Dolichol was prepared from yeast and chemically phosphorylated [6]. Generally, labelled lipids and polymers were extracted and washed as in [7]. β-Elimination and paper chromatography were done as in [7].

Abbreviations: Dol-P, dolichyl phosphate; GDP-Man, guanosine diphosphate mannose; UDP-Gal, uridine diphosphate galactose; UDP-GalNAc, uridine diphosphate *N*-acetylgalactosamine; UDP-GlcNAc, uridine diphosphate *N*-acetylgalactosamine

3. Results and discussion

Bovine submaxillary gland membranes catalyze the transfer of *N*-acetylgalactosamine into methanol-insoluble polymeric material upon incubation with UDP-[^{14}C]GalNAc (table 1). Incorporation is dependent on time and the amount of protein present in the assay system (not shown). Polymer synthesis seems to be effectively stimulated by Mn^{2+}, which can only partially be replaced by Mg^{2+} (table 1). An absolute requirement for Mn^{2+} was described in [2], where a purified polypeptide (*N*-acetylgalactosaminyl

transferase) and exogenously added mucin, pre-treated with glycosidases, have been used.

All attempts to achieve incoporation of radioactivity into chloroform–methanol soluble lipidic material were negative (table 1). Parameters like incubation time, amount of protein and metal dependence were varied in order to optimize incubation conditions with respect to glycolipid synthesis, but no appreciable radiolabelled material could be observed. The same is true for the experiment where exogenous dolichyl phosphate was present in addition; under these conditions, in other systems, e.g., yeast microsomes, a greatly stimulated incorporation of sugars into dolichyl saccharides has been obtained [8]. To get some insight into the nature of the polymeric material synthesized from UDP-[^{14}C]GalNAc, it was treated with 0.1 N sodium hydroxide at room temperature for 24 h. This treatment is known to split *O*-glycosidic bonds between hydroxyaminoacids (serine/threonine) and sugar attached to them (β-elimination). Upon paper chromatography as the only split product radioactive *N*-acetylgalactosamine (with some traces of galactosamine) is obtained (not shown) indicating that the amino sugar, presumably transferred directly via UDP-GalNAc, is indeed linked to serine/threonine residues of the polypeptide acceptor, as expected for mucin-type glycoproteins. The possibility, however, might exist that dolichyl saccharide synthesis in submaxillary gland microsomes occurs at a very high velocity, or that the intermediate lipid cofactors are required in minute concentrations, each preventing the direct demonstration of such a pathway. To circumvent at least the second problem we tried to synthesize GalNAc-containing dolichyl derivatives in 3 other eucaryotic in vitro systems. Yeast membranes are known effectively to catalyze dolichyl saccharide synthesis in vitro ([9] and references cited therein). If those membranes are incubated with UDP-GlcNAc or GDP-Man, glycosylation of dolichyl phosphate and formation of polymeric (glycoprotein) material occurs very effectively (table 2). No incorporation of *N*-acetylgalactosamine, either into chloroform/methanol-soluble or polymeric material is observed. (The small amount of radioactivity in the polymer fraction (table 2, expt. 1,2) might be explained by epimerization of UDP-GalNAc to UDP-GlcNAc, since *N*-acetylgalactosamine is not a known constituent of yeast glycopolymers.)

The dolichyl pathway was originally established using liver microsomal preparations [1]. Upon incubation of crude liver membranes with different radioactive nucleoside diphosphate sugars, labelled glycolipid is only obtained by using GDP-[^{14}C]mannose as donor, and its synthesis can be greatly stimulated by the addition of exogenous yeast dolichyl phosphate (table 3). Neither UDP-GalNAc nor UDP-Gal act in that

Table 1
Radioactive products formed from UDP-[^{14}C]GalNAc by submaxillary gland microsomes

Expt.	Incubation time (min)	Soluble in chloroform –methanol (2:1) (cpm)	Incorporated into methanol-insoluble fraction (cpm)
– Dol-P			
40 μg protein	60	15	5834
400 μg protein	60	76	20 582
+ Dol-P			
40 μg protein	60	29	5802
400 μg protein	60	52	23 667
100 μg protein	0.5	25	230
	2	51	388
100 μg protein			
–Mn, +Mg	10	51	272
+Mn, +Mg	10	61	1150

Microsomes were incubated with 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 20 mM $MnCl_2$, 11.6 μM UDP-[^{14}C]GalNAc (spec. act. 61.5 Ci/mol) in 0.07 ml total vol. When dolichyl phosphate (5 μg) was present, the incubations contained 2 μmol MgEDTA and 0.17% Triton X-100

Table 2
Radioactive products formed from different sugar nucleotides by yeast microsomal preparations

Expt.	[^{14}C]Sugar nucleotide	Soluble in chloroform –methanol (2:1) (cpm)	Incorporated into methanol-insoluble fraction (cpm)
1. – Dol-P	UDP-GalNAc	41	235
2. + Dol-P	UDP-GalNAc	87	552
3. + Dol-P	GDP-Man	16 227	58 860
4. + Dol-P	UDP-GlcNAc	10 153	4345
5. + Dol-P	UDP-GalNAc	55	25
6. + Dol-P	UDP-GalNAc(+ATP)	165	63
7. + Dol-P	GDP-Man	51 576	16 199

Microsomes (300 μg protein) were incubated as in [20] for 10 min; $MnCl_2$ (20 mM) was present in addition. In expt. 5–7, Tris–HCl buffer was replaced by 0.07 M Tris–maleate (pH 7.7) and 0.06 M β-mercaptoethanol, and $MnCl_2$ was omitted. In expt. 6, 0.1 mM ATP was added as pyrophosphatase inhibitor. Assays were performed with either 11.6 μM UDP-[^{14}C]GalNAc (spec. act. 61.5 Ci/mol), or 3.4 μM GDP-[^{14}C]Man (spec. act. 210 Ci/mol), or 4.8 μM UDP-[^{14}C]GlcNAc (spec. act. 300 Ci/mol)

way. Similar negative results for UDP-Gal have been reported, although galactosyl transfer from dolichyl-phosphate galactose (prepared with a bacterial system) to a dolichyl diphosphate oligosaccharide-like acceptor has been demonstrated [10]. The extent of incorporation of labelled sugars into polymer with either sugar donor is different. Upon addition of exogenous dolichyl phosphate, only in the case of GDP-mannose is the expected decrease in polymer synthesis observed, whereas in the case of UDP-GalNAc or UDP-Gal only insignificant changes occur (table 3). Biopolymers containing *N*-acetylgalactosamine or galactose have been reported to be formed in liver [11].

It is quite probable that the well-established sequence of reactions in the biosynthesis of *N*-glycosidically linked carbohydrate units in animals also occurs in plants [12]. Concerning *O*-glycosidic linkages between galactose and hydroxyproline and serine, respectively, no information is available [12], although galactosyl transfer from UDP-galactose to protein via

Table 3
Radioactive products formed from different sugar nucleotides by crude liver microsomes

Expt.	[^{14}C]Sugar nucleotide	Soluble in chloroform –methanol (2:1) (cpm)	Incorporated into methanol-insoluble fraction (cpm)
– Dol-P	UDP-GalNAc	2	2312
	UDP-GalNAc (+ATP)	30	2567
	UDP-Gal	76	13 011
	GDP-Man	8178	32 020
+ Dol-P	UDP-GalNAc	14	3089
	UDP-GalNAc (+ATP)	2	4742
	UDP-Gal	91	13 768
	GDP-Man	28 098	19 474

Microsomes (170 μg protein) were incubated in 0.1 M Tris–maleate (pH 7.7), 0.1 M β-mercaptoethanol, and either 16.3 μM UDP-[^{14}C]GalNAc (spec. act. 61.5 Ci/mol), or 4.8 μM GDP-[^{14}C]Man (spec. act. 210 Ci/mol), or 3.9 μM UDP-[^{14}C]Gal (spec. act. 254.5 Ci/mol) in 0.05 total vol. for 15 min. When indicated, 1 mM ATP was added. When dolichyl phosphate (5 μg) was present, the incubations contained 2 μmol MgEDTA and 0.6% Triton X-100

Table 4
Radioactive products formed from different sugar nucleotides by castor-bean endosperm microsomal preparations

Expt.	[^{14}C]Sugar nucleotide	Soluble in chloroform -methanol (2:1) (cpm)	Incorporated into methanol-insoluble fraction (cpm)
1. – Dol-P	UDP-GalNAc	7	12
2. + Dol-P	UDP-GalNAc	67	0
3. + Dol-P	GDP-Man	675	660
4. + Dol-P	UDP-Gal	695	218
5. + Dol-P	UDP-GalNAc	0	0
6. + Dol-P	UDP-GalNAc (+ATP)	0	0
7. + Dol-P	GDP-Man	324	330
8. + Dol-P	UDP-Gal	52	575

Microsomes (300 μg protein) were incubated as in yeast system in 0.09 ml total vol. for 10 min. In expt. 5–8, Tris–HCl buffer was replaced by 0.07 M Tris–maleate (pH 7.7) and 0.06 M β-mercaptoethanol, and $MnCl_2$ was omitted. In expt. 6, 0.1 mM ATP was added. Assays were performed with either 9.0 μM UDP-[^{14}C]GalNAc (spec. act. 61.5 Ci/mol), or 4.4 μM GDP-[^{14}C]Man (spec. act. 210 Ci/mol), or 2.2 μM UDP-[^{14}C]Gal (spec. act. 254.5 Ci/mol). When dolichyl phosphate was present, the incubations contained 2 μmol MgEDTA and 0.6% Triton X-100

a lipid-linked intermediate was postulated [13]. Glycosylation in plants from UDP-GalNAc has not yet been investigated, although galactosamine has been reported to occur in plants [12]. Incubation of castor-bean endosperm microsomes with GDP-mannose or UDP-galactose gives rise to the expected [1,13] formation of lipid-linked saccharides, whereas UDP-*N*-acetylgalactosamine does not seem to act as donor (table 4). In addition, transfer to polymer only via GDP-mannose and UDP-galactose is evident.

In conclusion, using 4 eucaryotic in vitro systems, efforts to synthesize lipid-linked *N*-acetylgalactosamine derivatives were negative, although the various systems were shown by control experiments with nucleoside diphosphate sugars other than UDP-GalNAc to be active in terms of lipid-saccharide synthesis. From these results it is suggested that the formation of serine/threonine–*N*-acetylgalactosaminyl linkages proceeds directly via nucleoside diphosphate sugar, which would infer that mucins are assembled by step-by-step addition of monosaccharides. The same conclusion has been drawn from experiments using a purified polypeptide: *N*-acetylgalactosaminyl transferase from bovine submaxillary glands [14,15]. The only case known where lipid-intermediates serve as glycosyl-donors in the formation of *O*-glycosidic carbohydrate–peptide linkages is the mannosylation of serine/threonine residues in fungal glycoprotein biosynthesis [16–19].

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