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Detection of the lipid-linked precursor oligosaccharide of *N*-linked protein glycosylation in *Drosophila melanogaster*

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The presence of a glycan of the same molecular size as the lipid linked precursor oligosaccharide ($Glc_3Man_9GlcNAc_2$) of the *N*-linked protein glycosylation pathway in mammalian cells has been detected in a glycolipid fraction of cultured *Drosophila melanogaster* cells. Oligosaccharide sequencing studies were consistent with the existence of a glucosylated high mannose containing structure, which may be the common precursor for *N*-linked protein glycosylation in insect cells.

N-Linked protein glycosylation; Glycolipid; Lipid-linked precursor oligosaccharide; Metabolic labelling; *Drosophila melagonaster*

1. INTRODUCTION

A consensus for the major pathway of *N*-linked glycosylation in vertebrates has been established [1]. This pathway involves the synthesis of the precursor oligosaccharide, $Glucose_3Mannose_9$*N*-$Acetylglucosamine_2$ ($Glc_3Man_9GlcNAc_2$), attached to dolichol, a long chain isoprenoid alcohol. The glycan is transferred *en-bloc* to nascent polypeptide chains in the endoplasmic reticulum. The precursor oligosaccharide is processed to give the diversity of final carbohydrate structures seen in mature glycoproteins. It is apparent that the initial synthesis and transfer reaction are the same in all eukaryotes analysed except for *Trypanosoma cruzi, Crithidia fasciculata* and *Leishmania mexicana* [2]. The precursor oligosaccharide transferred onto proteins in these organisms lacks the three glucoses. Non-vertebrate eukaryotes also differ in the processing of the precursor oligosaccharides [2–4].

In vertebrates processing is begun by the removal of the three glucoses from the precursor oligosaccharide by two enzymes. These are α-glucosidase I (E.C. 3.2.106) which removes the terminal α-1-2 linked glucose, and α-glucosidase II (E.C. 3.2.106, mannosyl-oligosaccharide glucosidase) which then removes the two remaining α-1-3 linked glucoses [1,4]. Our objective is to identify similar processing enzymes in *Drosophila melanogaster*. However, in the light of observations such as those on the three organisms lacking three terminal glucoses in their precursor, it is necessary to provide biochemical evidence for the existence of a glucosylated precursor oligosaccharide in this organism.

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2. MATERIALS AND METHODS

Bio-Gel P-4, Dowex AG50X12-100, Dowex AG3-X4A and Chelex 100 were obtained from Bio-Rad laboratories, radioactive mannose ([2-^{3}H]mannose 16.3 Ci/mmol) was purchased from Amersham Ltd, amino acids and organic buffers for cell culture were purchased from Sigma. QAE-Sephadex A-25 was obtained from Pharmacia Ltd. (Milton Keynes, UK). Foetal calf serum was supplied by Globepharm (Surrey, UK). Glutamine and trypsin were purchased from Gibco (Gibco/BRL, UK). Yeast extract powder was from Lab M, chloroform and methanol were 'analar' grade supplied by BDH.

Porcine liver α-glucosidase I was prepared from detergent extracts of isolated microsomes and purified using carboxypentyl deoxynojirimicyn-agarose affinity chromatography [5]. α-Glucosidase II from porcine liver microsomes was purified by concanavalin-A affinity chromatography and ion-exchange chromatography. *Canavalia ensiformis* (jack bean) α-mannosidase (E.C. 3.2.1.24) was prepared according to Li and Li [6].

The cell line DM3 [7] of *Drosophila melanogaster* was used for these experiments. The cells were grown at 25°C at atmospheric concentrations of carbon dioxide in Shields and Sang M3 medium [8]. The medium was supplemented with 12% heat-treated foetal calf serum and with fresh glutamine. In addition the medium contained penicillin and streptomycin.

For metabolic labelling, cells were grown in M3 medium containing $^1/_{10}$ the normal concentration of glucose (5 mM glucose). When the cells had reached approximately half the normal confluent concentration (2×10^6 floating cells/ml), the medium was removed and the floating cells recovered by centrifugation. The cells were resuspended in 5 mM glucose M3 medium containing 10 μCi/ml mannose and incubated for 1–2 h. The floating cells were recovered by centrifugation and the adhering cells were harvested by scraping. The ceils were washed once in ice-cold Dulbecco's PBS and frozen.

For the extraction of lipid linked oligosaccharides we used a method developed for CHO cells [9]. Briefly, the frozen cells were thawed, pelleted by centrifugation and washed twice with ice-cold Dulbecco's PBS. The cells were disrupted by sonication in chloroform/methanol

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(2:1) and centrifuged. The pellets were recovered and washed twice in chloroform/methanol (2:1), three times in distilled water and then dried, before being resuspended in chloroform/methanol/water (10:10:3). This suspension was centrifuged and the supernatant preserved. The pellet was washed again with chloroform/methanol/water (10:10:3) and the supernatant combined with that of the first wash. The chloroform/methanol/water washes were dried and resuspended in 0.1 N HCl. The lipid-linked oligosaccharides were released by incubating for 1 h at 60°C. The released oligosaccharides were recovered and desalted by gel filtration on a small Bio-Gel P-4 column (19 × 1.5 cm). The pooled activity from desalting was dried and resuspended in a saturated solution of $NaHCO_3$ and re-*N*-acetylated by adding acetic anhydride. (10 min at 0°C and 50 min at room temperature). The oligosaccharides were then desalted on a column of Dowex AG 50W-X12 (H^+ form).

Glycans were analysed via gel filtration chromatography on Bio-Gel P-4 (2 × 1.5 × 90 cm columns). Before each column run the samples were run through a mixed bed ion-exchange column. A dextran hydrolysate was added into each run to provide an internal oligoglucose standard [10]. The radioactivity eluting from the column was monitored by liquid scintillation counting. The elution of the dextran standard was detected using a refractive index monitor.

Sequencing was by enzymatic digestion and analysis of changes in molecular size [10]. For digestion, the appropriate peaks of radioactive material were collected, pooled and dried by evaporation under reduced pressure at 25°C before addition of enzyme.

α-glucosidase I and II digestion was carried out at pH 7.0 in 100 mM sodium phosphate buffer containing 0.4% lubrol-PX under a toluene atmosphere for 18 h at 37°C. Digestion with jack bean (*Canavalia ensiformis*) α-mannosidase was carried out at 37°C for 18 h under a toluene atmosphere in 10 μl of citrate buffer pH 4.5 containing 0.2 mM Zn^{2+} ions and 50 U/ml mannosidase. Digestions were stopped by heating to 100°C for 2 min and then analysed on Bio-Gel P-4 columns as described above.

3. RESULTS

The neutral lipid-linked oligosaccharide fraction contained 0.7% of the mannose incorporated (see Table I). The analysis of the oligosaccharides before enzymatic digestion gave a major peak at 15 glucose units (G.U.) see Fig. 1a. This is the elution position predicted for the precursor oligosaccharide and is identical with the elution position of radiolabelled oligosaccharide similarly obtained from calf thyroid microsomes (P. Scudder and T.D. Butters, unpublished results). This peak contained 46% of the activity loaded onto the column (see Table I). The fractions containing the peak were pooled and dried. The peak was subjected to digestion with α-glucosidase I and α-glucosidase II, an enzyme mixture which will remove the three terminal glucoses from the vertebrate oligosaccharide precursor. This digestion caused a shift to an elution position of 12 G.U., consistent with

Table I

Fraction	DPM values	% Values (per step)	% Recovery of radioactivity incorporated by cells
Total DPM	2.1×10^9	100	–
Incorporated by cells	3.25×10^7	1.5	100
In chloroform/methanol/water (10:10:3) fraction	9.19×10^4	0.28	0.283
After desalting	4.9×10^4	53	0.151
After re-*N*-acetylation	2.2×10^4	46	0.068

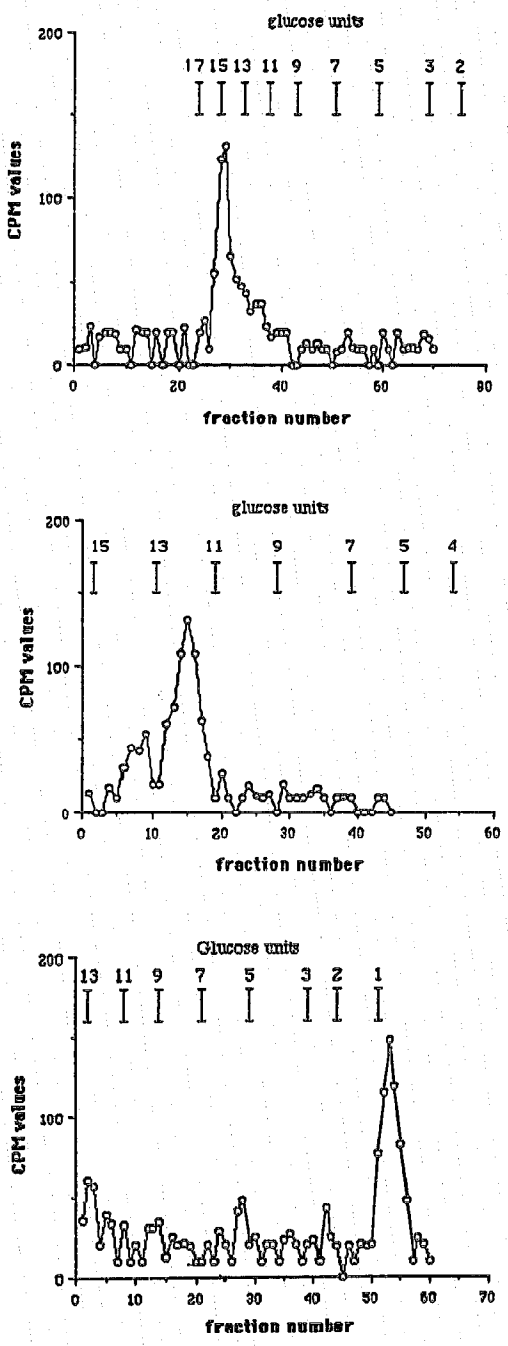

Fig. 1. Bio-Gel P-4 elution profiles of radiolabelled glycans, elution positions of the dextran hydrolysate standards are indicated by the numbered bars. (a) Analysis of glucans from DM3 cells after metabolic labelling and acid release of glycans. (b) Analysis of 15 G.U. glycan from (a) after digestion with α-glucosidase I and II. (c) Analysis of 12 G.U. glycan from (b) after digestion with α-mannosidase.

the removal of three hexose units. This peak contained 90% of the radioactivity from the initial 15 G.U. peak (see Fig. 1b).

After digestion with jack bean α-mannosidase the majority of the activity from 12 G.U. was detected at 1 G.U. (see Fig. 1c). A small peak of activity was detected at 5 G.U. This is consistent with the presence of the Man β $GlcNAc_2$ core which would remain after the removal of the eight α linked mannoses from the $Man_9GlcNAc_2$ structure expected in the 12 G.U. peak.

4. DISCUSSION

Observations consistent with the existence of the precursor oligosaccharide in dipterans using TLC analysis [11,12] and gel filtration [13] have already been made. In this report we have extended these findings by demonstrating the synthesis of a glycan of the predicted size in *Drosophila* cultured cells. On the basis of molecular size or TLC analysis it was formally possible that the glycan was not the same as the vertebrate precursor glycan. Our findings eliminate this conjecture. The glycan contains three α-glucoses in positions accessible to the oligosaccharide processing glucosidases isolated from mammalian tissue and probably contains eight accessible α-linked mannoses. The ability to produce a digest with α-mannosidase means that the majority of the mannose residues are also terminally located once the glucoses have been removed. This is the first structural biochemical evidence that a glucosylated, mannose containing lipid linked precursor oligosaccharide is synthesised in *Drosophila* cells. It provides evidence that the *N*-glycan structures associated with mature insect glycoproteins [14–17] and in particular those found by analysis of *Drosophila* tissues [18] arise from a glycosylation pathway that includes the formation of a precursor oligosaccharide common to most eukaryotes.

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