Hydrophobic glycosides of *N*-acetylglucosamine can act as primers for polylactosamine synthesis and can affect glycolipid synthesis *in vivo*

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Several hydrophobic glycosides of *N*-acetylglucosamine (GlcNAc) served as primers for polylactosamine synthesis when added to Chinese hamster ovary (CHO) cells. The modified glycosides, containing one to six lactosamine repeats in linear array, were sialylated and secreted into the culture medium. The relative efficiencies of the glycosides to serve as primers were dependent on the nature of the aglycone and on the anomeric configuration of the GlcNAc residue. The same compounds were

tested for their effects on glycolipid synthesis in CHO cells. All of the β -glycosides significantly inhibited the synthesis of the lacto-series glycolipid G_{M3} whereas the α -glycoside was inactive. The compound GlcNAc α 1-*O*-benzyl was the most efficient primer of polylactosamine synthesis and had no effect on glycolipid synthesis. This compound may have potential for the assay of the polylactosamine synthetic capacity of living cells.

INTRODUCTION

Hydrophobic glycosides have been used to study a variety of glycosylation processes *in vivo* and *in vitro*, in particular mucin and proteoglycan biosynthesis. Hydrophobic α -*N*-acetylgalactosaminides have been shown to inhibit endogenous mucin biosynthesis and to prime the formation of mucin-like O-linked oligosaccharides [1]. Similarly, hydrophobic β -xylosides inhibit endogenous proteoglycan synthesis and are secreted into the medium carrying chondroitin sulphate glycosaminoglycan chains ([2,3] and references therein). More recently, it has been shown that both α - and β -xylosides can affect glycolipid biosynthesis and that β -xylosides prime the formation of NANA α 2-3Gal β 1-4Xyl β 1-*O*-R in Chinese hamster ovary (CHO) cells via the action of glycosaminoglycan galactosyltransferase I followed by the action of the sialyltransferase G_{M3} -synthase [4].

In this paper, we show that α - and β -*N*-acetylglucosaminides can prime the formation of sialylated linear polylactosamine (PL) structures of up to six lactosamine repeats. We also show that the β -, but not the α -, *N*-acetylglucosamine (GlcNAc)-glycosides can inhibit the synthesis of the lacto-series glycolipid G_{M3} .

MATERIALS AND METHODS

Materials

The hydrophobic glycosides GlcNAc β 1-*O*-benzyl, GlcNAc α 1-*O*-benzyl, GlcNAc β 1-*O*-phenyl and GlcNAc β 1-*O*-*p*-nitrophenol (GlcNAc β 1-*O*-PNP) were obtained from Sigma. Cell culture media and trypsin/EDTA were obtained from Gibco-BRL. *Clostridium perfringens* neuraminidase was from New England Bio-Labs, all other exoglycosidases and endoglycosidases were from Oxford Glycosystems. *Streptomyces griseus* Pronase was from Boehringer and *Datura stramonium* lectin linked to agarose beads was from Vector-Laboratories. C18 reverse-phase columns (500 mg) were from Alltech Associates. High-performance TLC (HPTLC) plates and all other reagents and solvents were obtained from BDH-Merck, Poole, U.K. CHO-K1 cells were obtained

from the European Collection of Animal Cell Cultures (Salisbury, U.K.). Enhance spray and [6-³H]Gal (35–40 Ci/mmol) were obtained from Du Pont New England Nuclear. Ion-exchange resins (AG50X12 and AG3X4) and Bio-Gel P4 were obtained from Bio-Rad. Gangliosides G_{M3} , Lac-Cer and Glc-ceramide (cerebroside) were obtained from Sigma.

Cell culture and metabolic labelling

CHO-K1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 17.25 mg/l L-proline under a 5% CO₂ atmosphere. Cells were washed three times with glucose-free RPMI 1640 medium, supplemented with 25 mM Na-Hepes (pH 7.4), and pre-incubated for 2 h in the same medium (approximately 1 ml per 2×10^5 cells) containing the GlcNAc-glycosides at a final concentration of 2 mM. The cells were subsequently labelled by the addition of [³H]Gal (10 or 20 μ Ci/ml) and incubation for 20 h at 37 °C.

Purification of the [³H]Gal-labelled products of the GlcNAc-glycosides

The culture supernatants (10 to 25 ml, depending on the experiment) were centrifuged at 1500 *g* for 5 min at 4 °C to remove cell debris and adjusted to 1 M with respect to NaCl. The supernatants were subsequently applied under gravity to C18 reverse-phase columns that had been previously washed with 5 ml of methanol and pre-equilibrated with 10 ml of 1 M NaCl. The columns were washed with 10 ml of 1 M NaCl followed by 0.5 ml of water and the glycoside products were eluted with a further 10 ml of water. The columns were then washed with 5 ml of 40% (v/v) methanol in water followed by 5 ml of methanol. Radioactivity that eluted in these latter fractions was shown not to be associated with the glycosides and was not studied further. The elution of the glycoside products in the water eluate was confirmed using an authentic standard of [³H]Gal β 1-4GlcNAc β 1-

Abbreviations used: CHO, Chinese hamster ovary; Glc-Cer, Glc β 1-*O*-ceramide; GlcNAc, *N*-acetylglucosamine; Gu, glucose unit; HPTLC, high-performance TLC; Lac-Cer, Gal β 1-4Glc β 1-*O*-ceramide; LAMP, lysosome-associated membrane protein; PL, polylactosamine; PNP, *p*-nitrophenol; TCA, trichloroacetic acid.

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Figure 1 Bio-Gel P4 chromatography of the products of GlcNAc-glycosides

The [^3H]Gal-labelled products were separated on Bio-Gel P4 before (a) and after (b–e) desialylation with *C. perfringens* neuraminidase. The glycosides were GlcNAc β 1-*O*-benzyl (a and b), GlcNAc α 1-*O*-benzyl (c), GlcNAc β 1-*O*-phenyl (d) and GlcNAc β 1-*O*-PNP (e).

O-benzyl, prepared by the *in vitro* galactosylation of GlcNAc β 1-*O*-benzyl using UDP-[^3H]Gal and bovine β -galactosyltransferase [5]. The water eluate was concentrated to 2 ml by rotary evaporation and applied to a 1 m $\times$ 1.5 cm Bio-Gel P4 column (see below) to remove the unlabelled GlcNAc-glycosides and any remaining free [^3H]Gal. All of the radioactive fractions greater than 1 glucose unit (Gu) in size were pooled, passed through 2 ml of AG50X12(H^+) with water to remove sodium ions and freeze-dried twice from water.

Desialylation

For the experiments summarized in Figure 1 the samples were desialylated by digestion with 100 μl of *Clostridium perfringens* neuraminidase (4 units/ml) in 100 mM sodium acetate, pH 5.0, at 37 $^\circ\text{C}$ for 20 h. Digestions with Newcastle disease virus neuraminidase were performed using 20 μl of enzyme at 0.2 unit/ml in 50 mM sodium acetate, pH 5.5, at 37 $^\circ\text{C}$ for 16 h. For

Table 1 The relative efficiencies of hydrophobic glycosides as acceptors for [^3H]Gal *in vivo*

Glycoside	Relative efficiency (%)
GlcNAc α 1- <i>O</i> -benzyl	100*
GlcNAc β 1- <i>O</i> -benzyl	51
GlcNAc β 1- <i>O</i> -phenyl	10
GlcNAc β 1- <i>O</i> -PNP	< 2

* The total radioactivity associated with the glycoside-products of GlcNAc α 1-*O*-benzyl was 552000 c.p.m.

both neuraminidase digestions, samples were desalted by passage through a column of 0.2 ml of AG50X12(H^+).

Determination of the relative efficiencies of [^3H]Gal acceptance

For the experiments summarized in Table 1, 6 cm-diameter dishes of CHO-K1 cells (approximately 6×10^5 cells) were labelled with [^3H]Gal (20 $\mu\text{Ci}/\text{ml}$) for 20 h at 37 $^\circ\text{C}$ in the presence and absence of 2 mM GlcNAc-glycosides. The culture supernatants were processed as described above and desialylated with 1 ml of 25 mM H_2SO_4 (1 h, 100 $^\circ\text{C}$) followed by passage through 0.4 ml of AG3X4(OH^-) and elution with 2 ml of water. The products were passed through a column of 0.2 ml of AG50X12(H^+) over 0.5 ml of AG3X4(OH^-), eluted with 2 ml of water and analysed by Bio-Gel P4 chromatography. The control incubation, with no added glycoside, produced some radioactive components at the void volume and at 3.5 Gu, 2.5 Gu and 1 Gu; these components were ignored when integrating the glycoside-product peaks.

Estimation of incorporation of [^3H]Gal into total cellular glycoproteins and PL-containing glycoproteins

The amount of [^3H]Gal incorporated into cellular glycoproteins and cellular PL-containing glycoproteins in the absence of glycosides was estimated after incubating cells with [^3H]Gal as described above. The cells were collected from the culture dish by brief trypsinization (twice for 3 min at 37 $^\circ\text{C}$ with 0.5 ml of 0.5 mg/ml trypsin/0.2 mg/ml EDTA in PBS). The cell suspensions, plus PBS washes of the culture dish, were freeze-dried and redissolved in 600 μl of 2% (w/v) Triton X-100/1 mg/ml BSA.

An aliquot of 100 μl of the material was precipitated with an equal volume of ice-cold 20% trichloroacetic acid (TCA) and incubated at 4 $^\circ\text{C}$ for 16 h. The precipitated proteins and glycoproteins were collected on a 2.5 cm diameter Whatman GF/F filter that was washed exhaustively with ice-cold 10% TCA followed by ice-cold 70% ethanol. The filter was dried and subjected to scintillation counting.

The remaining 500 μl was precipitated with TCA as described above, except that the insoluble material was collected by centrifugation. The pellet was washed five times with 1 ml of 10% TCA and three times with 1 ml of 70% ethanol, dried under a stream of N_2 and resuspended in 200 μl of PBS containing 0.1% Triton X-100. *Streptomyces griseus* Pronase (40 μg in 20 μl of 5 mM CaCl_2) was added and incubated at 37 $^\circ\text{C}$ for 16 h. After the addition of a further 80 μg of Pronase and incubation at 37 $^\circ\text{C}$ for 6 h the digest was terminated by boiling for 30 min. After centrifugation, the Pronase digest, containing [^3H]Gal-labelled glycopeptides, was incubated for 12 h at 4 $^\circ\text{C}$ with 400 μl

(packed volume) of *Datura stramonium* lectin bound to agarose beads. The beads were washed exhaustively with PBS containing 0.1 mM CaCl_2 (with and without 0.2% Triton X-100 and additional 0.35 M NaCl). After the radioactivity in the washes reached background levels the beads were transferred to scintillation vials for scintillation counting.

Exoglycosidase and endoglycosidase digestions

Bacteroides fragilis endo- β -galactosidase digestions were performed at 0.083 unit/ml in 30 μl of 50 mM sodium acetate, pH 5.8, containing 250 $\mu\text{g/ml}$ BSA and 1 mM NaCl. *Diplococcus pneumoniae* β -galactosidase digestions were performed at 0.08 unit/ml in 50 μl of 100 mM sodium acetate, pH 6.0, containing 250 $\mu\text{g/ml}$ BSA. In both cases digestions were for 16 h at 37 °C and samples were desalted by passage through 0.2 ml of AG50X12(H^+). Digestions with jack bean β -hexosaminidase were performed at 5 units/ml in 50 μl of 100 mM citrate-phosphate buffer, pH 5.0, for 16 h at 37 °C and samples were desalted by passage through 0.2 ml of AG50X12(H^+) over 0.2 ml of AG3X4(OH^-).

Bio-Gel P4 chromatography

The chromatography shown in Figure 1 was performed using a 1 m $\times$ 1.5 cm column of Bio-Gel P4 (–400 mesh) held at 55 °C and eluted with water at 0.3 ml/min. Fractions (1 ml) were collected for scintillation counting and co-injected glucose oligomers were detected with a refractive index monitor. The sizes of the radioactive components are expressed in glucose units (Gu) by linear interpolation of their elution positions between adjacent glucose oligomer standards. The chromatography shown in Figures 3 and 5 was performed using an Oxford Glycosystems GlycoMap apparatus and 0.25 ml fractions were collected for scintillation counting.

Glycolipid extractions

Cell pellets (approximately 6×10^5 cells) were freeze-dried, resuspended in 65 μl of water (with vortexing and sonication) and extracted with 435 μl of chloroform/methanol (1:1, v/v) with vortexing, sonication and incubation overnight at 4 °C. After centrifugation in a microfuge, the supernatant was recovered and the pellet was re-extracted with 500 μl of chloroform/methanol/water (10:10:3, by vol.) for 2 h at 4 °C. The combined supernatants were dried under a stream of N_2 and resuspended in 400 μl of 1-butanol saturated with water. The 1-butanol phase was washed with 200 μl of water saturated with 1-butanol and the lower water phase was back-extracted with 400 μl of 1-butanol saturated with water. The combined upper 1-butanol phases were extracted twice with 200 μl of water saturated with 1-butanol, dried and redissolved in 20 μl of 1-butanol saturated with water.

High-performance thin layer chromatography (HPTLC)

Oligosaccharides and glycoside products were fractionated on silica gel-60 HPTLC plates using 1-butanol/ethanol/water (4:3:3, by vol.) and glycolipids were fractionated on the same plates using chloroform/methanol/0.02% CaCl_2 in water (65:25:4, by vol.). Radioactive components were detected by fluorography, at –70 °C using Kodak XAR-5 film, after spraying the plates with Enhance. Unlabelled glycolipid standards were visualized by orcinol- H_2SO_4 staining.

RESULTS

Hydrophobic GlcNAc-glycosides act as acceptors for [^3H]Gal and sialic acid *in vivo*

The hydrophobic glycosides GlcNAc β 1-*O*-benzyl, GlcNAc α 1-*O*-benzyl, GlcNAc β 1-*O*-phenyl and GlcNAc β 1-*O*-PNP were incubated with CHO cells in the presence of [^3H]Gal. After 20 h the radiolabelled products were recovered from the culture supernatants and processed as described in the Materials and methods section. Before desialylation the majority of the radioactivity eluted in the void volume of the Bio-Gel P4 column (indicative of negative charge, see for example Figure 1a) whereas after desialylation with *C. perfringens* neuraminidase a series of neutral products were resolved in each case (Figures 1b–1e). Quantitative desialylation of the products could also be achieved using Newcastle disease virus neuraminidase (data not shown), indicating that all of the sialic acid was in α 2-3 linkage [6].

The differences in the hydrodynamic volumes of the desialylated products (Figures 1b–1e) appear to be dependent on the nature of the aglycone and its interaction with the Bio-Gel P4 column packing. The radioactivity that eluted as a sharp peak at about 19 Gu (e.g. Figures 1d and 1e) contained oligosaccharyl-glycoside products that co-eluted with Phenol Red carried over

Scheme 1 Summary of the characterization of [^3H]Gal-labelled desialylated products of the GlcNAc β 1-*O*-benzyl glycoside

The sizes of the individual glycoside products are indicated in brackets in glucose units (Gu), as measured by Bio-Gel P4 chromatography. The ^3H -labelled Gal residues are indicated by an asterisk. Only radiolabelled enzyme digestion products are shown. The enzyme digests used were: *D. pneumoniae* β -galactosidase, Dp β G; jack bean β -hexosaminidase, JB β H and *B. fragilis* endo- β -galactosidase, Endo β G.

Figure 2 Characterization of the 2.1 Gu product of the GlcNAc β 1-*O*-benzyl glycoside

HPTLC analysis of the [^3H]Gal-labelled 2.1 Gu product purified by Bio-Gel P4 chromatography (Figure 1b) before (lane 1) and after (lane 2) digestion with *D. pneumoniae* β -galactosidase. The positions of radiolabelled dextran oligomer standards (Dex) and [^3H]Gal (Gal) are shown.

from the culture medium. The radioactivity found at the void volume after desialylation (Figures 1b–1e) was insensitive to endo- β -galactosidase and was not studied further.

Of the compounds tested, the GlcNAc α 1-*O*-benzyl and GlcNAc β 1-*O*-benzyl glycosides were the most efficient acceptors of [^3H]Gal, whereas GlcNAc β 1-*O*-phenyl and GlcNAc β 1-*O*-PNP were relatively poor acceptors (Table 1). Assuming no dilution of the specific activity of the [^3H]Gal label, the amount of Gal found attached to the GlcNAc α 1-*O*-benzyl acceptor and secreted into the medium was about 15 pmol, from 6×10^5 cells in 20 h. Using the same assumption, the comparable amounts of Gal incorporated, in the absence of glycosides, into total cellular glycoproteins and into PL-containing glycopeptides that bind to *Datura stramonium* lectin [7] were about 60 pmol and 1 pmol respectively.

The labelled materials produced from the GlcNAc α 1-*O*-benzyl, GlcNAc β 1-*O*-benzyl, GlcNAc β 1-*O*-phenyl and GlcNAc β 1-*O*-PNP acceptors were found to be similar, based on their sensitivities to β -galactosidase, β -hexosaminidase and endo- β -galactosidase. Representative results from experiments using GlcNAc β 1-*O*-benzyl are described below.

The galactosylated GlcNAc-glycosides belong to a PL series

The desialylated glycoside products were characterized by exoglycosidase and endoglycosidase digestions as summarized in Scheme 1.

The smallest of the desialylated glycoside products, eluting at 2.1 Gu, co-chromatographed with authentic Gal β 1-4GlcNAc β 1-*O*-benzyl. Digestion of the product with *Diplococcus pneumoniae* β -galactosidase liberated the [^3H]Gal label as free [^3H]Gal (Figure

Figure 3 Microsequencing of the 5.6 Gu product of the GlcNAc β 1-*O*-benzyl glycoside

The [^3H]Gal-labelled 5.6 Gu product purified by Bio-Gel P4 chromatography (Figure 1b) was analysed as follows: (a) Bio-Gel P4 analysis of the 5.6 Gu product after digestion with *D. pneumoniae* β -galactosidase; (b) Bio-Gel P4 analysis of the 5.6 Gu product after digestion with *D. pneumoniae* β -galactosidase followed by jack bean β -hexosaminidase; (c) Bio-Gel P4 analysis of the 2.1 Gu material from (b) after digestion with *D. pneumoniae* β -galactosidase.

2). The specificity of the *D. pneumoniae* β -galactosidase [8] suggests that the smallest desialylated product is [^3H]Gal β 1-4GlcNAc β 1-*O*-benzyl.

The next largest desialylated glycoside product, eluting at 5.6 Gu, was digested with *D. pneumoniae* β -galactosidase to give two labelled products of equal radioactivity eluting at 1.0 Gu and 4.4 Gu respectively (Figure 3a). Digestion of this mixture with jack bean β -hexosaminidase resulted in the conversion of the 4.4 Gu product into a 2.1 Gu labelled product (Figure 3b). Finally, digestion of the 2.1 Gu material with *D. pneumoniae* β -galactosidase produced free [^3H]Gal eluting at 1.0 Gu (Figure 3c). Taken together, these data define the 5.6 Gu material as [^3H]Gal β 1-4GlcNAc β 1-? [^3H]Gal β 1-4GlcNAc β 1-*O*-benzyl, where the GlcNAc-Gal linkage is most likely β 1-3.

The remaining desialylated glycoside products, ranging in size from 9.1 Gu to 19.0 Gu, were digested individually with *Bacteroides fragilis* endo- β -galactosidase to yield two radiolabelled products (Figure 4). Analysis of the products from the 9.1 Gu desialylated glycoside by Bio-Gel P4 showed the presence of two labelled oligosaccharides at 4.0 and 3.0 Gu (Figure 5a). Each of these oligosaccharides was sequenced by sequential exoglycosidase digestions (Figures 5b–5d), as summarized in Scheme 1, and shown to correspond to [^3H]Gal β 1-4GlcNAc β 1-3[^3H]Gal and GlcNAc β 1-3[^3H]Gal, respectively. The ratio of the disac-

Figure 4 HPTLC analysis of the endo- β -galactosidase digests of the 9.1 Gu to 19.0 Gu products of the GlcNAc β 1-*O*-benzyl glycoside

The individual [^3H]Gal-labelled products, purified by Bio-Gel P4 chromatography (Figure 1b), were digested with *B. fragilis* endo- β -galactosidase and analysed by HPTLC. Lanes 1 to 4 represent the digestion products of the 9.1 Gu, 13.0 Gu, 15.6 Gu and 19.0 Gu species respectively. The positions of radiolabelled dextran oligomer standards (Dex) are shown.

charide (upper band) to the trisaccharide (lower band) was found to increase with the size of the desialylated glycoside substrate as expected (Figure 4).

Taken together, these data suggest that the desialylated products belong to the linear PL series: ([^3H]Gal β 1-4GlcNAc β 1-3) $_n$ -[^3H]Gal β 1-4GlcNAc β 1-*O*-benzyl, where $n = 0$ to 5.

The effects of GlcNAc-glycosides on glycolipid synthesis in CHO cells

The radiolabelled glycolipids extracted from the cell pellets of the experiment described in Table 1 were analysed by HPTLC (Figure 6). The total radioactivity recovered in each glycolipid fraction, and the relative proportions of each of labelled glycolipid species, are shown in Table 2. The GlcNAc α -glycoside had almost no effect on glycolipid synthesis (Table 2 and compare Figure 6, lanes 1 and 3). However, all of the GlcNAc β -glycosides caused a reduction in total glycolipid synthesis and an increase in the ratio of Glc β 1-*O*-ceramide (Glc-Cer) to AcNeu α 2-3Gal β 1-4Glc β 1-*O*-ceramide (G_{M3}) (Table 2 and compare Figure 6, lane 1 with lanes 2, 4 and 5). The accumulation of Glc-Cer suggests that the GlcNAc β -glycosides can partially inhibit and/or compete for the galactosyltransferase responsible for the formation of the Gal β 1-4Glc β 1-*O*-ceramide (Lac-Cer) intermediate.

In addition to Glc-Cer, Lac-Cer and G_{M3} , some other minor glycolipid species were observed (running above Lac-Cer and between Lac-Cer and G_{M3}). The synthesis of these unidentified glycolipids was not affected by any of the glycosides, suggesting that they do not belong to the lacto-series of glycolipids. The apparent increase in labelling of these species in lanes 2, 4 and 5 is an illusion due to the loading of equal numbers of counts to the HPTLC plate in this experiment.

DISCUSSION

The ability of hydrophobic glycosides to cross membranes allows them to be used as probes of cellular glycosylation processes and/or as inhibitors of glycoconjugate biosynthesis [1–3]. In this

Figure 5 Microsequencing of the endo- β -galactosidase digestion products

(a) The endo- β -galactosidase digest of the [^3H]Gal-labelled 9.1 Gu material was separated into trisaccharide (4.0 Gu) and disaccharide (3.0 Gu) products by Bio-Gel P4 chromatography. These structures were subjected to sequential exoglycosidase digestions and reanalysis by Bio-Gel P4 chromatography as follows: (b) *D. pneumoniae* β -galactosidase digest of the 4.0 Gu trisaccharide from (a); (c) Jack bean β -hexosaminidase digest of the 3.0 Gu product from (b); (d) Jack bean β -hexosaminidase digest of the 3.0 Gu disaccharide from (a).

study, we demonstrate that both the α - and β -hydrophobic glycosides of GlcNAc enter the secretory pathway of living CHO cells, where they prime the formation of lactosamine and linear PL chains that are sialylated and secreted into the culture medium. The efficiency of the GlcNAc-glycosides to incorporate Gal into lactosamine and PL structures is dependent on the nature of the aglycone (benzyl > phenyl > PNP) and, in the case of the benzyl glycosides, the α -anomer was found to be more efficient than the β -anomer. The nature of the aglycone also appeared to affect the ratio of the primary product (i.e. Gal β 1-4GlcNAc1-*O*-R) to the PL-containing products [i.e. (Gal β 1-4GlcNAc) $_n$ β 1-3Gal β 1-4GlcNAc1-*O*-R] (see Figure 1). Thus, although the β -phenyl and β -PNP glycosides were relatively poor acceptors of total Gal, their primary products appeared to be elongated by lactosamine units quite efficiently.

The synthesis of PL structures on the GlcNAc-glycoside primers was unexpected because CHO cells normally express only low levels of PL structures. The O-linked oligosaccharides of CHO cells are almost exclusively short sialylated Gal β 1-3GalNAc structures [9,10] and the largest major glycolipid species

Figure 6 HPTLC glycolipid analysis of CHO-K1 cells labelled with [3 H]Gal in the presence of GlcNAc-glycosides

CHO cells were labelled with [3 H]Gal following pre-incubation with and without 2 mM GlcNAc-glycosides. Radiolabelled glycolipids were extracted and equal numbers of counts were analysed by HPTLC. The pre-incubations were as follows: lane 1, no glycoside; lane 2, GlcNAc β 1-*O*-benzyl; lane 3, GlcNAc α 1-*O*-benzyl; lane 4, GlcNAc β 1-*O*-phenyl; lane 5, GlcNAc β 1-*O*-PNP. The positions of authentic glycolipid standards are indicated on the left.

is G_{M3} [11], see also Figure 6. Thus, most of the PL structures of these cells are presumably present on N-linked glycoprotein oligosaccharides, such as those described by Li et al. [12]. These authors suggested that the PL-containing N-linked oligosaccharides represent only 1.3% of the total pool of N-linked oligosaccharides in CHO cells. Most of these PL structures are associated with the lysosome-associated membrane proteins (LAMPs) [13]. Some linear PL structures have been detected at the surface of CHO cells, using anti-i antibody staining [14], but this could be due to the small quantities of LAMPs expressed at the cell surface [15,16]. Thus, the GlcNAc-glycosides may be priming the PL pathway normally utilized by LAMP N-linked oligosaccharide biosynthesis in CHO cells.

The amount of Gal incorporated into the GlcNAc α 1-*O*-benzyl glycoside products was estimated to be at least 750 000 residues/cell per h, of which about one-third was found in PL structures. These figures can be compared with our estimates for the normal incorporation of Gal into total cellular glycoproteins (approximately 3 000 000 residues/cell per h) and into PL-containing glycopeptides that bind to *Datura* lectin (approximately 50 000 residues/cell per h). From these figures it would

appear that exogenously added GlcNAc α 1-*O*-benzyl can prime the synthesis of more PL structures than the endogenous cellular LAMP glycoproteins, suggesting that CHO cells have an excess capacity for PL-biosynthesis. A similar conclusion might be reached from the observation that recombinant erythropoietin expressed in CHO cells contains a greater proportion of PL-containing N-linked oligosaccharides (34–53% of the oligosaccharides) than does natural erythropoietin (7.5% of the oligosaccharides), reviewed in [17].

PL-chain initiation on N-linked oligosaccharides is largely dependent on the action of GlcNAc transferase V, which leads to the generation of the so-called 6-arm of the 2,6-branched mannose of certain tri- and tetra-antennary complex oligosaccharides ([18,19] and references therein). The GlcNAc-glycosides might prime this pathway by acting as mimetics of the product of GlcNAc transferase V. Alternatively, since hydrophobic GlcNAc-glycosides are known to be good substrates for UDP-Gal:GlcNAc β 1-4 Gal transferase *in vitro* [5,20], it is possible that Gal β 1-4GlcNAc1-*O*-R generated *in situ* is sufficient to act as an acceptor for the UDP-GlcNAc:Gal β 1-4GlcNAc β 1-3 GlcNAc transferase (iGlcNAc transferase) of the PL pathway [18]. In either case, it is surprising that GlcNAc α 1-*O*-benzyl is a better acceptor for the formation of Gal β 1-4GlcNAc1-*O*-benzyl than GlcNAc β 1-*O*-benzyl, since the soluble UDP-Gal:GlcNAc β 1-4 Gal transferase isolated from bovine milk shows a marked preference for β GlcNAc glycosides *in vitro* [21]. The reasons for this apparent anomaly are unclear. The fact that all of the structures isolated terminate in NANA α 2-3Gal or in Gal β 1-4GlcNAc, and not in GlcNAc β 1-3Gal, suggests that the action of the iGlcNAc transferase is the rate-limiting step for each lactosamine repeat addition.

The high levels of PL expression in fetal and neonatal cells and the up-regulation of PL expression in cancer cells and during cell differentiation (reviewed in [22,23]) indicate that PL synthesis is carefully regulated and associated with cell phenotype. Based on the results of this study, it is possible that GlcNAc α 1-*O*-benzyl and GlcNAc β 1-*O*-benzyl could find applications in assaying the lactosamine and PL synthetic capacity of living cells. In addition, they could be used as a marker of bulk flow from the *trans*-Golgi to the plasma membrane (by analogy with octyl-Asn-Tyr-Thr-amide, which is used as a marker of bulk flow from the endoplasmic reticulum to the plasma membrane [24]).

The effects of the GlcNAc-glycosides on glycolipid biosynthesis were also investigated. The α -glycoside had little effect (quantitatively and qualitatively) on glycolipid biosynthesis. In contrast, all of the β -glycosides showed a significant inhibition in total glycolipid biosynthesis, an accumulation of Glc-Cer and a reduction in the formation of G_{M3} . These data are consistent with inhibition of, and/or competition for, the UDP-Gal:Glc-Cer β 1-

Table 2 The effects of GlcNAc-glycosides on glycolipid biosynthesis in CHO cells

Glycoside	Radioactivity incorporated* (c.p.m.)	Inhibition (%)	Glycolipid species content (%)			
			Glc-Cer	Lac-Cer	G_{M3}	Non lacto-series†
No glycoside control	129 000	0	28	7	56	9
GlcNAc α 1- <i>O</i> -benzyl	111 000	14	30	7	57	7
GlcNAc β 1- <i>O</i> -benzyl	55 000	57	47	6	31	16
GlcNAc β 1- <i>O</i> -phenyl	88 000	32	47	5	36	12
GlcNAc β 1- <i>O</i> -PNP	68 000	47	49	5	33	16

* Radioactivity incorporated into the glycolipid fraction from 6×10^5 cells.

† The non-lacto-series glycolipids are those found migrating above Lac-Cer and between Lac-Cer and G_{M3} in Figure 6.

4Gal transferase of the lacto-series glycolipid biosynthetic pathway. Presumably the β -glycosides are acting as analogues of the Glc β 1-*O*-ceramide acceptor. In contrast to the structural requirements for priming PL synthesis, the inhibition of glycolipid synthesis appears to be relatively independent of the nature of the aglycone but highly specific for the β -anomeric linkage. Thus, GlcNAc α 1-*O*-benzyl could be used to assay PL synthesis without concomitantly affecting glycolipid synthesis.

This work was supported by an SERC/DTI biotransformations LINK programme involving Celltech Ltd., Glaxo Group Research, The Wellcome Foundation and Zeneca Pharmaceuticals. We thank Pascal Schneider and Lucia Güther for helpful comments. M.A.J.F. is a Howard Hughes International Research Scholar.

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