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Postnatal changes in dolichol-pathway enzyme activities in rat liver

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The activity of hepatic protein *N*-glycosylation was compared in rats of different ages by incubating UDP-[^{14}C]glucose with liver microsomes. Dolichyl-phosphate [^{14}C]glucose, [^{14}C]glucosyl-oligosaccharide–lipid and [^{14}C]glycoproteins formed were increased after birth to maximal levels at 2 weeks; thereafter dolichyl-phosphate [^{14}C]glucose remained constant, while [^{14}C]glucosyl-oligosaccharide–lipid and [^{14}C]glycoproteins were decreased to constant levels at 4 weeks. The postnatal change in the formation of [^{14}C]glycoproteins was similar to the change in the hexosamine content of *N*-glycans in liver microsomes and plasma, suggesting that the *N*-glycosylation of proteins in rat liver increases after birth to a maximum at 2 weeks, and thereafter decreases to a constant level at 4 weeks. The possibility of a regulatory role for dolichyl phosphate in glycoprotein synthesis in rat liver during postnatal development was eliminated by demonstrating the inefficiency of exogenous dolichyl phosphate on the postnatal changes in [^{14}C]glycoprotein formation. The transfer of [^{14}C]glucose from UDP-[^{14}C]glucose to denatured α-lactalbumin in liver microsomes increased to a maximum at 2 weeks and then decreased to a constant level, as with transfer to endogenous proteins (i.e. the formation of [^{14}C]glycoproteins). On the other hand, the transfer of oligosaccharide from exogenous [^{14}C]glucosyl-oligosaccharide–lipid to denatured α-lactalbumin reached a maximum at 2 weeks and then remained constant. These results strongly suggest that oligosaccharide–lipid available for *N*-glycosylation is limiting in rat liver after 2 weeks *post partum*. The activities of dolichyl-phosphate glucose, dolichyl-phosphate mannose and dolichyl-pyrophosphate *N*-acetylglucosamine synthases increased until 2 weeks *post partum*. Thereafter, the activity of dolichyl-pyrophosphate *N*-acetylglucosamine synthase decreased to a constant level at 4 weeks, while the activities of dolichyl-phosphate glucose and dolichyl-phosphate mannose synthases remained constant. These results suggest that *N*-glycosylation of proteins in rat liver increases until 2 weeks *post partum*, and that this depends on the activities of dolichol-pathway enzymes as a whole rather than on the activity of specific enzymes. *N*-Glycosylation then decreases to a constant level at 4 weeks due to decreases in the activities of enzymes responsible for oligosaccharide assembly on lipids, including dolichyl-pyrophosphate *N*-acetylglucosamine synthase.

INTRODUCTION

N-Glycosylation is thought to be responsible for many biologically significant features of glycoproteins [1,2]. Changes in the *N*-linked oligosaccharides of glycoproteins may alter the expression of various cellular functions during development, growth and differentiation [3–5]. Therefore, it seems interesting to study changes in the composition and synthesis of *N*-linked oligosaccharides of glycoproteins in growing liver. We have previously reported that glycoprotein synthesis was decreased in regenerating rat liver [6,7]. This decrease was caused by the decreased activity of oligosaccharyltransferase [8], and the glycoproteins contained a higher proportion of complex-type oligosaccharides during regeneration [9,10]. These changes may be related to the controlled growth of liver cells. Thus, we studied glycoprotein synthesis in the rat liver during postnatal development and found that the proportion of complex-type oligosaccharides in the glycoproteins increased after birth [11], as in regenerating liver. This paper presents the postnatal changes observed in the activity of *N*-glycosylation in liver microsomes. The evidence suggests that *N*-glycosylation of proteins in rat liver is regulated by the assembly of oligosaccharide on lipids during postnatal development.

MATERIALS AND METHODS

Chemicals

Dolichyl phosphate (Dol-*P*) and bovine α-lactalbumin were obtained from Sigma, St. Louis, MO, U.S.A. UDP-[U-^{14}C]glucose (295 mCi/mmol), GDP-[U-^{14}C]-mannose (203 mCi/mmol) and UDP-*N*-acetyl-[U-^{14}C]glucosamine (229 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of analytical grade and obtained from commercial sources.

Abbreviations used: Dol, dolichol; Dol-*P*, dolichyl phosphate; Dol-*PP*, dolichyl pyrophosphate; Dol-*PP*-GlcNAc synthase, UDP-*N*-acetylglucosamine–dolichyl-phosphate *N*-acetylglucosaminephosphotransferase (EC 2.7.8.15); Dol-*P*-Man synthase, dolichyl-phosphate mannosyltransferase (EC 2.4.1.83); Dol-*P*-Glc synthase, dolichyl-phosphate β-glucosyltransferase (EC 2.4.1.117); oligosaccharyltransferase, dolichyl diphospho-oligosaccharide–protein glycosyltransferase (EC 2.4.1.119); CM fraction, fraction extracted with chloroform/methanol (2:1, v/v).

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Animals

Pregnant Wistar rats (Nihon Rat Co., Urawa, Japan) were fed *ad libitum* with laboratory chow in a room at a constant temperature (23.5 °C) with 12 h each of light (6:30–18:30) and darkness. Neonatal rats within 15 h *post partum*, rats aged 1 and 2 weeks, of either sex, taken from the mother, and male rats aged 3, 4 and 5 weeks from different litters (Wistar strain, Nihon Rat Co.) were killed by decapitation between 9:00 and 10:30.

Preparation of liver microsomes and plasma from rats

Liver microsomes [7] and plasma [12] were prepared from 3–12 rats as described previously. Protein was determined by the method of Lowry *et al.* [13] with bovine serum albumin as a standard.

Assay for the transfer of glucose from UDP-glucose to lipid intermediates and glycoproteins

The incorporation of radioactivity from UDP-[^{14}C]glucose into lipid intermediates and glycoproteins was measured in the presence of 0.1% Triton X-100, with or without 30 μg of Dol-*P* as previously reported [8]. The incubation time was 5 min in order to obtain maximal incorporation into glycoproteins.

Determination of hexosamine in *N*-glycans of liver microsomes and plasma

Portions of liver microsomes and plasma were added to 10 vol. of 10% (w/v) trichloroacetic acid and boiled for 5 min to hydrolyse Dol-bound saccharides. The hydrolysate was chilled on ice and centrifuged at 1000 *g* for 10 min at 4 °C. The pellet was washed twice with 4 ml of 10% trichloroacetic acid, and successively with 4 ml of ethanol, ethanol/diethyl ether (1:1, v/v) and diethyl ether. The resulting pellet was suspended in 4 ml of 0.1 M-NaOH and was incubated at 37 °C for 48 h to release *O*-linked oligosaccharides [14]. This solution was precipated again with 10% trichloroacetic acid and washed as above. The hexosamine in the acid-stable, alkali-stable and acid-precipitable fractions of liver microsomes and plasma was released by acid hydrolysis and was measured as reported previously [12].

Assays for the transfer of glucose and oligosacharide to denatured α-lactalbumin

Denatured α-lactalbumin prepared as previously described [8] was used as an exogenous acceptor protein. [^{14}C]Glucose transfer from UDP-[^{14}C]glucose was measured using the same incubation mixture as for glucose transfer to lipid intermediates and endogenous proteins in the presence of Dol-*P*, except that 1.8 mg of denatured α-lactalbumin was added. After incubation at 37 °C for 5 min, the reaction was stopped by adding 1 ml of 5 mM-EDTA (sodium salt, pH 7). The mixture was cooled in an ice bath and centrifuged at 15000 *g* for 20 min at 4 °C. Trichloroacetic acid-precipitable fractions of the supernatant were obtained and the radioactivity was determined as previously [8].

[^{14}C]Glucosyl-oligosaccharide–lipid was prepared using liver microsomes of 5-week-old rats, and oligosaccharide transfer from [^{14}C]glucosyl-oligosaccharide–lipid to denatured α-lactalbumin was measured as previously [8].

Assays for Dol-*PP*-GlcNAc, Dol-*P*-Man and Dol-*P*-Glc synthases

Dol-*P* (1 μg) in chloroform/methanol (2:1, v/v) (referred to as CM) was added to tubes with Triton X-100 (final concentration 0.1%) and dried under N_2. These tubes were used for the following assays.

The assay mixture for Dol-*PP*-GlcNAc synthase contained liver microsomes (0.1 mg of protein), 50 mM-Tris/maleate buffer, pH 7.7, 10 mM-$MgCl_2$, 0.3 mM-ATP, 5 mM-dimercaptopropanol, 5 mM-AMP and 22 μM-UDP-*N*-acetyl[^{14}C]glucosamine (68 mCi/mmol) in a total volume of 50 μl [15]. The assay mixture for Dol-*P*-Man synthase contained microsomes (0.1 mg of protein), 50 mM-Tris/maleate buffer, pH 7.1, 10 mM-$MgCl_2$, 3 mM-AMP, 3 mM-NaF and 7 μM-GDP-[^{14}C]mannose (150 mCi/mmol) in a total volume of 50 μl [8]. For Dol-*P*-Glc synthase, the assay mixture contained microsomes (0.1 mg of protein), 50 mM-Tris/maleate buffer, pH 6.7, 5 mM-$MnCl_2$ and 48 μM-UDP-[^{14}C]glucose (31 mCi/mmol) in a total volume of 50 μl [8].

All assay incubations were carried out at 37 °C for 1 min. The reaction was stopped by adding 1 ml of CM. CM extract, chloroform/methanol/water (10:10:3, by vol.) extract and residue were prepared by the method of Waechter *et al.* [16]. The radioactivity in the CM extract was measured as reported previously [8]. No radioactivity was detectable in either the chloroform/methanol/water extract (oligosaccharide–lipid) or the residue (glycoproteins). In the Dol-*PP*-GlcNAc synthase assay, the CM extract was hydrolysed by mild acid [17], and *N*-acetylglucosamine and *NN'*-diacetylchitobiose were separated by paper chromatography. The radioactivity in *NN'*-diacetylchitobiose was negligibly low (less than 5% of that of *N*-acetylglucosamine).

RESULTS AND DISCUSSION

A structural requirement for the transfer of oligosaccharide from oligosaccharide–lipid to proteins is the presence of three glucose residues at the non-reducing end of the oligosaccharide moiety of oligosaccharide–lipid [18]. In order to evaluate the *N*-glycosylating activity, we assayed the transfer of [^{14}C]glucose from UDP-[^{14}C]glucose to lipid intermediates and to glycoproteins using liver microsomes from rats aged 0–5 weeks. The transfer of [^{14}C]glucose to Dol-*P*-glucose, oligosaccharide–lipid and glycoproteins was increased after birth with maximum rates at 2 weeks *post partum* (Fig. 1); thereafter the transfer to Dol-*P*-glucose remained constant (Fig. 1*a*), while that to oligosaccharide–lipid (Fig. 1*b*) and glycoproteins (Fig. 1*c*) decreased, and levelled off at 4 weeks. These assays were done in the presence of Triton X-100; however, similar age-dependent profiles were also observed in the absence of Triton X-100 (results not shown). The exogenously added Dol-*P* increased the incorporation of radioactivity without affecting the age-dependent profiles in the transfer of [^{14}C]glucose to lipid intermediates and glycoproteins (Figs. 1*a*, 1*b* and 1*c*). Therefore, in rat liver during postnatal development, it is unlikely that microsomal Dol-*P* plays a regulatory role in *N*-glycosylation as reported in other systems [19–21]. Alternatively, the activities of α-glucosidases may influence the transfer of glucose to oligosaccharide–lipid and to glycoproteins. As reported previously, the activities of two α-glucosidases were

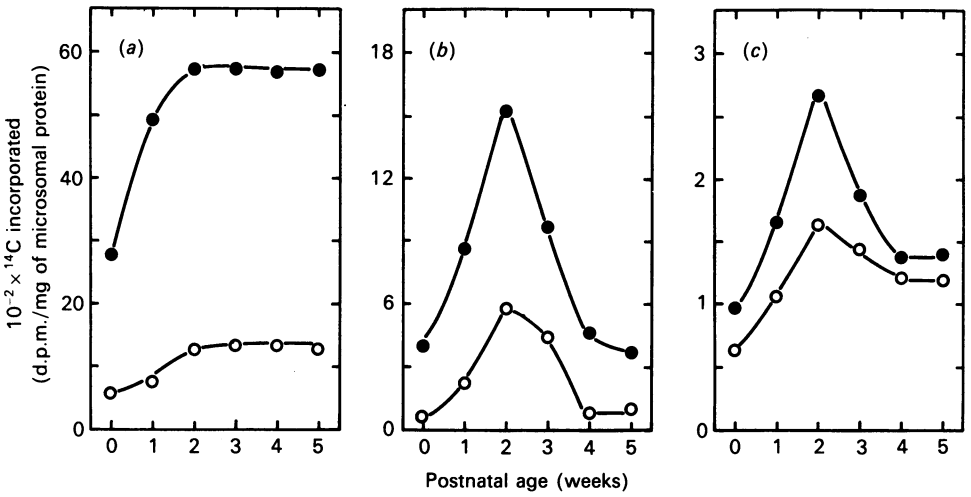

Fig. 1. Transfer of [^{14}C]glucose from UDP-[^{14}C]glucose to Dol-*P*-glucose (*a*), oligosaccharide–lipid (*b*) and glycoproteins (*c*)

The assays were done in the presence (●) or the absence (○) of 30 μg of Dol-*P*. Each point represents the mean value from three separate experiments; the S.E.M. values did not exceed 5%.

increased after birth, with their maximal values at 2 weeks, and then decreased [11]. In spite of the highest activities of two α-glucosidases, the transfer of glucose to oligosaccharide–lipid and to glycoproteins showed maximal values at 2 weeks *post partum*. This indicates that the age-dependent changes shown in Figs. 1(*b*) and 1(*c*) did not result from the altered α-glucosidase activities. All of the oligosaccharide moieties in [^{14}C]glucosyl-oligosaccharide–lipids prepared using liver microsomes from rats of different ages showed similar chromatographic behaviour on paper to the moiety from 5-week-old rats, which is considered to contain three glucose residues [8].

The content of hexosamine in *N*-glycans from liver microsomes (Fig. 2*a*) and plasma (Fig. 2*b*) increased until 2 weeks *post partum* and then decreased to constant levels at 4 weeks. These results, together with those described above, strongly suggest that *N*-glycosylation of proteins in rat liver increases after birth with a maximum at 2 weeks, and then decreases to a constant level at 4 weeks.

We measured the transfer of sugars to denatured α-lactalbumin, an exogenous sugar-accepting protein, from UDP-[^{14}C]glucose and [^{14}C]glucosyl-oligosaccharide–lipid. If the age-dependent change in [^{14}C]glycoprotein formation (Fig. 1*c*) is caused by the change in the acceptor capacity of endogenous proteins, the postnatal difference should be abolished by the addition of a sufficient amount of denatured α-lactalbumin. However, this was not the case. As shown in Fig. 3(*a*), the transfer of glucose from UDP-[^{14}C]glucose to denatured α-lactalbumin changed in a similar way as that to endogenous proteins (Fig. 1*c*). On the other hand, the transfer of oligosaccharide from exogenous [^{14}C]glucose-labelled oligosaccharide–lipid to denatured α-lactalbumin; that is, the activity of oligosaccharyltransferase, increased after birth and levelled off at 2 weeks *post partum* (Fig. 3*b*). After the assay incubation, the degradation of

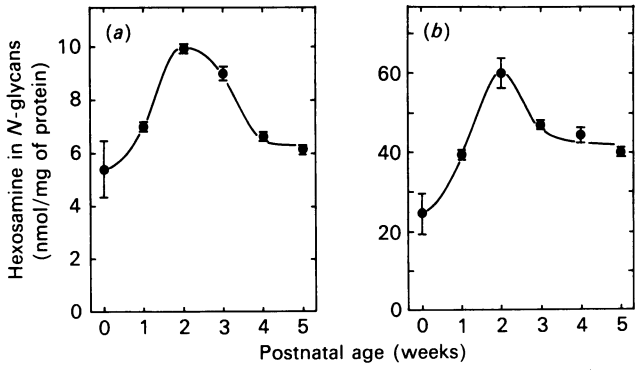

Fig. 2. Hexosamine contents in *N*-linked oligosaccharides of glycoproteins in liver microsomes (*a*) and plasma (*b*)

The amounts of hexosamine per mg of protein in liver microsomes and in plasma are indicated. Each point represents the mean value ± S.E.M. from three separate experiments.

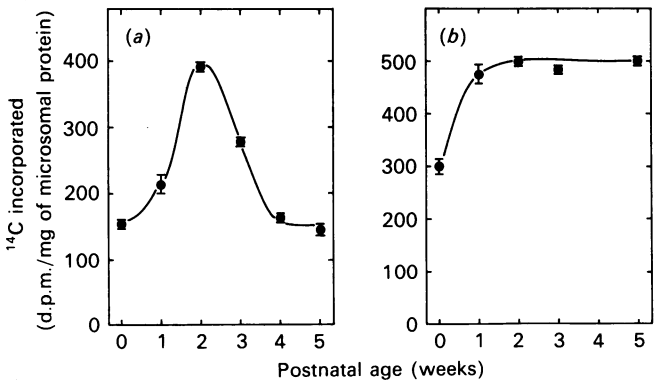

Fig. 3. Incorporation of radioactivity from UDP-[^{14}C]glucose (*a*) and [^{14}C]glucosyl-oligosaccharide-lipid (*b*) into denatured α-lactalbumin

The assay conditions were described in the Materials and methods section. Each point represents the mean value ± S.E.M. from three separate experiments.

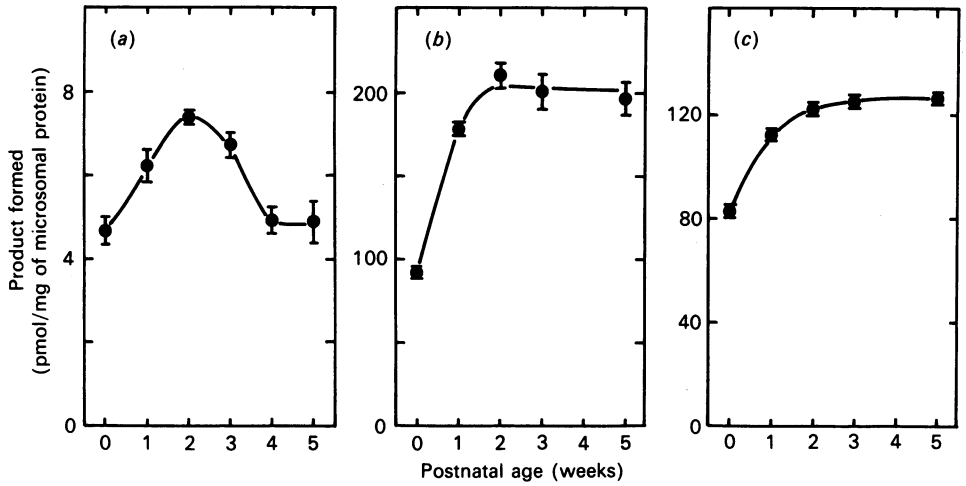

Fig. 4. Activities of Dol-*PP*-GlcNAc synthase (*a*), Dol-*P*-Man synthase (*b*) and Dol-*P*-Glc synthase (*c*)

The assay conditions were described in the Materials and methods section. The radioactivities in CM extracts were determined. All activities are given as pmol of the sugars transferred per mg of microsomal protein for comparison. Each point represents the mean value ± S.E.M. from three separate experiments.

[^{14}C]glucosyl-oligosaccharide–lipid was negligibly low regardless of the age of the rats. It should be noted that the transfer did not decrease after 2 weeks *post partum* in the presence of sufficient amounts of glucosyl-oligosaccharide–lipid. These results suggest that the decrease of *N*-glycosylation after 2 weeks *post partum* is caused by the decreased availability of glucosyl-oligosaccharide–lipid. We have previously reported that oligosaccharyltransferase regulates *N*-glycosylation in the early period of rat liver regeneration [8]. In rat liver, during postnatal development, oligosaccharyltransferase may contribute only to the increase in *N*-glycosylating activity during the first 2 weeks *post partum*. After 2 weeks *post partum*, *N*-glycosylating activity appears to depend on the assembly of oligosaccharide on lipids.

The assembly of oligosaccharide on lipids is essentially a process controlled by the level of microsomal Dol-*P* and enzyme activities involved in the process. As shown in Fig. 1(*b*), addition of Dol-*P* had no effect on the postnatal changes in the formation of [^{14}C]glucosyl-oligosaccharide–lipid. Thus, the activities of the enzymes involved in the assembly of oligosaccharide on lipids were determined. Dol-*P* monosaccharide derivatives, i.e. Dol-*PP*-*N*-acetylglucosamine, Dol-*P*-mannose and Dol-*P*-glucose, act as sugar donors for the assembly of oligosaccharide on lipids. The activities of synthases which catalyse the formation of these Dol-*P* monosaccharide derivatives were measured by a short-term incubation assay. As shown in Fig. 4, the activities of all three synthases were increased after birth until 2 weeks *post partum*; thereafter the activity of Dol-*PP*-GlcNAc synthase was decreased, while those of Dol-*P*-Man synthase and Dol-*P*-Glc synthase remained constant. The dependency of these three synthases on sugar nucleotide concentration was measured using the microsomes from the livers of neonatal, 2- and 5-week-old rats. For each synthase, the apparent K_m values for the substrate sugar nucleotides were similar regardless of age (Table 1). Therefore, the changes in the activities of the enzymes could not be caused by the age-dependent changes in their properties.

Table 1. Apparent K_m values for the substrate sugar nucleotides of Dol-*PP*-GlcNAc, Dol-*P*-Man and Dol-*P*-Glc synthases

The activity of each synthase was measured using liver microsomes of neonatal, 2-week- and 5-week-old rats as described in the Materials and methods section, except that the concentrations of substrate sugar nucleotides tested were varied. K_m values were calculated from Lineweaver–Burk plots. Each K_m value is the average of three separate experiments, ± S.E.M.

Age of rats (weeks)	K_m (μM)		
	Dol-*PP*-GlcNAc synthase (for UDP-*N*-acetyl-glucosamine)	Dol-*P*-Man synthase (for GDP-mannose)	Dol-*P*-Glc synthase (for UDP-glucose)
Neonate	5.0±0.2	1.0±0.1	6.5±0.2
2	8.0±0.5	1.5±0.1	8.3±0.3
5	6.3±0.2	1.8±0.1	9.9±0.3

The increased activity of these three synthases during the first 2 weeks *post partum* probably contributes to the increase in the assembly of oligosaccharide on lipids. Neither Dol-*P*-Man synthase nor Dol-*P*-Glc synthase could be responsible for the decrease in oligosaccharide assembly on lipids after 2 weeks *post partum*. Dol-*PP*-GlcNAc synthase has been considered to be a key enzyme in the assembly of oligosaccharide on lipids in other developing tissues, because this enzyme catalyses the initial reaction of oligosaccharide–lipid synthesis, and exhibits low activity as compared with Dol-*P*-Man and Dol-*P*-Glc synthases [22,23]. We also found that Dol-*PP*-GlcNAc synthase showed lower activity than Dol-*P*-Man and Dol-*P*-Glc synthases (Fig. 4). Moreover, it should be noted that the activity of Dol-*PP*-GlcNAc synthase decreased after 2 weeks *post partum* (Fig. 4*a*), as the formation of [^{14}C]glucosyl-oligosaccharide–lipid de-

creased (Fig. 1*b*). It is most likely that the decreased activities of the enzymes involved in oligosaccharide–lipid synthesis, including Dol-*PP*-GlcNAc synthase, should be responsible for the decrease in *N*-glycosylating activity after 2 weeks *post partum*. On the other hand, the increase of *N*-glycosylating activity until 2 weeks *post partum* depends not on the increased activity of a specific dolichol-pathway enzyme, but rather on the collective increase in the activities of dolichol-pathway enzymes.

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