Participation of an endogenous inhibitor of fucosyltransferase activities in the developmental regulation of intestinal fucosylation processes

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During the rat weaning period (about day 19 after birth) the intestinal maturation is accompanied by a drastic increase in the fucose content of mucosal glycoconjugates, concomitant with an increase in fucosyltransferase activities. The regulation of this fucosylation process appears to be a rather complex phenomenon, which involves several systems controlling fucosyltransferase activity or substrate availability. An endogenous protein inhibitor of the fucosyltransferase activities displays an opposite developmental pattern to that of fucosyltransferase activities, since its activity is high before weaning and is decreased 5-fold after weaning. Similarly, the GDP-fucose pyrophosphatase activity markedly decreases at weaning. The transformation of GDP-mannose into GDP-fucose increases early, at day 18, preceding the increase in fucosyltransferase activities. Before weaning, and especially at days 14 and 18, high levels of GDP-4-dehydro-6-deoxymannose, the product of the GDP-mannose 4,6-dehydratase activity, are produced during the transformation of GDP-mannose into GDP-fucose, even in excess of reduced coenzyme. This fact indicates that the second step of the transformation (epimerase–reductase reaction) could be a limiting factor for GDP-fucose availability before weaning, but not after weaning. The inverse relationship between the mucosal fucose content (or the fucosyltransferase activity) and the endogenous protein inhibitor during normal postnatal development supports the hypothesis of a physiological role for this inhibitor.

INTRODUCTION

The rat small intestine undergoes a number of profound morphological and enzymic changes between weeks 2 and 3 after birth, corresponding to the weaning period. These developmental modifications result in a functionally mature intestine containing the digestive enzymes necessary to cope with the carbohydrate-rich diet of adulthood, such as sucrase, maltase or isomaltase [1], as well as other enzymes, e.g. aminopeptidase and alkaline phosphatase [2,3]. All these enzymes are glycoproteins located in the brush-border membranes. Their developmental patterns have been often studied in terms of enzymic activity; however, little is known about the synthesis of their glycan chains. Different means of regulation of the metabolism of these glycoproteins are possible, among which post-translational modifications of synthesized polypeptides, including glycosylation, are of great interest. It has been demonstrated that in microvillus membranes the sialic acid content of alkaline phosphatase and γ -glutamyl-transferase is different in fetal and adult forms [4–6]. Changes have also been observed between newborn and adult animals in glycoprotein fucosylation or sialylation of intestinal mucus glycoproteins [7] and microvillus membrane glycoproteins [8–11]. Several studies have shown that the terminal sugars of both *N*-linked and *O*-linked oligosaccharide chains of the lactase-phlorizin hydrolase shift from a predominantly sialylated form during the suckling period to a fucosylated one in adulthood, and that these changes are concomitant with the decline in the intestinal lactase activity at the weaning period [12–14]. Moreover, in duodenal absorptive cells, the biosynthesis and the intracellular transport of glycoproteins were demonstrated to be age-dependent [15].

The mechanisms of these profound developmental variations of intestinal glycosylation patterns are poorly known. Most of

the studies done until now were directed to the intestinal glycosyltransferase activities as being responsible for the modifications of the intestinal glycosylations. During rat postnatal development, modifications of the activities of the intestinal enzymes responsible for the glycosylation of glycoproteins, such as fucosyl-, sialyl-, galactosyl- and *N*-acetylgalactosaminyl-transferase have been demonstrated, with a particularly important variation stage near the weaning period [9,16,17]. The fucosyl-, galactosyl- and *N*-acetylgalactosaminyl-transferase activities, at a low level during the suckling period, were enhanced near weaning and rather rapidly reached a plateau until adulthood, whereas the sialyltransferase activity decreased from birth to weaning. The fucose and galactose contents of microvillus or microsomal membranes, like their sialic acid content, underwent modifications parallel with the corresponding enzymic activities [8,9,17]. Previously, we demonstrated that glycosyltransferase activities in rat intestine were very sensitive to dietary modifications [18,19] and that their developmental variations could be delayed by prolonged nursing or prematurely induced by early weaning [20].

Intestinal glycosylation is a complex pathway in which glycosyltransferase activities are the result of the combination of several factors, such as substrate availability, enzyme quantity and activity and presence of regulatory effectors. We have previously demonstrated the existence of a powerful endogenous protein inhibitor of the glycoprotein: α (1–2)- and glycoprotein: α (1–3)-fucosyltransferase activity in rat small-intestinal mucosa. This inhibitor was characterized as a soluble protein with a molecular mass of 60 kDa, essentially localized in the digestive tract [21]. Modifications of the fucosyltransferase activities during development might be the consequence of changes in this inhibitory activity, leading to a specific regulation mechanism of the fucosylation process. On the other hand, the

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regulation of fucosylation may depend on the availability of GDP-fucose, which is the donor substrate of fucosyltransferase. The GDP-fucose cellular pool is accurately regulated in the intestine by two opposing enzymic systems: the glycosyl-nucleotide pyrophosphatases are responsible for GDP-fucose degradation; GDP-mannose 4,6-dehydratase and GDP-4-dehydro-6-deoxymannose epimerase-reductase are responsible for the synthesis of 90 % of the GDP-fucose cellular pool [22,23]. Obviously, modifications of the activities of these enzymic systems would also modify intestinal fucosylation processes.

The purpose of the present study was to gain insight into the regulation mechanisms of the intestinal fucosylation processes, focusing on the participation of the endogenous protein inhibitor of the fucosyltransferase activity and on the enzymes involved in GDP-fucose metabolism.

MATERIALS AND METHODS

Chemicals

Radioactive nucleotide-sugars were obtained from New England Nuclear (Boston, MA, U.S.A.); non-radioactive GDP-mannose was obtained from Sigma (St. Louis, MO, U.S.A.) and non-radioactive GDP-fucose was from BioCarb (Lund, Sweden). All the other reagents were of the best grade commercially available.

Animals

All the rats were of the Sprague-Dawley strain (IFFA-CREDO, L'Arbresle, France). They were housed under controlled conditions of temperature (21 °C) and lighting (daily cycle of 12 h light/12 h darkness). The diet given to dams in our laboratory was similar to that on which dams were fed at the supplier house (Souriffarat, IFFA-CREDO). Adult male rats, dams and young male pups (weaned at day 19) were fed on this standard non-purified diet, used as compact pieces (Souriffarat, IFFA-CREDO). The number of litters and animals will be detailed for each experiment.

In four independent experiments, suckling male rats (7, 10, 14 and 18 days of age) were treated as four different litters of 11 pups at each age. The 11 small intestines of each litter were pooled for subcellular fractionation. For weaned rats (23 and 28 days of age) each fraction was obtained from six or four pooled intestines respectively and, for older weaned rats (42 days of age), two intestines were used to produce each subcellular fraction.

When litters and dams are maintained on the non-purified diet *ad libitum* in normal cages, suckling pups begin to ingest spontaneously some solid food around 16 days of age, and weaning is complete about 1 week later. Thus, in order to obtain a controlled weaning at day 19, litters were housed at day 15 in special feeder cages with a wire-netting floor to avoid coprophagy, as previously described [20]. In order to avoid completely intake of solid diet by pups, the cages were heightened so that the feeding troughs containing the dams' non-purified diet could not be reached by the pups.

Cell fractionation

The animals were killed by decapitation between 08:00 and 09:00 h. The small intestines were removed, washed with cold 0.9 % NaCl and weighed. For suckling rats, the intestines were opened, cut into small pieces and directly homogenized with a Potter-Elvehjem homogenizer in 10 mM-Tris/HCl/10 mM-KCl/10 mM-MgCl₂/250 mM-sucrose buffer, pH 7.4 (9 ml/g wet

wt. of tissue). The muscular tissue (which was not destroyed by homogenization) was removed by filtration on cheese-cloth. For older rats (22–42 days old), the mucosa was harvested by scraping before homogenization. All the homogenates were centrifuged at 30 000 *g* for 30 min. The supernatants were then centrifuged for 90 min at 200 000 *g* leading to sedimentation of a microsomal fraction and giving cytosol; these fractions were stored at –20 °C until use.

Determination of fucosyltransferase activities

The soluble α -(1–2)-fucosyltransferase activity was determined in rat intestinal cytosol with asialofetuin as exogenous acceptor, prepared as described by Ko & Raghupathy [24]. The assay system included, in a total volume of 240 μ l, 0.6 mg of cytosolic protein, 5 mM-MnCl₂, 0.2 mg of asialofetuin, 0.8 kBq of GDP-[¹⁴C]fucose (sp. radioactivity 8.8 GBq/mmol) at 0.38 μ M final concentration and 10 mM-AMP (used as an inhibitor of the glycosyl-nucleotide pyrophosphatase activity).

Microsomal α -(1–2)-fucosyltransferase activity was determined on the microsomal pellets homogenized in 10 mM-Tris/HCl/10 mM-KCl/10 mM-MgCl₂ buffer, pH 7.4. The enzymic assay contained, in a total volume of 250 μ l, 0.6 mg of microsomal protein, 5 mM-MnCl₂, 0.2 mg of asialofetuin, 0.25 % Triton X-100, 0.8 kBq of GDP-[¹⁴C]fucose (sp. radioactivity 8.8 GBq/mmol) and 10 mM-AMP.

Both enzymic assay mixtures were incubated for 20 min at 23 °C (kinetic assays were linear for 1 h) and stopped by adding 1 ml of 20 % (w/v) trichloroacetic acid. Trichloroacetic acid precipitates were filtered on Whatman GF/B glass-fibre filters, and the radioactivity was counted in a Minaxi scintillation counter (Packard) in the presence of 5 ml of toluene scintillator (Packard).

Determination of the fucosyltransferase endogenous inhibitor activity

Intestinal cytosol was diluted 4-fold with cold distilled water and incubated for 30 min at 4 °C with 5 g of DEAE-cellulose (Whatman, DE 52) equilibrated in 10 mM-Tris/HCl buffer, pH 7.6. After filtration on a glass filter, cellulose that retained the inhibitory activity was recovered and poured into a glass column. The column was rinsed with 50 ml of 200 mM-KCl/10 mM-Mes buffer, pH 5.5. Elution of the inhibitory fraction was performed with 50 ml of 300 mM-KCl/10 mM-Mes buffer, pH 5.5, as described previously [21]. The eluate was dialysed overnight against 10 mM-Mes buffer, pH 5.5. The fucosyltransferase inhibitory activity was assayed against a partially purified α -(1–2)-fucosyltransferase preparation obtained from intestines of adult rats [21]. The incubation mixture contained 40 μ l of partially purified fucosyltransferase, 160 μ l of 10 mM-Mes buffer contained in the dialysis bath (for the controls) or dialysed inhibitory fractions diluted with the same buffer in order to adjust the protein concentrations at the same values. The inhibitory activity was assayed at four different protein concentrations, i.e. 0.5, 1, 2.5 and 5 μ g of protein of the inhibitory fraction per assay. Then 0.2 kBq of GDP-[¹⁴C]fucose (sp. radioactivity 8.8 GBq/mmol) and 0.2 mg of asialofetuin were added. The incubations were carried out for 30 min at 23 °C and stopped with 1 ml of 20 % trichloroacetic acid. Radioactivity was determined in the same way as for fucosyltransferase activities. An inhibitor unit was defined as the amount of protein giving 50 % inhibition in this standardized assay.

Partial purification of the protein inhibitor

DEAE-cellulose eluates of corresponding groups from the four independent experiments were pooled. The protein inhibitor of each pool was partially purified by (NH₄)₂SO₄ fractionation and gel filtration. (NH₄)₂SO₄ was added to the inhibitory fraction

eluted from DEAE-cellulose to give 50 % saturation; the mixture was stirred at 4 °C for 30 min, and centrifuged at 5000 *g* for 20 min. The precipitate was discarded and the supernatant was dialysed against several changes of water, then freeze-dried. The resulting material was dissolved in 5 ml of 10 mM-Mes buffer, pH 5.5, and applied to a Sephacryl HR-100 S (Pharmacia) column (2.5 cm × 100 cm) equilibrated with the same buffer. Elution was performed at a flow rate of 10 cm/h, and 8 ml fractions were collected. The A_{280} of the eluate was monitored with an absorbance-fluorescence monitor (Isco, model UA-5), and the inhibitory activity was assayed as described above in each collected fraction, and then in the pooled active fraction. A gel-filtration calibration kit for low-molecular-mass proteins (Pharmacia) was used for molecular-mass estimations.

Determination of GDP-fucose breakdown by glycosyl-nucleotide pyrophosphatase activity

Degradation of GDP-fucose by glycosyl-nucleotide pyrophosphatase activity was measured in an assay system containing, in a final volume of 200 μ l, 0.03 mg of cytosolic proteins and 0.7 kBq of GDP-[14 C]fucose (sp. radioactivity 8.8 GBq/mmol), added with non-radioactive GDP-fucose to give 7 μ M final concentration. Incubation was performed for 10 min at 23 °C. GDP-fucose was separated from its degradation products by anion-exchange h.p.l.c. as previously described [25].

Determination of GDP-fucose formation from GDP-mannose

The transformation of GDP-mannose to GDP-fucose was measured in a reaction mixture containing, in a final volume of 250 μ l, 0.15 mg of cytosolic protein, 10 mM-2,3-dimercapto-propanol as glycosyl-nucleotide pyrophosphatase inhibitor, 40 μ M- or 80 μ M-NADPH and 0.7 kBq of GDP-[14 C]mannose (sp. radioactivity 8.8 GBq/mmol) added with non-radioactive GDP-mannose to give 10 μ M final concentration. Incubations were performed for 20 min at 30 °C. The reaction products (free sugars, sugar phosphates, GDP-mannose, GDP-fucose and GDP-4-dehydro-6-deoxymannose) were separated by reverse-phase h.p.l.c. [25].

Other chemical determinations

Proteins were determined by the method of Lowry *et al.* [26], with BSA as standard, or by the Amino Black staining method of Schaffner & Weissmann [27] for the inhibitory fraction. The fucose content of the glycoconjugates of the whole mucosa homogenates was determined, after trichloroacetic acid precipitation, hydrolysis and purification by ion-exchange chromatography, by using the cysteine/ H_2SO_4 reaction of Dische [28].

Statistical analysis

Results are expressed as means $\pm$ s.d. of determinations obtained from four different samples of independent subcellular fractions. In one sample, activities were linear with respect to time and protein concentration: coefficients of variation did not exceed 5–10 %. In similar samples prepared from rats in different experiments, variations were obviously higher (up to 60 %), though the developmental pattern was similar for the four independent experiments. Therefore the pooled values of the four experiments were submitted to one-way analysis of variance. When the 'F' test was statistically significant, the Newman-Keuls test was used to determine the significance of the differences between mean values of each group [29].

RESULTS AND DISCUSSION

Previous studies dealing with glycosylation changes in the intestinal mucosa at the weaning period reported a drastic increase in the fucose content of glycoconjugates in subcellular fractions such as brush borders [8–11] or mucins [7] of weaned rats as compared with suckling ones. Until now, this increase has been related exclusively to changes in fucosyltransferase activities. It has been suggested that the regulation of the expression of glycosyltransferase activities was at the level of transcription [30]. Though this mechanism could be actually implicated also in the intestine, it would not be exclusive to the weaning period, as demonstrated below. In the groups of rats used in these experiments, we have verified that the total fucose content of the intestinal mucosa varied from 0.64 ± 0.20 μ mol/g wet wt. of tissue ($n = 16$) before weaning to 2.60 ± 0.40 μ mol/g wet wt. of tissue ($n = 12$) after day 23 and that fucosyltransferase activities were also increased (Fig. 1). The study of the activity of the different systems involved in the fucosylation process during normal intestinal postnatal development provides further insight in the regulation of this phenomenon.

Postnatal changes in the protein inhibitor of fucosyltransferase activity

As seen in Fig. 1, high levels of inhibitory activity were present in the small intestine until the age of 18 days, with no significant changes from day 7 to day 18. After the weaning period, the inhibitory activity significantly decreased by 5-fold to an adult level at day 23 ($P < 0.01$), whereafter no further significant changes were observed. Thus the evolution of the inhibitory activity appears to be inverse of that of fucose content and fucosyltransferase activities.

In order to determine possible postnatal changes in the structure or the nature of the endogenous protein inhibitor, which was first characterized in adult tissue [21], an additional purification was achieved on the pooled inhibitory fractions obtained from the six groups of 7–28-day-old rats in the four experiments. Though specific activities were high, total activities in young rats were not sufficient to allow inhibitor purification for each experiment. This pooling was also necessary to obviate activity losses during purification, so that no statistical result could be obtained. The results are reported in Table 1. Fractionation with $(NH_4)_2SO_4$ led to a purification factor of about 5 as compared with the inhibitory fraction eluted from DEAE-cellulose. After freeze-drying the total fraction was analysed by gel filtration on Sephacryl HR-100 S. For all the groups, the inhibitor was eluted as a single peak; the maximum inhibitory activity was eluted in the same tube, corresponding to an apparent molecular mass of 60 kDa, identical with that reported for 8-week-old rats [21]. At this step, the purification factor was of the same order of magnitude for all groups, except the day-18 group. The specific activities of the pooled tubes of these peaks exhibit a developmental pattern which was essentially the same as that reported in Fig. 1. The similar results obtained during purification of the endogenous protein inhibitor before or after weaning suggest that the inhibitory activity would likely be present in the same protein fraction which does not undergo major changes during development. More complete information would be obtained after complete purification of this inhibitory fraction.

Postnatal changes of enzymes involved in GDP-fucose metabolism

The availability of GDP-fucose may be also regulated during development by the activities of synthesizing or degradative enzymes as shown in Fig. 2.

Fig. 1. Developmental patterns of the protein inhibitor of fucosyltransferase activities and of microsomal fucosyltransferase activity

Each point represents the mean $\pm$ S.D. of duplicate determinations, in four independent experiments. After one-way analysis of variance, the significance of the differences was analysed by the Newman-Keuls test at $P < 0.01$. For the protein inhibitor activity, the mean values for the pre-weaning groups are significantly (*) higher than those of the post-weaning groups. For the fucosyltransferase activities, the mean values for the post-weaning groups are significantly different (**) from those of the pre-weaning groups.

Table 1. Partial purification of the endogenous protein inhibitor during post-natal development

Corresponding DEAE-cellulose eluates from the four experiments of Fig. 1 were pooled and submitted to partial purification by 50 %-(NH₄)₂SO₄ fractionation and chromatography on Sephacryl HR 100-S.

Age (days)	DEAE-cellulose eluate		(NH ₄) ₂ SO ₄ supernatant		Sephacryl pool		Purification factor
	Activity (units/mg)	Protein (mg)	Activity (units/mg)	Protein (mg)	Activity (units/mg)	Protein (mg)	
7	748	18.8	4816	3.25	8824	0.161	11.8
10	669	31.8	4393	6.81	6820	0.464	10.2
14	873	37.6	4302	14.03	7853	0.739	9.0
18	860	29.9	3243	15.66	4225	1.604	4.9
23	144	77.8	676	22.49	1050	2.464	7.3
28	120	98.5	341	48.47	1258	1.201	10.5

The level of GDP-fucose pyrophosphatase (Fig. 2a) was the highest in 7-day-old rats and decreased significantly ($P < 0.01$) at day 10. Then it remained high until weaning, with no significant difference in activity from day 10 to day 18. At weaning, the activity fell quickly and significantly ($P < 0.01$) between days 18 and 23 to reach a low level in adult rats.

In contrast, the transformation of GDP-mannose exhibited a complex pattern (Fig. 2b). The production of GDP-fucose was significantly ($P < 0.01$) increased at day 18 as compared with values obtained between days 7 and 14. This increase preceded the increase in the fucosyltransferase activities. It reached its highest levels at days 23 and 28. The production of GDP-4-dehydro-6-deoxymannose (the result of GDP-mannose 4,6-dehydratase activity) was detectable from day 7 to day 18, and displayed a significantly ($P < 0.01$) high level at days 14 and 18 as compared with the levels at days 7 and 10, whereas it was not detectable after weaning. During the week preceding weaning, the production of the intermediate was similar at concentrations of 40 and 80 μ M for the reduced coenzyme NADPH; higher concentrations were not assayed, since Chang *et al.* [22] reported an inhibitory effect of higher concentrations of the coenzyme on the

epimerase-reductase of the thyroid gland. Under the conditions for the determination of GDP-fucose synthesis, we found that spontaneous degradation of NADPH by the enzymic fractions was only 0.28 ± 0.04 nmol/min ($n = 16$) before weaning and 0.38 ± 0.03 nmol/min ($n = 12$) after weaning. Thus NADPH degradation was significantly higher in the fractions where no accumulation of intermediate product was found, so that NADPH degradation did not appear as a limiting factor for the reaction. Therefore the accumulation of the intermediate product in the transformation suggests that the activity of the epimerase-reductase would be a limiting factor in the production of GDP-fucose before weaning. The biochemical characterization of the intestinal system was not as complete as for the thyroid, so that alternative mechanisms could not be excluded. However, after weaning, this activity is sufficiently high to transform all the intermediate, even at a 100 μ M-GDP-mannose concentration.

The results of the study of normal weaning in rats support the idea that the fucose content of intestinal glycoconjugates depends not only on the expression or the activity of the transferases but also on the co-ordinate activity of several systems. On the one hand, there is obviously a close parallelism between the fucose

Fig. 2. Developmental patterns of the enzymes involved in GDP-fucose metabolism

(a) GDP-fucose pyrophosphatase activity. (b) Transformation of GDP-mannose into GDP-fucose: ----, GDP-fucose production by the two-enzyme system (GDP-mannose 4,6-dehydratase and epimerase-reductase); —, production of GDP-4-dehydro-6-deoxymannose, the intermediate product resulting from the action of the GDP-mannose 4,6-dehydratase. Results were analysed by one-way analysis of variance and means were compared by the Newman-Keuls test at $P < 0.01$. The significant differences are described in the text.

content or fucosyltransferase activities and the activity of the system responsible for the production of the major part of the available GDP-fucose. On the other hand, there is an inverse relationship between these parameters and the systems that are opposed to fucosylation (endogenous protein inhibitor and glycosyl-nucleotide pyrophosphatase).

Recently, we reported that the changes in the fucosyltransferase activity observed at the weaning period could be prematurely induced by early weaning or delayed by prolonged nursing of the pups [20], underlining the importance of nutritional factors in the regulation of fucosylation changes. Indeed, the changes observed at the weaning period may be due to the effects of age and/or nutritional changes. Dietary manipulation at weaning would allow estimation of the influence of each of these factors. On the other hand, the hypothesis of the involvement of the endogenous protein inhibitor in the physiological fucosylation process would be strengthened if the inverse changes observed during normal weaning between the inhibitory activity and the fucosyltransferase activity or the mucosal fucose content could be reproduced by dietary modifications during the weaning period.

The inverse modifications observed for the fucose content (or the fucosyltransferase activities) and the endogenous protein inhibitor activity during normal weaning argue for a physiological role of the endogenous protein inhibitor of fucosyltransferase activities, the exact mechanism of which remains to be elucidated. We previously reported that the variations of fucosyltransferase activities *in vitro* during postnatal development

were not due to the presence of inhibitors [20]; however, this conclusion was deduced from mixing experiments and not from direct determinations of this inhibitory activity, which cannot be performed directly on the cytosol. On the other hand, several endogenous inhibitors of glycosyltransferase activities have been described for sialyl- [31,32], glucosyl- and galactosyl- [33] and *N*-acetylgalactosaminyl-transferases [34]. Their role in the regulation of glycosylation has not yet been characterized, but their potential importance has been recently suggested by the demonstration that an endogenous inhibitor of the *N*-acetylgalactosaminyltransferase inhibits retina neuron differentiation in culture [35].

Thus the development of intestinal fucosylation appears to be complex, involving the participation of several enzymes and probably of an endogenous regulatory protein.

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