Implication of Insulin and Nutritional Factors in the Regulation of Intestinal Galactosyltransferase Activity During Postnatal Development

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In the rat small intestine, galactosyltransferases are the enzymes implicated in the biosynthesis of glycoproteins of the brush-border membranes and mucins. During postnatal development, the circulating insulin level increased at weaning in parallel with the activities of intestinal galactosyltransferases on O-glycans and N-glycans. This study deals with the role of insulin in the regulation of galactosyltransferase activities during postnatal development. The treatment of immature suckling rats with insulin induced a precocious increase in the activities of the O-glycan and N-glycan galactosyltransferases, partly reproducing the increase in galactosyltransferase activity normally found at weaning, since the O-glycan galactosyltransferase activity increased more quickly than the N-glycan galactosyltransferase activity. The sensitivity of the two galactosyltransferase activities to insulin disappeared after weaning, a period when drastic diet changes occur. In 22-day-old rats submitted to prolonged nursing (high-fat diet), the activities of the O-glycan and N-glycan galactosyltransferases were lower than those found in age-matched normally weaned rats (high-carbohydrate diet), indicating a delay in the maturation of the intestine of prolonged-nursing rats. The circulating insulin level of these animals stayed lower than that of the age-matched weaned rats. When the prolonged-nursing animals were treated with insulin, the O-glycan and N-glycan galactosyltransferase activities reached levels similar to those of the weaned rats. These observations suggest that insulin is one of the maturation factors for intestinal glycoprotein galactosylation and may be partly responsible for the natural enhancement of intestinal galactosyltransferase activities observed during postnatal development in relation to the dietary changes at weaning.

DURING THE THIRD WEEK of postnatal life, important ontogenic changes occur in the young rat small intestine, including changes in the activity of many digestive enzymes that enable the animal to cope with the solid high-carbohydrate diet of adulthood.[1] Most of the enzymes of the intestinal brush-border membranes of enterocytes are glycoproteins (lactase, sucrase, maltase, aminopeptidase, and alkaline phosphatase,[2-6] and mucins are glycoproteins secreted by goblet cells as a constituent of mucus.[7] Despite the fact that the role of the glycan chains of these glycoproteins in their biological activity is largely unknown, the intestinal glycosylation processes are also drastically modified during postnatal development. Between birth and adulthood, a shift is observed from sialylation to fucosylation in brush-border membrane glycoproteins and mucins,[8-12] and in the activity of the glycosyltransferases involved in sialylation or fucosylation processes.[13-15] Sialic acid and fucose are generally linked to the nonreducing termini of the external sugars of the glycan chains (often onto galactose residues). In the small intestine, two galactosyltransferases have been described that are responsible for galactose linkage to glycoproteins, a GalNAc:β-1,3-galactosyltransferase onto O-glycans[16] and GlcNAc:β-1,4-galactosyltransferase onto N-glycans.[17] In the adult rat intestine, their activities are differentially regulated along the intestine and along the crypt-villus axis.[5] Ozaki et al[18] have shown that these galactosyltransferase activities are modified in small intestine after the third week of life. The nature of the signals for the developmental regulation of the small intestine is largely unknown.

The involvement of hormones or growth factors in the regulatory mechanism of intestinal maturation has been evoked often.[19,20] Insulin, a hormone found in milk and colostrum[21] whose plasma level increases in the rat toward the end of the third week,[22] is a factor that may influence differentiation and a possible hormonal candidate in glycosylation regulation during postnatal development. In a previous study,[23] we have shown that insulin is a maturation factor for the intestinal glycoprotein fucosylation process. The present study examines the effect of insulin on galactosyltransferase activity in rat small intestine to determine if the normal increase in the hormone level at the time of weaning may explain the large increase in galactosyltransferase activity that occurs at this time.

MATERIALS AND METHODS

Animals and Treatments

Rats of the Sprague-Dawley strain (IFFA CREDO, L'Arbresle, France) were maintained in controlled conditions of temperature (21°C) and lighting (12-hour light/dark cycle). One day after birth, the pups were redistributed as litters of 10 male rats and each litter was maintained in special cages, where they received no solid food before weaning, as previously described.[24] Some male rats were weaned at 19 days of age (after removal of the dams) with a solid commercial diet (Souriffarat; UAR, Villemoisson sur Orge, France) and maintained with this food until adulthood. For experiments with 14-day-old rats, in each litter of ten 10-day-old suckling rats, a first group of pups received intraperitoneal injections for 4 days of 16 mU porcine insulin/g body weight in 0.9% NaCl twice per day at 8 and 18 hours (insulin group). A second group of rats received only 0.9% NaCl in the same conditions (control group). The experiments were performed at 14 days of age. A second set of experiments on 22-day-old animals involved either prolonged nursing (high-fat diet) without solid diet until day 22 in conditions previously described[24] or an abrupt weaning with a solid high-carbohydrate diet (Souriffarat) at 19 days of age (weaning group). In each diet group, some rats received insulin in the conditions described earlier between days 18 and 22, while the others received 0.9% NaCl. For postweaning treatment, rats were weaned on the 19th day and received insulin (16 mU/g body weight) or 0.9% NaCl twice

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per day between the 24th and 28th day, and were then killed at 28 days of age.

Cell Fractionation

The animals were killed by decapitation, and the small intestine was removed, flushed with cold 0.9% NaCl, and opened. The mucosae from 1 (adult) to 3 (young) rat small intestines for each control or test group were harvested with a glass slide and homogenized in 10 mmol/L Tris hydrochloride, 10 mmol/L KCl, 10 mmol/L $MgCl_2$, and 250 mmol/L sucrose, pH 7.4, buffer with a Potter-Elvehjem homogenizer (9 mL/g wet tissue). Cytosols and microsomal pellets were prepared after centrifugation of the homogenate at 30,000× *g* for 30 minutes and the supernatant at 140,000× *g* for 1.5 hours.

Determination of Galactosyltransferase Activity

The glycoprotein:galactosyltransferase activities were determined using two different exogenous acceptors, respectively, asialomucin for a β-1,3-galactosyltransferase that links galactose to N-acetylgalactosamine residue in O-glycans, or asialoagalactofetuin for galactosyltransferase that links galactose to N-glycans (asialofetuin can link galactose residue to the terminal N-acetylglucosamine of its N-glycan chains by either β-1,3 or β-1,4 linkages). The two acceptors were prepared according to the method of Ko and Raghupathy[25] from bovine submaxillary gland mucin and fetuin (Sigma, St Louis, MO). Galactosyltransferase activities were determined after subtraction of the endogenous activity (determined without exogenous acceptor). Microsomal pellets were suspended in 10 mmol/L Tris hydrochloride, 10 mmol/L KCl, and 10 mmol/L $MgCl_2$, pH 7.4, buffer. The reaction mixture (250 μL) contained 400 to 500 μg proteins, 0.8 mg/mL asialomucin or asialoagalactofetuin, 1 mmol/L $MnCl_2$, 10 mmol/L AMP, 0.25% Triton X-100, and 50 μmol/L UDP-^{14}C-galactose (specific activity, 10.1 GBq/mmol; New England Nuclear, Boston, MA). The incubations were performed at 37°C for 30 minutes. The reactions were stopped by precipitation with 20% trichloroacetic acid, and the radioactive products were collected on GF/B fiberglass filters (Whatman, Maidstone, UK). Radioactivity was then determined with a Toluene Scintillator (Packard, Downers Grove, IL).

Protein, Insulin, and Glucose Assays

The protein content was determined according to the method of Lowry et al[26] and serum insulin was determined by radioimmunoassay using an insulin RIA-GNOST kit from Behring (Marburg, Germany). Glucose was automatically determined by the glucose oxidase method with Technicon RA systems reagents (Technicon Diagnostics, Tarrytown, NY).

Statistical Analysis

The results are expressed as the mean ± SE. For developmental variations, the data were submitted to a 1-factor ANOVA. When the F test indicated a significant effect, differences between mean values were analyzed by the Newman-Keuls test. Differences were considered significant at a P level less than .05. For comparisons between 2 groups, Student's t test or the Mann-Whitney U test was used. For studies on variations due to 2 factors (diet and insulin treatment for 22-day-old rats), the results were submitted to a 2-factor ANOVA and mean values were compared with the Newman-Keuls test.

RESULTS

Developmental Changes in Circulating Insulin and Intestinal Galactosyltransferase Activity

A large and significant increase in circulating insulin was observed during postnatal development just after the abrupt weaning (Fig 1a) concomitantly with the increase in galactosyltransferase activities. Indeed, the β-1,3-galactosyltransferase activity specific for galactose linkage to O-glycans, as well as galactosyltransferase activity specific for galactose linkage to N-glycans, were largely and significantly enhanced at the end of the third week immediately after weaning on the 19th day of life (Fig 1b).

Effect of Insulin Treatment on Immature Suckling Rats

To study the role of insulin in intestinal maturation, we treated 10-day-old immature suckling rats with exogenous insulin for 4 days, and its effect on the activity of galactosyltransferases was determined in the small intestine. After each injection of insulin, the circulating insulin level was transitorily

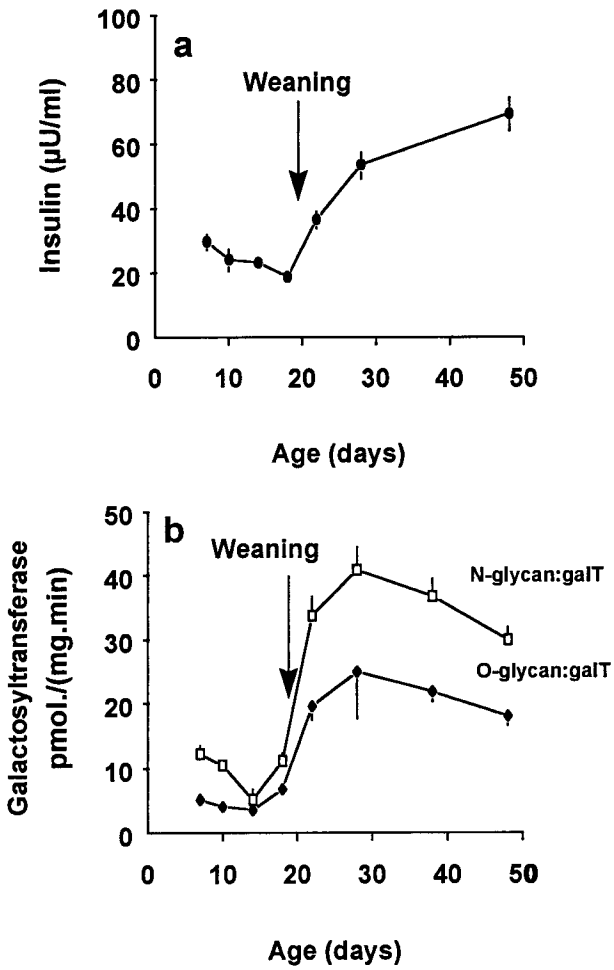

Fig 1. Variations of (a) circulating insulin and (b) intestinal O-glycan and N-glycan galactosyltransferase activities during postnatal development. The animals were weaned at 19 days of age. Insulin was determined in serum and galactosyltransferases in intestinal microsomal pellets of rats between 7 and 48 days of age. O-glycan and N-glycan galactosyltransferase activities were determined respectively with asialomucin or asialoagalactofetuin. Results are the mean ± SE for 8 independent values for insulin and 12 values for galactosyltransferases. Results were submitted to 1-factor ANOVA and means were compared by Newman-Keuls test. For insulin and galactosyltransferase activity, all values for weaned rats were significantly different *v* suckling rats at $P < .05$.

increased for 4 hours at a level near that observed for adult rats, but it returned to the basal level 8 hours after injection.[23] After insulin treatment, O-glycan β-1,3-galactosyltransferase activity was progressively increased; after 2 days of treatment, the activation factor was 1.3-fold, and 1.7-fold after 4 days (Fig 2a). N-glycan galactosyltransferase activity (Fig 2b) was less sensitive to insulin, since a significant increase in its activity was observed after 4 days of treatment only (activation factor, 1.4).

Effect of Insulin Treatment After Weaning

In weaned rats treated with insulin for 4 days from the 24th day and killed at 28 days of age, the hormone did not alter the two galactosyltransferase activities (Table 1).

Effect of Dietary Change at Weaning

To study the impact of the drastic diet changes that occur at the time of weaning, we measured the insulin level and galactosyltransferase activity in 22-day-old rats either submitted to prolonged nursing or weaned at 19 days of age. In 22-day-old prolonged-nursing rats (high-fat diet), circulating insulin stayed at a lower level than in age-matched weaned rats (high-carbohydrate diet), whereas the glucose level was not significantly modified by the diet (Table 2). The increase, normally observed at the end of the third week of life, in intestinal galactosyltransferase activity was delayed by prolonged nursing. The activity of the galactosyltransferases on O- and N-glycans was significantly lower in the intestine of 22-day-old rats submitted to prolonged nursing versus age-matched weaned rats. The introduction of the solid diet induced an increase (activation factor, nearly 1.5) in the two activities (Table 2).

Effect of Insulin Treatment at Weaning

After insulin treatment of the 22-day-old rats submitted to prolonged nursing, intestinal O-glycan and N-glycan galactosyltransferase activities (Fig 3a and b) were significantly increased

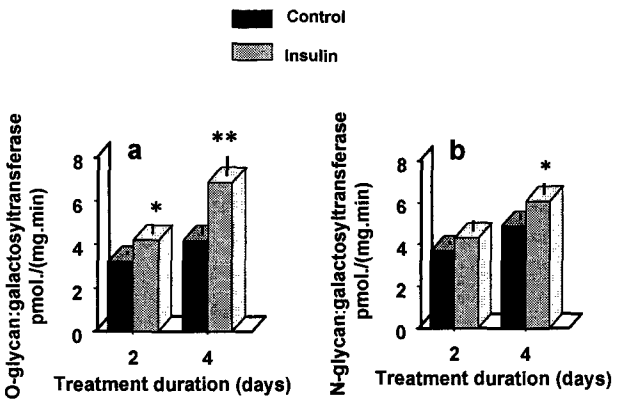

Fig 2. Insulin effect on galactosyltransferase activities in suckling rats. (a) O-glycan galactosyltransferase activity (determined with asialomucin), (b) N-glycan galactosyltransferase activity (determined with asialoagalactofetuin). In the insulin group, rats were treated twice per day with insulin (16 mU/g body weight) from the 10th day for either 2 or 4 days and were then killed at 12 or 14 days of age. In the control group, rats were treated with 0.9% NaCl in the same way. Results are the mean ± SE for 10 values, and means were compared by pairs with Student's *t* test: *$P < .05$, **$P < .01$.

Table 1. Effect of Insulin on Galactosyltransferase Activity After Weaning

Activity (pmol/mg protein · min)	Control Group	Insulin Group
O-glycan galactosyltransferase	22.24 ± 1.11	23.77 ± 3.39
N-glycan galactosyltransferase	26.50 ± 1.66	27.59 ± 1.00

NOTE. In the insulin group, rats were treated with insulin (16 mU/g body weight in 0.9% NaCl) twice per day from the 24th to the 28th day of life and killed at 28 days of age. In the control group, they were treated with 0.9% NaCl. Galactosyltransferase activities on O-glycans or N-glycans were determined respectively with asialomucin or asialoagalactofetuin as exogenous acceptors. The mean ± SE for 10 values were compared by Student's *t* test (no significant difference).

compared with the control prolonged-nursing rats and reached a level similar to that of the age-matched weaned rats. In 22-day-old weaned rats treated with insulin compared with control weaned rats, insulin treatment was without effect on the two galactosyltransferase activities. These results indicate that insulin treatment reversed the delay caused by prolonged nursing of the normal increase in O-glycan and N-glycan galactosyltransferase activity at weaning, and confirm the loss of insulin responsiveness in the weaned rats whether they were 22 or 28 days old.

DISCUSSION

This study deals with the role of insulin in the regulation of rat intestinal galactosyltransferase activities during postnatal development.

During postnatal development, the insulin level and the activity of a β-1,3-galactosyltransferase on O-glycans and a N-glycan galactosyltransferase were largely increased in the small intestine just after weaning, and the elevation of galactosyltransferase activity was accompanied by parallel increases in α-1,2-fucosyltransferase[23] and N-acetylgalactosaminyltransferase activities.[14] The increase in the activity of the O-glycan galactosyltransferase (an enzyme that preferentially links galactose residue in β-1,3 to free N-acetylgalactosamines of O-glycan chains) could be in relation to the evolution of the mucin glycannic composition. By chemical determination, Shub et al[11]

Table 2. Effect of Diet Change at Weaning on Circulating Insulin and Glucose and Intestinal Galactosyltransferase Activity†

Parameter	PN Group	W Group	W/PN
Insulin (μU/mL)	20.9 ± 3.9	43.3 ± 4.2*	2.07
Glucose (μmol/mL)	6.96 ± 0.22	8.02 ± 0.30	1.15
Galactosyltransferase (pmol/mg protein · min)			
O-glycan	15.64 ± 1.38	22.96 ± 2.60†	1.53
N-glycan	12.76 ± 0.95	19.02 ± 2.40†	1.49

NOTE. Animals aged 22 days were either submitted to prolonged nursing (PN group) or weaned at age 19 days (W group). Insulin and glucose were determined in serum and galactosyltransferase activity on O- and N-glycans in intestinal microsomal pellets, respectively, with asialomucin or asialoagalactofetuin as acceptors. The mean ± SE for 5 values for insulin and glucose were compared with the Mann-Whitney *U* test (*$P < .050$) and the mean for 11 values for galactosyltransferases were compared by Student's *t* test (†$P < .025$).

Abbreviation: W/PN, ratio of activities of weaned rats *v* prolonged-nursing rats.

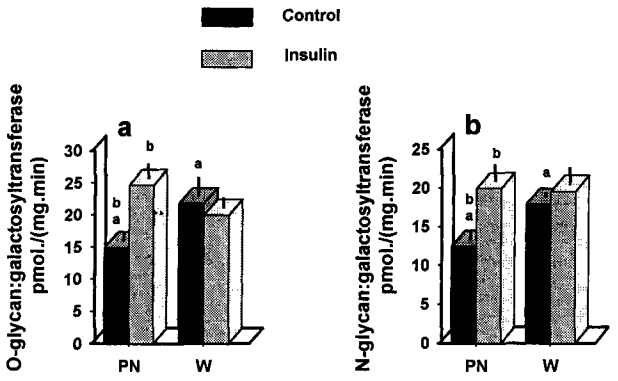

Fig 3. Insulin effect on galactosyltransferase activities in relation to weaning. (a) O-glycan galactosyltransferase activity, (b) N-glycan galactosyltransferase activity. The 22-day-old animals were submitted to either prolonged nursing (PN) or prolonged nursing and treatment twice daily with insulin (16 mU/g body weight) or were abruptly weaned at age 19 days (W) or weaned and treated with insulin. The mean ± SE (on 8 values) were compared by the Newman-Keuls test after a 2-factor ANOVA. Means with an "a" superscript are significantly different according to diet and those with a "b" superscript according to treatment, $P < .05$.

have shown that the galactose content of newborn rat mucins tends to be lower (but not significantly) than that of adult rat mucins and the fucose and N-acetylgalactosamine levels were very significantly lower in mucins of newborn rats versus adult rats. These results suggest that mucins of newborn rats might have either fewer carbohydrate side chains or a lower percentage of side chains with N-acetylgalactosamine able to accept galactose residues, and perhaps fewer galactose residues able to link fucose external residue, than adult mucins. The appearance after weaning of many membrane-bound glycoproteins with terminal α-1,2-fucose residues linked to galactose in glycoprotein N-glycannic chains of the brush-border membranes[27] might be related to the concomitant increase in the activity of a galactosyltransferase that links galactose residues preferentially to N-acetylglucosamine in N-glycans (probably a β-1,4-galactosyltransferase[17]) and an α-1,2-fucosyltransferase,[23] both observed at weaning. The involvement of galactosyltransferases in intestinal differentiation and maturation seems important in the intestine. It has been recently shown that a N-glycan β-1,4-galactosyltransferase plays a critical role in regulation of the proliferation and differentiation of intestinal epithelial cells after birth, since, in β-1,4-galactosyltransferase knockout mice, cell proliferation and differentiation are abnormal in intestinal villi.[28] However, the mechanism by which galactosyltransferase activities are enhanced during postnatal development remains to be elucidated. The developmental changes in these activities could be due to transcriptional regulation of the enzymes, but until now, no insulin response element has been found in the murine galactosyltransferase gene,[29] although it has been described in the promoter of a human N-acetylglucosaminyltransferase.[30]

The treatment of immature suckling rats with insulin increased the two galactosyltransferase activities, but galactosyltransferase activity on O-glycans was quickly and more highly increased than galactosyltransferase activity on N-glycans. A parallel increase was also shown for an α-1,2-fucosyltransferase activity.[23] The fact that the levels of galactosyltransferase activity found in the weaned rats were not reached exactly may be due to the fact that insulin injection twice daily only temporarily reproduces the insulin level found after weaning.

Insulin is known to be involved in the control mechanisms for the maturation of rodent small intestinal mucosa, since it was found to stimulate the activities of several glycoproteinic digestive enzymes[20,31,32] in the brush-border membrane when injected in suckling rats. The common increase in intestinal glycosyltransferase activities (galactosyltransferases and fucosyltransferases) after insulin treatment of the immature rat provides evidence that insulin is a regulator of the glycoprotein maturation, although the role of the glycannic chains in the biological activity of the digestive enzymes is not known.

Our results show that immature intestinal cells in suckling rats (14-day-old) respond to insulin, whereas mature intestinal cells in weaned rats (28-day-old) do not, since the treatment of weaned rats with insulin (between the 24th and 28th day) induced no change in the two galactosyltransferase activities. This phenomenon was also observed for the enzymes of the brush-border membrane.[20] The mechanism by which insulin affects intestinal maturation is not well known. In the small intestine, developmental differences in insulin degradation cannot be evoked, since the level of an insulin-degrading enzyme is similar in the small intestine of suckling and adult rats.[33] The role of insulin receptors can be evoked. Studies by Buts et al[34,35] indicated that in immature enterocytes, the glycosidase response to insulin is mediated by the binding of the hormone to its receptors. The circulating level of insulin is low in the suckling growing rat and markedly increased just after weaning, whereas the concentration of insulin receptors follows an inverse ontogenic pattern[36] and the capacity of the hormone to bind intestinal receptors decreases with age.[36] These observations may explain the loss of sensitivity to insulin of the galactosyltransferases and α-1,2-fucosyltransferase[23] observed in the rat small intestine after weaning.

At the time of weaning, the animals ingest a standard laboratory diet rich in carbohydrate and poor in fat. The normal increase in insulin depends on the diet, since it was delayed in prolonged-nursing rats (high-fat diet) compared with age-matched weaned rats (high-carbohydrate diet), as were the increases in galactosyltransferase and fucosyltransferase activity.[23] It also has been demonstrated that administration of a high-fat diet at the end of the weaning period produces a decrease in plasma insulin.[22] Craig et al[37] have described a decrease in insulin binding in the intestine of adult rats fed a high-fat diet compared with rats fed a high-carbohydrate diet, indicating that changes in the macronutrient composition of the diet can alter insulin receptors in the gastrointestinal tract. Nevertheless, the proposition that the insulin responsiveness of galactosyltransferases and fucosyltransferases may be related to the receptor status (number or sensitivity) of this hormone remains to be demonstrated. After insulin treatment of the 22-day-old prolonged-nursing rats, the activity of O-glycan and N-glycan galactosyltransferases reaches levels similar to those of all of the 22-day-old weaned rats. These results indicate that insulin is likely one of the factors in the regulation of intestinal galactosyltransferase activities in relation to diet changes at weaning. Similar results have been observed for α-1,2-

fucosyltransferase activity.[23] In prolonged-nursing rats treated with insulin, we previously also showed that the appearance in the brush-border membranes of many α-1,2-fucoproteins, delayed by prolonged nursing, becomes similar to that of weaned rats.[23] This phenomenon was due to variation in the activity of the α-1,2-fucosyltransferase, but it might be favored by the similarity of behavior of galactosyltransferase and fucosyltransferase activities versus insulin, since α-1,2-fucose residues are linked to galactose residues. In rats underfed during the suckling period, Jaswal et al[38] have also shown that the maturational development of the intestine is delayed and intestinal membrane glycoproteins have characteristics of the immature tissue (increased sialylation and decreased fucosylation). Moreover, insulin treatment of these undernourished pups reversed the sialylation and fucosylation pattern of these membranes, as we have observed for prolonged-nursing rats, but these investigators have not studied the impact of undernutrition on galactosyltransferase activities.

In conclusion, the insulin responsiveness of the two galactosyltransferases in immature suckling rats, concomitant with that of the α-1,2-fucosyltransferase, obviously provides evidence that insulin participates in the maturation processes of the rat intestine for the regulation of not only digestive enzymes but also glycoprotein glycosylation. Regarding the parallel evolution of the circulating insulin and the activities of galactosyltransferases on O- and N-glycans and α-1,2-fucosyltransferase during postnatal development, and the fact that the intestinal galactosyltransferase and fucosyltransferase activities can be modified by the dietary changes (milk or solid diet) in this period, as can the circulating insulin level, insulin may be involved in a concerted regulation of the linkage of galactose and fucose to glycoproteins in relation to the diet variations at the time of weaning.

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