Spontaneous galactosylation of agalactoglycoproteins in colostrum

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Received 15 February 2000; received in revised form 13 April 2000

Edited by Guido Tettamanti

Abstract We have found that spontaneous galactosylation of GlcNAc residues occurs in bovine colostrum, but not in dialyzed colostrum, without adding UDP-Gal as a donor substrate. UDP-Gal was shown to be present in bovine colostrum at a level ranging from 200 to 600 μM. When a tracer UDP-[^{14}C]Gal was added to the dialyzed colostrum together with a Gal β1,4-specific β-galactosidase, remarkable incorporation of radioactivity into 24–28 kDa and 33 kDa RCA1-positive glycoproteins was demonstrated by SDS–PAGE/autoradiography. Some 100–140 kDa agalactoglycoproteins of a CHO mutant cell line were also galactosylated on a blotted membrane by the incubation in the colostrum.

Key words: β1,4-Galactosyltransferase; Galactosylation; UDP-Gal; Glycoprotein; Colostrum; β-Galactosidase

1. Introduction

In most cells β1,4-galactosyltransferase (GalT) is expressed as a type II membrane-bound protein in the *trans*-Golgi apparatus. However, GalT also occurs as a soluble form in several biological fluids such as serum [1], semen [2], and milk [3]. Although the mechanism of production and secretion of soluble GalT is not fully understood [4], soluble GalT is thought to result from the proteolytic cleavage of the stem region, the catalytic domain thus being released from the membrane [5]. The physiological significance of such soluble GalT in extracellular fluids has remained unsolved [4,6].

It is well known that bovine colostrum is rich in soluble GalT and a good source for GalT purification. The presence of UDP-Gal in milk and colostrum was also reported for several animal species [7,8]. Thus, the presence of both GalT and UDP-Gal in colostrum appears to be a common feature among mammals. However, the question has never been raised whether galactosylation of glycoconjugates takes place in colostrum. In the course of assessing milk GalT activity using an enzyme-linked immunosorbent assay (ELISA)-based assay method [9], we found that galactosylation of GlcNAc residues of a synthetic acceptor substrate occurred in colostrum without any exogenous supply of UDP-Gal. In the present study, we report that in bovine colostrum galactosylation by GalT of acceptor agalactoglycoproteins takes place using endogenous UDP-Gal even in the presence of α-lactalbumin.

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2. Materials and methods

2.1. Materials

Bovine milk GalT, UDP-Gal, *Ricinus communis* agglutinin 1 (RCA1), rabbit anti-RCA1 antibody, peroxidase-conjugated anti-rabbit IgG antibodies and enzymes (UDP-Gal 4-epimerase and UDP-Glc dehydrogenase) were purchased from Sigma (USA). UDP-[^{14}C]Gal (326 mCi/mmol) was purchased from NEN (USA). The Gal β1,4-specific *Diplococcus pneumoniae* β-galactosidase was obtained from Boehringer Mannheim (USA). The polyacrylamide-type glycopolymer PAP(GlcNAcβ) was a generous gift from Dr. K. Kobayashi (Nagoya University). Fresh milk and colostrum of Holstein cows were kindly gifted by the Shimizu farm in Aichi prefecture (Japan).

2.2. Preparation of milk and colostrum samples

Fresh milk and colostrum samples were cooled on ice immediately after milking, frozen and kept at −20°C. After being thawed, the samples were centrifuged at 5000×*g* at 4°C for 20 min. Cream and precipitate fractions were removed. The defatted milk or colostrum was centrifuged at 20 000×*g* at 4°C for 15 min to precipitate most of the caseins, and the resulting supernatant fraction referred to as whey was used for further analyses. In the tracer experiments using UDP-[^{14}C]Gal described below, whey samples were dialyzed against phosphate-buffered saline (PBS) at 4°C for 3 days to remove endogenous UDP-Gal. Dialyzed whey samples were appropriately diluted with PBS, and used directly for the measurement of GalT activity.

2.3. Assay for GalT activity

The GalT activity in colostrum samples and their fractions was determined by the recently developed ELISA-based method [9] under optimal conditions. Briefly, a polystyrene microtiter plate (Nunc, Denmark) was coated with a polyacrylamide derivative having a β-linked GlcNAc on each monomeric unit, PAP(GlcNAcβ) (1 μg/ml). The sample solution was added to each well of the plate and incubated in the presence or absence of UDP-Gal (0.32 mM) and Mn^{2+} (10 mM). The Gal residues transferred to the polyacrylamide-bound GlcNAc were detected using a β1,4 Gal-specific lectin, RCA1, rabbit anti-RCA1 and peroxidase-conjugated anti-rabbit IgG. The ELISA value was converted into the enzymatic activity (mU/ml) by using the standard curve obtained for pure GalT. The coefficient of analytical variations was smaller than 10%.

2.4. Measurement of UDP-Gal concentration in milk and colostrum samples

The amounts of UDP-Gal in each colostrum sample and pooled milk from five cows at different lactation stages were determined based on the combined enzymatic assay using UDP-Glc dehydrogenase and UDP-Glc 4-epimerase [10] with slight modifications according to the method of Arthur et al. [11]. Proteins were removed from two times diluted whey samples by centrifugal ultrafiltration at 3000×*g* for 2 h at 4°C [11]. The ultrafiltrate was 100 times diluted with ultrapure water, and used for the UDP-Gal determination by the combined enzymatic assay [10].

2.5. Assay for spontaneous galactosylation of glycoproteins in colostrum

The colostrum whey was dialyzed as described above to remove endogenous UDP-Gal. Aliquots (8 μl) of the dialyzed colostrum whey were supplemented with 0.25 μCi UDP-[^{14}C]Gal (77 μM final concentration) and 100 mM Mn^{2+}, and incubated alone or with *D. pneumoniae* β-galactosidase at varying concentrations at 37°C for 2 h. The reaction mixtures were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) [12], and the dried

PII: S0014-5793(00)01523-4

gel was exposed to an imaging plate (Fuji Photo Film Co. Ltd., Japan) for 36 h. The radiolabeled proteins were visualized by an imaging analyzer (BAStation system, Fuji Photo Film Co. Ltd., Japan). The dialyzed colostrum was also incubated with or without the bacterial β-galactosidase and subjected to SDS–PAGE and the RCA1-lectin blotting analysis described below. The gel was stained with Coomassie brilliant blue R-250 (CBB).

2.6. Cell culture of Chinese hamster ovary (CHO) cells and preparation of cell lysates

A CHO cell line, wild type and its mutants, Lec 8 and 6B2 [13] with agalacto- and asialo-glycans, respectively, were kindly provided by Dr. R. Gerardy-Schahn (Medizinische Hochschule, Hannover, Germany). These cells were cultured in modified Eagle's medium (α-MEM) supplemented with 10% fetal calf serum (FCS) [13]. The cultured cells were lysed by adding 500 μl per dish (9 cm in diameter) of the lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin), and the cell lysates were centrifuged at 20 000×*g* for 10 min at 4°C. The supernatants were used as a source of agalactoglycoproteins as potential acceptor substrates for the galactosylation assay described below.

2.7. Galactosylation assay for agalactoglycoproteins blotted on a polyvinylidene difluoride (PVDF) membrane

The glycoproteins from the CHO cells were separated by SDS–PAGE [12], and then transferred to PVDF membranes [14]. After blocking with 3% bovine serum albumin (BSA) in PBS, the membrane was incubated at 37°C for 2 h with 1 ml of dialyzed colostrum supplemented with 1.5 μCi (4.6 μM) UDP-[^{14}C]Gal and 20 mM Mn^{2+}. After being washed with PBS–Tween the membrane was analyzed using the imaging analyzer as described above.

2.8. Lectin blotting

The glycoproteins transferred to PVDF membranes were subjected to RCA1-lectin blotting as described previously [15]. Briefly, the membranes were incubated successively with RCA1 solution (1.6 μg/ml of 1% BSA in PBS), rabbit anti-RCA1 and peroxidase-conjugated anti-rabbit IgG antibody. Finally, the membranes were incubated with the chromogenic peroxidase substrate, 4-chloronaphthol, to detect RCA1-bound glycoproteins.

3. Results

3.1. A synthetic glycopolymer acceptor substrate was galactosylated in colostrum

The GalT activity of colostrum was assayed by the ELISA-based method after dialysis or ultrafiltration using Microcon YM-3 (molecular cut-off 3 kDa, Millipore, USA). As shown in Fig. 1, no galactosylation was detected in colostrum after these treatments and, furthermore, the ultrafiltrate contained UDP-Gal and Mn^{2+} sufficient in amount for GalT reaction, suggesting the occurrence of enzymatic galactosylation in colostrum. These results also suggest that the galactosylation occurs enzymatically in colostrum whenever acceptor substrates are present at appropriate concentrations.

Table 1
UDP-Gal and GalT concentrations in bovine colostrum and milk

	Colostrum					Milk[a]
Time post-partum (h)	0.5	13.5	19.5	23	61	
UDP-Gal (μM)	2600	300	420	590	230	70
GalT[b] (mU/ml)	150	154	162	152	180	16

[a]A pooled milk sample from five cows at different stages (20–200 days) of lactation.
[b]GalT was determined by measuring its enzymatic activity in each sample, which had been dialyzed and supplemented with 0.32 mM UDP-Gal and 10 mM Mn^{2+}.

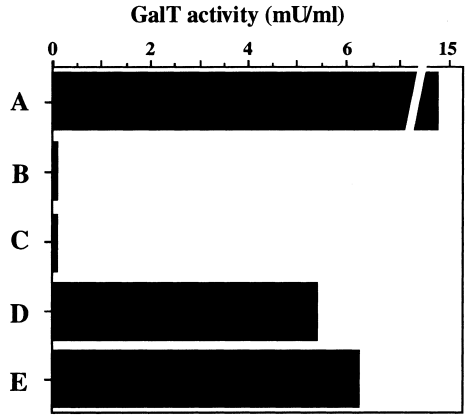

Fig. 1. GalT activity in dialyzed and ultrafiltered colostrum. GalT activity in the colostrum whey (A), its dialysate (B) and ultrafiltrate (C) was measured without the addition of UDP-Gal and Mn^{2+} by the ELISA-based method using the glycopolymer, PAP(GlcNAcβ), as an acceptor substrate. The activity of pure GalT (6.8 mU/ml) supplemented with either the ultrafiltrate (D) or UDP-Gal plus Mn^{2+} (E) was also measured. For the GalT activity measurement the colostrum samples were diluted with PBS 10 times for A and B and two times for C and D. GalT activity is expressed in mU/ml.

3.2. Colostrum contains UDP-Gal at a level higher than the K_m for GalT

The concentrations of UDP-Gal in the colostrum and milk samples were measured by the enzymatic method [10]. As shown in Table 1, the UDP-Gal concentrations determined for colostrum were about 4–40 times that of milk, and about 2–20 times the K_m (125 μM) [16] required by GalT. The GalT concentration in colostrum as measured by the enzymatic activity was also revealed to be about 10 times that of the pooled milk analyzed.

3.3. Some 24–28 and 33 kDa glycoproteins were spontaneously galactosylated in colostrum in the presence of β-galactosidase

Since enzymatic galactosylation using the endogenous donor substrate was shown to occur in colostrum, galactosylation of endogenous glycoproteins in colostrum was analyzed next by a radiotracer experiment using UDP-[^{14}C]Gal. Colostrum whey was dialyzed to remove endogenous UDP-Gal, supplemented with UDP-[^{14}C]Gal and Mn^{2+}, and then incubated without or with the bacterial Gal β1,4-specific β-galactosidase at varying concentrations. As shown in Fig. 2, although no radiolabeling was detected in the absence of β-galactosidase, when a trace of β-galactosidase was added to the colostrum, remarkable radiolabeling of a broad band of 24–28 kDa and another of 33 kDa was observed. As little as 0.5 mU/ml of β-galactosidase was sufficient for the galactosylation of these glycoproteins. The galactosylation increased with the increase in the β-galactosidase concentration, and the maximum galactosylation of a wide range of glycoproteins was attained at a β-galactosidase concentration of 50 mU/ml.

The dialyzed colostrum was incubated with or without β-galactosidase, and subjected to SDS–PAGE and RCA1-lectin blotting analysis (Fig. 2). No protein band corresponding to the galactosylated glycoproteins was detected in a gel stained with CBB, whereas some weakly stained bands of about 28 and 33 kDa, as well as several strongly stained bands

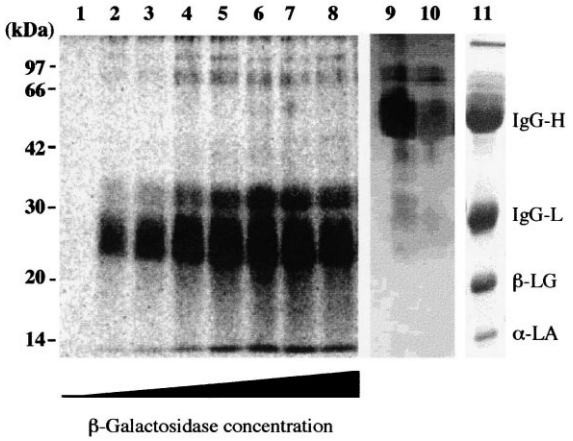

Fig. 2. Galactosylation of some endogenous glycoproteins in colostrum. Various amounts of a Gal β1,4-specific β-galactosidase from *D. pneumoniae* (lanes 1–8: 0, 0.5, 1, 5, 10, 50, 100 and 200 mU/ml final concentrations) were added to the colostrum whey, which had been dialyzed and supplemented with UDP-[^{14}C]Gal (0.25 μCi) and Mn^{2+}. After being incubated at 37°C for 2 h, these samples (15 μl) were applied to SDS–PAGE (15% acrylamide gel) followed by autoradiography. The dialyzed colostrum whey was incubated in the absence (lane 9) and presence (lane 10) of the bacterial β-galactosidase (100 mU/ml), subjected to SDS–PAGE and RCA1-lectin blotting analyses. The CBB staining pattern of the colostral whey separated on the same gel is also shown (lane 11) with the names of major colostral whey proteins: heavy (IgG-H) and light (IgG-L) chains of IgG, β-lactoglobulin (β-LG), α-lactalbumin (α-LA) on the right. The migration positions of molecular size markers are shown on the left.

of higher molecular masses, were detected by RCA1 staining. The RCA1-positive reaction of the 28 and 33 kDa bands was reduced or lost by incubation in colostrum containing β-galactosidase. This result suggests the presence of β-galactosidase-sensitive terminal Gal residues in these colostrum glycoproteins and the absence of GalT reaction in the dialyzed colostrum devoid of UDP-Gal and Mn^{2+}.

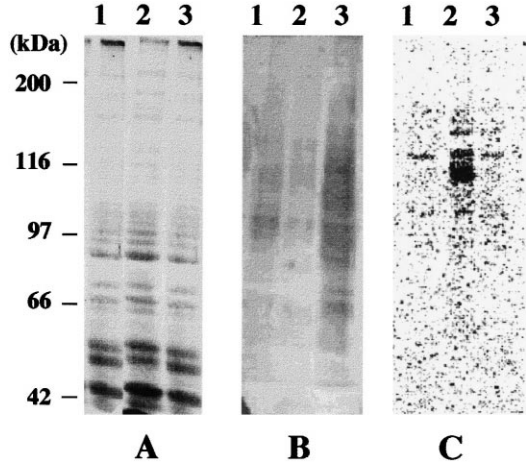

Fig. 3. Galactosylation of some agalactoglycoproteins from Lec8, a CHO mutant cell line, by incubation with colostrum. Aliquots (10 μg protein) of the cell lysates from three CHO cell lines, the wild type (lane 1), Lec8 (lane 2) and 6B2 (lane 3) mutants, were applied to SDS–PAGE (7.5% gel). A gel sheet was stained with CBB (A), and the others were used for electroblotting onto PVDF membranes. One membrane was subjected to RCA1-lectin staining (B), and the other to incubation with the colostrum whey, which had been dialyzed and supplemented with UDP-[^{14}C]Gal/Mn^{2+} (C).

3.4. Some agalactoglycoproteins from CHO cells were galactosylated in colostrum

The cell lysates from CHO cells and its mutants were used as a source of possible acceptor glycoproteins that carry glycans with different terminal structures: [NeuAcα2,6-Galβ1,4GlcNAc-] (the wild type), [GlcNAc-] (Lec8), and [Galβ1,4GlcNAc-] (6B2) [13]. Strong and moderate stainings were observed for the 6B2 mutant with asialoglycans and the wild type having sialylated ones, respectively. On the other hand, Lec8 glycoproteins with agalactoglycans showed almost no reactivity to RCA1 as expected (Fig. 3B) and, therefore, could be a good substrate for colostral GalT. As shown in Fig. 3C, the incubation of the blotted membrane in the colostrum dialyzed and supplemented with UDP-[^{14}C]Gal/Mn^{2+} resulted in specific radiolabeling of some 100–140 kDa glycoproteins only from Lec8 glycoproteins.

4. Discussion

The presence of UDP-Gal in colostrum was previously reported by other groups [7,8] and the reported UDP-Gal concentrations in bovine colostrum agree with those obtained in the present study (Table 1). Besides the donor substrate, colostrum has also been reported to contain Mn^{2+} [17], which is indispensable for GalT reaction [18]. Altogether, we conclude that colostrum contains the enzyme, the donor substrate, and Mn^{2+} in concentrations sufficient for the catalysis of GalT reaction. Despite the presence of GalT and its donor substrate, no acceptor glycoprotein appeared to be present in colostrum unless exogenous β-galactosidase was added (Fig. 2). The strong radiolabeling of some colostral glycoproteins in the presence of the β-galactosidase indicates that both the degalactosylation by exogenous β-galactosidase and the regalactosylation by endogenous GalT took place simultaneously in colostrum. The 24–28 kDa and 33 kDa galactosylated glycoproteins remain to be identified. Glycan chains of these glycoproteins might be good substrates for colostrum GalT as well as for β-galactosidase.

It seems reasonable that all of the GalT substrate glycoproteins in colostrum have already been galactosylated, assuming that spontaneous galactosylation took place in colostrum. Therefore, GalT reaction could be observed in colostrum only when agalactoglycoproteins were produced. Inflammation of the mammary gland due to its invasion by pathogenic bacteria is a widespread disease in cows [19] and in such instances, the invading bacteria may release β-galactosidase [20] that would remove terminal Gal residues of glycoproteins, resulting in the production of agalactoglycoproteins. Gal-terminated glycoproteins in milk were reported to be important in the protection against bacterial pathogens and their toxins [21]. Therefore, a possible role of colostrum GalT might be to repair or supplement the carbohydrate chains of some hypogalactosylated milk glycoproteins.

It is of interest to note that galactosylation of GlcNAc residues occurred even in the presence of α-lactalbumin. The concentration of α-lactalbumin in colostrum is about 2 mg/ml, which might be insufficient for the complete shift of GalT acceptor specificity from GlcNAc to Glc. Lactose biosynthesis in colostrum seems unlikely because of the low concentration of glucose (0.1–0.2 mM) [22], which is quite lower than the K_m for glucose (5 mM) [23].

Acknowledgements: We thank Dr. R. Gerardy-Schahn (Medizinische Hochschule, Hannover, Germany) for providing CHO cell lines, and Dr. K. Kobayashi (Graduate School of Engineering, Nagoya University) for preparing the glycopolymers. This research was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan and from The Ryoshoku Foundation.

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