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Expression of β-N-Acetylgalactosaminylated N-Linked Sugar Chains Is Associated with Functional Differentiation of Bovine Mammary Gland[1]

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During lactation the mammary gland synthesizes a large amount of glycoproteins including those composing milk fat globule membrane (MFGM). Our previous study showed that N-linked sugar chains with GalNAcβ1→4GlcNAc structure appears to increase in bovine MFGM glycoproteins during early lactation [Ujita *et al.* (1993) *FEBS Lett.* 332, 119-122]. Western blot analysis of membrane glycoproteins from lactating and post-lactating bovine mammary glands using *Wistaria floribunda* agglutinin (WFA), which binds oligosaccharides terminating with β-N-acetylgalactosamine, and *Ricinus communis* agglutinin-I (RCA-I), which binds oligosaccharides preferentially terminating with β-1,4-galactose, showed that the number and reactivity of protein bands to WFA but not to RCA-I decrease drastically in the post-lactating mammary sample. Establishment of primary cultured epithelial cells from lactating bovine mammary gland and their culture on collagen-coated dishes in the presence of a mixture of lactogenic hormones revealed that N-linked sugar chains with GalNAcβ1→4GlcNAc structure are expressed in the functionally differentiated cells without altering the apparent β-galactosylation of the oligosaccharides. These results strongly suggest that the expression of GalNAcβ1→4GlcNAc structure on N-linked sugar chains is associated with the mammary gland differentiation.

Key words: bovine mammary gland, functional differentiation, N-linked sugar chains, β-N-acetylgalactosamine.

The mammary gland is a unique organ in which a series of functional differentiations is repeated upon each pregnancy throughout the reproductive period. In association with the functional differentiation, dramatic changes are induced in the morphology and physiology of the mammary gland (*1, 2*). One of the most prominent physiological changes in the lactating mammary gland is synthesis and storage of a large amount of milk proteins including α-lactalbumin, transferrin, and MFGM glycoproteins, most of which are N-glycosylated (*3-7*). In accordance with this, elevated activities of several glycosyltransferases involved in the initial steps of N-linked sugar chain biosynthesis have been observed in the functionally developed mammary gland (*8-10*).

Our previous studies showed that most MFGM glycoproteins from bovine mature milk contain the GalNAcβ1→4GlcNAc structure in addition to the Galβ1→4GlcNAc structure in the outer chain moieties of N-linked sugar chains (*11, 12*), and that expression levels of the β-N-acetylgalactosaminylated oligosaccharides in the MFGM glycoproteins appear to increase during an early stage of lactation (*13*). About 30% of the total oligosaccharides released by hydrazinolysis from MFGM glycoproteins prepared from bovine mature milk contained the GalNAcβ1→4GlcNAc structure (*12*). However, few glycoproteins have been reported to contain this disaccharide structure (reviewed in Refs. *12* and *14*), probably due to the unique glycosylation mechanism underlying the β-N-acetylgalactosaminylation of glycoproteins (reviewed in Ref. *14*). The biological significance of the GalNAcβ1→4GlcNAc structure is not well understood, but the sulfated form of the disaccharide found in pituitary glycohormone sugar chains appears to be involved in the clearance mechanism of the glycoproteins from the circulation by binding to the lectin present on hepatic reticuloendothelial cells (*15*), and the sialylated and fucosylated form could be effective in inhibition of sperm-egg binding (*16*).

Because higher levels of β-N-acetylgalactosaminylated N-linked sugar chains were detected in membrane glycoproteins from lactating bovine mammary gland than from other bovine tissues (Sakiyama, T., and Furukawa, K., unpublished data), the mammary gland was considered to be a better source to study the mechanism of this unique glycosylation. As the first step, we investigated the rela-

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Abbreviations: BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks buffered salt solution; MFGM, milk fat globule membrane; PAGE, polyacrylamide gel electrophoresis; RCA-I, *Ricinus communis* agglutinin-I; SDS, sodium dodecyl sulfate; WFA, *Wistaria floribunda* agglutinin.

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tionship between the expression of β-N-acetylgalactosaminylated N-linked sugar chains and functional differentiation of the mammary gland in the present study.

MATERIALS AND METHODS

Preparation of Membrane Glycoproteins from Bovine Mammary Gland—Lactating and post-lactating bovine mammary glands were obtained from a local slaughter house. Each tissue was homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA. The homogenates were centrifuged at 8,000 × *g* for 30 min at 4°C, then the pellets were suspended in a mixture of acetone and water (1 : 1, v/v) and homogenized with a Polytron homogenizer. The defatted samples, referred to as membrane glycoprotein samples, were solubilized in a sample buffer [50 mM Tris-HCl buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue] by heating at 100°C for 5 min. Protein was determined with a bicinchonic acid protein assay reagent kit (Pierce Chemical, Rockford, IL) using bovine serum albumin (BSA) as a standard.

Preparation of Primary Cultured Cells from Bovine Mammary Gland—Primary cultured epithelial cells were established from lactating bovine mammary gland by the method described previously (*17*). In brief, the sliced tissue was minced into smaller pieces and treated with 0.05% collagenase in a Hanks buffered salt solution (HBSS) containing penicillin-streptomycin (5 units/ml) and gentamycin (50 μg/ml) at 37°C for 14 h. The digests were further treated with 0.05% pronase E at 37°C for 30 min, then filtered through a stainless steel mesh. The filtrates were centrifuged at 80 × *g* for 1 min at 4°C. The single cells thus prepared were washed with HBSS several times. Mammary epithelial cells were isolated from the single cells by Percoll gradient centrifugation and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin-streptomycin (5 units/ml). To induce the cells to differentiate, they were cultured on collagen-coated dishes in media containing a mixture of insulin (10 μg/ml), hydrocortisone (1 μg/ml), and prolactin (4 μg/ml) for six days as described previously (*18-21*). The spent media were dialyzed against water and lyophilized, and the resulting materials were used for immunoblot analysis in order to detect casein, one of the milk proteins secreted by functionally differentiated mammary epithelial cells.

SDS-PAGE, and Lectin and Immuno Blot Analyses—The membrane proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (*22*) using a Mini Protean II Electrophoresis Cell (Bio-Rad, Hercules, CA) and transferred to nitrocellulose filters with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). Western blot analysis using WFA or *Ricinus communis* agglutinin-I (RCA-I) was performed as described previously (*12*). The filter was incubated with Coomassie Brilliant Blue (CBB) for detecting proteins or blocked with 1% BSA prior to incubation with lectin or antibody. In brief, the filters were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween 20), then incubated with peroxidase-conjugated lectin at 4°C for 1 h. The filters were then washed five times with PBS-Tween 20, and the lectin-glycoprotein complexes were visualized by incubating with 0.05% 4-chloro-1-naphthol and 0.03% hydrogen peroxide in PBS. In some experiments, the filters blocked with BSA were treated with 0.5 unit of *Arthrobacter ureafaciens* sialidase in 150 μl of 0.5 M acetate buffer (pH 5.0), 5.0 units of N-glycanase in 150 μl of 0.1 M phosphate buffer (pH 8.2) or 1.5 units of jack bean β-N-acetylhexsosaminidase in 150 μl of 0.3 M citrate-phosphate buffer (pH 4.0) at 37°C for 24 h prior to incubation with lectin. For detection of antibody-protein complexes the filters were incubated with peroxidase-conjugated anti-rabbit IgG antibody followed by visualization as described above.

Liberation of N-Linked Sugar Chains from Membrane Glycoproteins—Membrane glycoprotein samples (10 mg each), which were dried thoroughly over P_2O_5 *in vacuo*, were subjected to hydrazinolysis for 10 h as described previously (*23*). After N-acetylation, the liberated oligosaccharides were reduced with NaB^3H_4 to obtain tritium-labeled oligosaccharides. The radioactive oligosaccharides were treated with *A. ureafaciens* sialidase, then subjected to WFA-agarose column chromatography (*24*) in order to estimate the carbohydrate structures (*25*). More than 90% of the bound materials were recovered from the column. Fractionation of oligosaccharides by Bio-Gel P-4 column chromatography and exoglycosidase digestion were conducted as described previously (*26, 12*, respectively). Oligosaccharides were digested with 0.1 unit of *Bacillus* sp. AT173-1 β-N-acetylgalactosaminidase in 50 μl of 50 mM acetate buffer (pH 6.0) containing 0.1% dithiothreitol (*27*).

Chemicals, Enzymes, and Lectins—Peroxidase-conjugated WFA and RCA-I were obtained from E.Y. Labs (San Mateo, CA). 4-Chloro-1-naphthol, hydrogen peroxide and sialidase from *A. ureafaciens* were obtained from Nacalai Tesque (Kyoto). *Flavobacterium meningosepticum* recombinant N-glycanase and *Bacillus* sp. AT173-1 β-N-acetylgalactosaminidase were kindly supplied by Dr. T. Kaizu, Genzyme Japan (Tokyo) and Dr. A. Tanaka, Daiwa Kasei (Shiga), respectively. Insulin, hydrocortisone, prolactin, casein (α-, β-, and κ-) and peroxidase-conjugated goat anti-rabbit IgG antibody were obtained from Sigma Chemical (St. Louis, MO). NaB^3H_4 (1,000 mCi/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA). Polyclonal antibodies against casein were purified from serum of immunized rabbit with mixted types of casein.

Oligosaccharides—GalNAcβ1→4GlcNAcβ1→2Manα1→6 or 3(Galβ1→4GlcNAcβ1→2Manα1→3 or 6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (GalNAc·Gal·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$), and Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) were prepared from bovine CD36 (*11*). GalNAcβ1→3Galα1→4Galβ1→4Glc$_{OT}$ (GalNAc·Gal·Gal·Glc$_{OT}$) was prepared as described previously (*28*).

RESULTS

Expression Levels of β-N-Acetylgalactosaminylated N-Linked Sugar Chains—In our previous study, most glycoproteins consisting of MFGM prepared from bovine mature milk were shown to contain N-linked sugar chains with the GalNAcβ1→4GlcNAc structure (*12*). To detect the N-

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linked sugar chains with the disaccharide structure, membrane glycoprotein samples were prepared from lactating and post-lactating bovine mammary glands, since there is no milk available from post-lactating mammary gland for preparing MFGM glycoproteins. The membrane glycoprotein samples (20 μg protein) were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose filters. Western blot analysis was performed by using peroxidase-conjugated WFA, which interacts with oligosaccharides terminated with the disaccharide structure (*24*). Since some of the β-*N*-acetylgalactosamine residues of bovine MFGM glycoproteins were sialylated (*11-13, 29*), the blotted filters were treated with *A. ureafaciens* sialidase prior to incubation with lectin. Lanes A and B, C-H, and I and J in Fig. 1 show filters incubated with CBB, WFA, and RCA-I, respectively. As protein components synthesized in the mammary gland change drastically during lactation (*3, 7*), CBB-staining patterns of membrane proteins were different between the lactating (lane A) and post-lactating (lane B) mammary glands (Fig. 1). When filters were incubated with WFA, many lectin-positive bands were observed in the lactating mammary gland sample (lane C), while only two weak lectin-reactive bands with molecular sizes of 80 K and 82 K were detected in the post-lactating mammary gland sample (lane D) (Fig. 1). The glycoprotein samples on filters were treated with jack bean β-*N*-acetylhexosaminidase, then incubated with WFA. No lectin binding was observed in both samples from lactating and post-lactating mammary glands (lanes E and F in Fig. 1, respectively). Upon digestion with *N*-glycanase, no WFA binding was also observed in both samples (lanes G and H in Fig. 1, respectively). However, when filters were incubated with RCA-I, which preferentially interacts with oligosaccharides terminated with Galβ1→4GlcNAc/Glc group(s) (*30*), many protein bands in both samples reacted to the lectin although the patterns of lectin-positive bands were different between the samples (lanes I and J in Fig. 1, respectively).

Structural analysis of the sugar chains released by hydrazinolysis from membrane glycoprotein samples by sequential digestion of exoglycosidases including *Bacillus* sp. AT173-1 β-*N*-acetylgalactosaminidase, which only cleaves β-*N*-acetylgalactosaminyl linkages found in glycoconjugates (*27*), revealed that oligosaccharides, that bound to a WFA-agarose column contain GalNAcβ1→4GlcNAc structure in the outer chain moieties of complex-type sugar chains (data not shown), most of which are the same as those of the MFGM glycoproteins described previously (*12*).

These results indicated that levels of β-*N*-acetylgalactosaminylation of *N*-linked sugar chains are reduced when the bovine mammary gland becomes involuted.

Changes in Morphology and Casein Production of Primary Cultured Cells—To elucidate the relationship between the expression of the β-*N*-acetylgalactosaminylated oligosaccharides and the differentiation of the mammary gland, primary cultured cells were established from lactating bovine mammary gland. It is well documented that the primary cultured mammary epithelial cells grown on plastic dishes lose their ability to synthesize and secrete most milk proteins immediately, but the cells grown on proper substrates in the presence of lactogenic hormones can maintain production of milk proteins (*19-21*). Therefore, in order to induce the primary cultured mammary epithelial cells to differentiate, they were cultured on collagen-coated dishes, then treated at a subconfluent stage with a mixture of insulin, hydrocortisone, and prolactin for six days. The hormone-treated cells

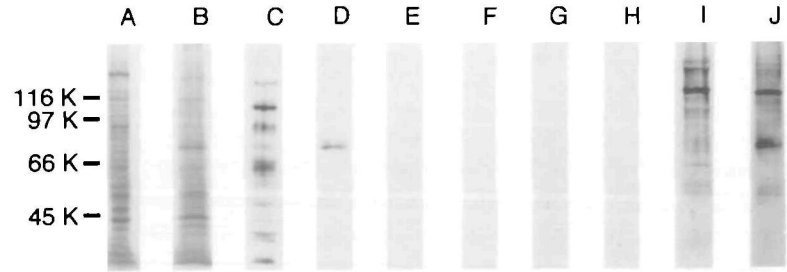

Fig. 1. **Lectin blot analysis of mammary gland membrane glycoproteins.** Each filter contained 20 μg of membrane glycoproteins prepared from lactating (A, C, E, G, and I) and post-lactating (B, D, F, H, and J) bovine mammary glands. Filters A and B were stained with CBB. Filters were incubated either with WFA before (C and D) and after (E and F) treatment with jack bean β-*N*-acetylhexosaminidase or *N*-glycanase (G and H) or with RCA-I (I and J), followed by visualization as described in "MATERIALS AND METHODS."

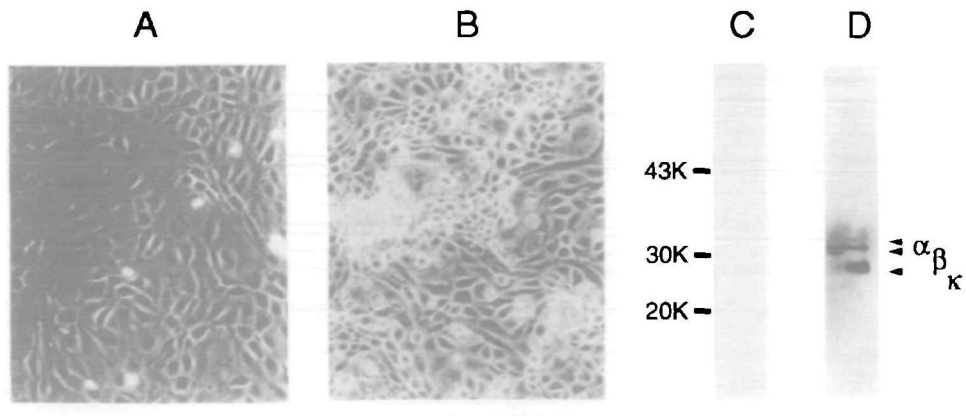

Fig. 2. **Morphological and biochemical changes of cultured mammary epithelial cells.** The cells in panel A were cultured on plastic dishes and those in panel B were grown on collagen-coated dishes in the presence of lactogenic hormones for six days as described in "MATERIALS AND METHODS." Glittering materials in panel B indicate fat globules secreted into spent media. Secretion of casein into spent media from the cells with (lane D) or without (lane C) treatment of lactogenic hormones was monitored by immunoblot analysis using anti-casein antibody.

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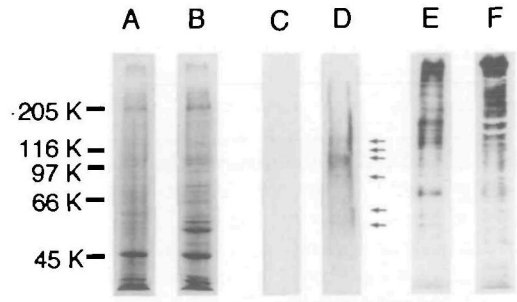

Fig. 3. **Lectin blot analysis of membrane glycoproteins from cultured mammary epithelial cells.** Each filter contained 20 μg of membrane glycoproteins prepared from the hormone-treated (B, D, and F) and untreated (A, C, and E) cells. Filters A and B were stained with CBB. Filters C, D, E, and F were initially treated with sialidase, then filters C and D were incubated with WFA and E and F with RCA-I, followed by visualization as described in "MATERIALS AND METHODS." Arrows on lane D indicate the WFA-positive protein bands.

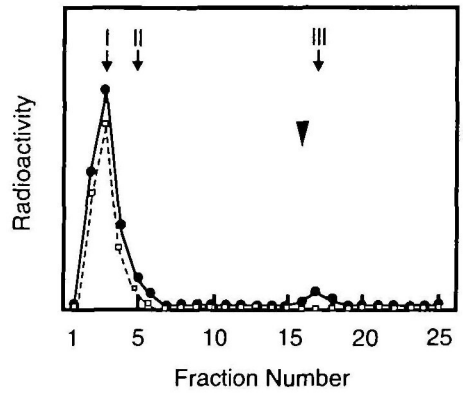

Fig. 4. **WFA-agarose column chromatography of sialidase-treated oligosaccharides from cultured mammary epithelial cells.** Solid and dotted lines indicate oligosaccharides obtained from membrane glycoprotein samples of the hormone-treated and untreated cells, respectively. The arrowhead indicates the change of the elution buffer to one containing the haptenic sugar. Arrows indicate the elution positions of authentic oligosaccharides: (I) $Gal_2 \cdot GlcNAc_2 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$; (II) GalNAc·Gal·Gal·$Glc_{OT}$; (III) GalNAc·Gal·$GlcNAc_2 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$.

secreted fat globules (oil droplets) into media (Fig. 2B) while untreated cells did not (Fig. 2A).

Upon immunoblot analysis, the milk protein was not detected in spent media of the primary cultured cells grown on plastic dishes (Fig. 2C), but three types (α-, β-, and κ-) of casein molecules were detected in spent media of the cells grown on collagen-coated dishes in the presence of lactogenic hormones (Fig. 2D). Since production of casein is a marker of the functionally differentiated mammary gland (*18, 21*), these results indicated that the hormone-treatment on collagen-coated dishes induces the change in primary cultured mammary epithelial cells from undifferentiated to functionally differentiated cells.

Expression of β-N-Acetylgalactosaminylated N-Linked Sugar Chains in Functionally Differentiated Cultured Cells—CBB-staining of the blotted filters showed markedly different patterns of protein bands before and after treatment of the primary cultured mammary epithelial cells with lactogenic hormones (Fig. 3, A and B, respectively). In the hormone-treated cells, amounts of proteins with molecular sizes of 56 K, 59 K, 80 K, and 97-116 K increased (Fig. 3, lane B), indicating that a pattern of proteins synthesized is changed by the hormone treatment. When the blotted filters were treated with *A. ureafaciens* sialidase and then incubated with WFA, no bands were detected in the sample from the untreated cells (Fig. 3, lane C). However, WFA-binding was observed in protein bands with molecular sizes of 58 K, 63 K, 90 K, 105 K, 116 K, and 140 K, as indicated by arrows in Fig. 3, lane D, from the hormone-treated cells grown on collagen-coated dishes. When the filter was treated with *N*-glycanase or jack bean β-*N*-acetylhexosaminidase prior to incubation with WFA, no bands were detected (data not shown). In contrast, no significant difference in apparent RCA-I-reactivity was observed between the untreated and hormone-treated cells (Fig. 3, lanes E and F, respectively).

Cultures of the cells on collagen-coated dishes without the hormone treatment or on plastic dishes in the presence of lactogenic hormones showed no apparent production of fat globules and casein in spent media, and no WFA-positive bands were detected in membrane glycoprotein samples prepared from these cells (data not shown).

The tritium-labeled *N*-linked sugar chains prepared from the membrane protein samples were subjected to WFA-agarose column chromatography. No radioactivity was detected in the bound fraction when the oligosaccharides were obtained from the control cells. However, when the oligosaccharides were prepared from the hormone-treated differentiated cells and applied to the column, about 4% of the total oligosaccharides bound to the column and were eluted with 100 mM *N*-acetylgalactosamine (Fig. 4). Bio-Gel P4 column chromatography in combination with sequential digestion of exoglycosidases including *Bacillus* sp. AT173-1 β-*N*-acetylgalactosaminidase and jack bean β-*N*-acetylhexosaminidase revealed that the oligosaccharides that bound to the WFA-agarose column contain GalNAcβ1→GlcNAc structure. *N*-Acetylgalactosamine is presumed to be present in the GalNAcβ1→4GlcNAc sequence, since oligosaccharides that terminated with this structure but not the GalNAcβ1→3Gal structure were bound to a WFA-agarose column (Fig. 4 and *25*). These results suggest that the expression of GalNAcβ1→4GlcNAc structure in *N*-linked sugar chains is associated with the functional differentiation of the mammary epithelial cells.

DISCUSSION

During lactation, the mammary gland secretes a large amount of glycoproteins (*1-3, 7*) and thereby provides an excellent source for studying the biosynthetic and developmental regulation of *N*- and *O*-linked sugar chains. In fact, several groups have reported the developmental regulation of *N*-linked sugar chain biosynthesis by lactogenic hormones (*8-10*). The activities of three key glycosyltransferases, UDP-GlcNAc:dolichol-phosphate *N*-acetylglucosamine-1-phosphate transferase, GDP-Man:dolichol-phosphate mannosyltransferase and UDP-Glc:dolichol-phosphate glucosyltransferase, for formation of lipid-linked oligosaccharides, and of glucosidase-I, which is involved in the processing of *N*-linked sugar chains, in mouse mam-

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mary gland are regulated during the development.

In the present study we demonstrated that the β-*N*-acetylgalactosaminylation, one of the sub-terminal glycosylations of *N*-linked sugar chains, is also under the influence of lactogenic hormones. This is supported by the facts that many WFA-positive bands are present in the membrane glycoprotein sample from lactating bovine mammary gland but only a few WFA-positive bands are detected in that from the involuted mammary gland, and that WFA-positive bands are obtained only from the glycoprotein sample of the hormone-treated primary cultured mammary epithelial cells. The presence of a few WFA-positive bands in the involuted mammary gland may indicate that some of the mammary epithelial cells are still partially differentiated. It has been shown that differentiation of cultured mammary epithelial cells is induced by synergistic actions of insulin, glucocorticoid, and prolactin (*19-21*). In the presence of a mixture of these hormones the highest synthesis of milk proteins, lipid-linked oligosaccharides, and glycoproteins was achieved in bovine and mouse mammary gland explants or cell cultures (*8-10, 31-33*). When the primary cultured cells established from lactating bovine mammary gland were grown on plastic dishes, no fat globules or casein were secreted into the media. However, when the cells were cultured on collagen-coated dishes in the presence of insulin, hydrocortisone and prolactin, they started to secrete fat globules and casein into the spent media, indicating that the hormone-treatment is effective for the cells to differentiate functionally. Lectin blot analysis in combination with exo- and endo-glycosidase digestion revealed that several glycoprotein bands from the functionally differentiated cells but not from the undifferentiated cells contain β-*N*-acetylgalactosaminylated *N*-linked sugar chains. The amount of *N*-linked sugar chains with the GalNAcβ1→4GlcNAc structure was at most 4% of the total oligosaccharides released from the membrane glycoprotein sample of the hormone-treated cells, possibly because differentiation of the primary cultured mammary epithelial cells was not maximal under the present conditions and/or because the established cells were heterogenous. Since no WFA-positive bands were detected in the glycoprotein sample from the cells grown simply on collagen-coated dishes, lactogenic hormones could be essential for inducing this glycosylation.

In contrast, the apparent RCA-I-reactivity to protein bands was not significantly different between membrane glycoprotein samples from lactating and post-lactating bovine mammary glands, or between those from the primary cultured mammary epithelial cells with or without treatment with lactogenic hormones. Therefore, only β-*N*-acetylgalactosaminylation of *N*-linked sugar chains is strongly affected by differentiation of the mammary epithelial cells, although β-1,4-*N*-acetylgalactosaminyltransferase and β-1,4-galactosyltransferase compete with each other for the same sugar-acceptor molecules during the biosynthesis of *N*-linked sugar chains.

Since β-*N*-acetylgalactosaminylated *N*-linked sugar chains appeared in the hormone-treated cells but were absent in the untreated cells, the gene expression of β-*N*-acetylgalactosaminyltransferase could be also regulated by lactogenic hormones. In support of this, the expression of the Neu5Acα2→6GalNAcβ1→4GlcNAc structure on *N*-linked sugar chains of prolactin/growth hormone family members produced by rat spongiotrophoblasts has recently been shown to be regulated developmentally (*34*). This and the present study strongly suggest that the expression of β-*N*-acetylgalactosaminyltransferase is developmentally regulated at least in the mammary gland and placenta. The occurrence of two types of β-*N*-acetylgalactosaminyltransferase, one with and one without peptide-specificity, both of which are involved in formation of GalNAcβ1→4GlcNAc structure, has been described in mammalian tissues (*35, 36*), although neither has yet been purified. It is, therefore, of interest to investigate which of the transferases is under the influence of the cellular differentiation. Accordingly, further work is necessary to elucidate the exact control mechanism of this unique glycosylation at a molecular biological level by obtaining cDNA which encodes β-*N*-acetylgalactosaminyltransferase.

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