Novel induction of α -lactalbumin-mediated lacdiNAc-R expression *in vivo*

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α -Lactalbumin (α -LA) is a regulatory protein by which the mammalian β 1,4-galactosyltransferase (β 1,4-galT) is induced to utilize glucose as an acceptor instead of *N*-acetylglucosamine (GlcNAc) during lactose synthesis in mammary gland. α -LA can also modulate β 1,4-galT to utilize UDP-*N*-acetylgalactosamine (UDP-GalNAc) as a donor towards GlcNAc acceptor substrate with high efficiency *in vitro* [Do, Do and Cummings (1995) J. Biol. Chem. 270, 18447–18451]. In the present study we transfected cDNA encoding bovine α -LA into Lec8 cells and examined whether nucleotide sugar switching of UDP-galactose (UDP-

Gal) into UDP-GalNAc occurred *in vivo* and whether the neo-glycosylation of GalNAc β 1,4GlcNAc-R structure was synthesized in α -LA-stable transfectants. Our studies demonstrate that the stable expression of α -LA in Lec8 cells induces the formation of GalNAc β 1,4GlcNAc-R *in vivo* through the nucleotide sugar switching of β 1,4-galT.

Key words: nucleotide sugar switching, Lec8 cells, β 1,4-galactosyltransferase, β 1,4-*N*-acetylgalactosaminyltransferase.

INTRODUCTION

Since the glycan epitope of GalNAc β 1,4GlcNAc-R (lacdiNAc-R) structure was first reported [1], substantial efforts to identify the occurrence of lacdiNAc-R sequence have been made [2–6]. It is now known that the lacdiNAc-R sequence is widespread, because diversified structures of sulphated, fucosylated and sialylated forms in many glycoproteins are synthesized not only in invertebrates [7–10] but also in vertebrates including human beings [11–15]. The formation of the lacdiNAc-R structure inside the cell has been considered to be a novel glycosylation pathway because it is distinct from the common pathway of gal β 1,4GlcNAc-R synthesis [16]. Until recently this lacdiNAc-R Glycan epitope was known to be involved in many biological functions, such as a ligand for the hepatic receptor, anti-inflammatory activity in the immune response, immunosuppressive activity, and contraceptive properties [17–19]. The synthesis of the lacdiNAc-R structure in pituitary glycoproteins seems to be catalysed by β 1,4-*N*-acetylgalactosaminyltransferase (β 1,4-GalNAcT), which recognizes the specific amino acid motif of Pro-Xaa-Arg/Lys [20]. However, the widespread occurrence of lacdiNAc-R in many glycoproteins suggests the possible existence of either other types of β 1,4-GalNAcTs or some unusual mechanisms to generate the lacdiNAc-R sequence. Recent studies have shown that the lacdiNAc (GalNAc β 1,4GlcNAc) sequence can be formulated under certain conditions by a conventional enzyme, β 1,4-galactosyltransferase (β 1,4-galT) [21,22]. Furthermore, we reported that modifier protein α -lactalbumin (α -LA) could modulate the nucleotide sugar specificity of β 1,4-galT from UDP-Gal into UDP-GalNAc *in vitro*, by which lacdiNAc synthesis was greatly enhanced [23]. At present, the underlying mechanism of β 1,4-galT modulation by α -LA at the nucleotide sugar level is not known. We have also investigated the presence of an endogenous lacdiNAc-R sequence in Chinese hamster ovary (CHO) cells and its genetic variant line Lec8. However, we

failed to obtain convincing evidence for lacdiNAc-R modification in these cells, although both cells contained endogenous β 1,4-GalNAcT activity [24]. To explore the α -LA-induced neo-glycosylation of lacdiNAc-R modification *in vivo*, we selected Lec8 cells as a host system for the stable expression of α -LA [25] and examined the effects of α -LA expression on lacdiNAc-R modification at the cellular level. Here we report that the stable expression of α -LA in Lec8 cells initiates lacdiNAc-R synthesis *in vivo* and that this neo-glycosylation of lacdiNAc-R sequence is induced by α -LA-associated endogenous β 1,4-galT. These results also demonstrate that the association of β 1,4-galT with α -LA is able to generate additional β 1,4-GalNAcT activity that is distinct from endogenous β 1,4-GalNAcT activity.

MATERIALS AND METHODS

Materials

Polyclonal rabbit anti-(bovine α -LA) IgG was purchased from Bethyl Laboratories (Montgomery, TX, U.S.A.). Protein A-agarose was obtained from Santa Cruz. Jack-bean β -hexosaminidase, Sephadex G-50, nucleotide sugars and GlcNAc were from Sigma Chemical Co. Pronase was purchased from Calbiochem; D-[6-³H]glucosamine (40 Ci/mmol), UDP-[³H]Gal (50 Ci/mmol) and UDP-[³H]GalNAc (6.3 Ci/mmol) were obtained from American Radiolabeled Chemicals and NEN DuPont.

Cell culture and metabolic radiolabelling with sugars

The wild-type Lec8 cell line was obtained from the American Type Culture Collection (A.T.C.C., Manassas, VA, U.S.A.) and was cultured in Eagle's α -minimal essential medium containing 10% (v/v) fetal bovine serum as described [25]. For metabolic radiolabelling, cells grown to 80% confluence were

Abbreviations used: CHO, Chinese hamster ovary; β 1,4-GalNAcT, β 1,4-*N*-acetylgalactosaminyltransferase; β 1,4-galT, β 1,4-galactosyltransferase; α -LA, α -lactalbumin; lacdiNAc-R, GalNAc β 1,4GlcNAc-R; RT-PCR, reverse-transcriptase-mediated PCR.

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Figure 1 Detection of α -LA expression in stable cells

(A) The expression of α -LA at mRNA level was analysed by RT-PCR with total RNA prepared from five independent stable cell lines, Lec8-L7 to Lec8-L5 (lanes 1–5) and mock-treated cells (lane m, vector only transfected), with bovine mammary gland (lane mg) as a positive control. (B) Western blot analysis was performed to detect α -LA protein expression in culture medium from stable cells, Lec8-L2, Lec8-L4 and Lec8-L5 (lanes 2, 4 and 5) and from mock-treated cells (lane m), and in medium only (lane M).

incubated with D-[6- 3 H]glucosamine (200 μ Ci/ml) for 24 h at 37 $^{\circ}$ C in complete medium as described [24]. Single cells stably expressing α -LA were maintained in complete medium containing 1 mg/ml G418 antibiotics (Gibco-BRL).

Plasmid construction and DNA transfection

The full-length bovine cDNA for α -LA was prepared by reverse-transcriptase-mediated PCR (RT-PCR) with mRNA isolated

from bovine mammary gland [26] and was subcloned into the unique *Eco*RI site of the pCDNA6 expression vector (Invitrogen) with the sense primer 5'-CAGCGAATTCATATGATGTC-CTTTGTCTCTCTG-3' and the anti-sense primer 5'-GCAC-CTCGAGTCACAACCTTCTCACAGAG-3'. Stable Lec8 cells expressing simian virus 40 T-antigen, designated Lec8-T cells, were isolated by gene transfection of simian virus 40 T-antigen cDNA subcloned into pCDNA3 [27] followed by selection with G418 antibiotics. Gene transfection was performed with Lipofectamine in accordance with the manufacturer's instructions (Life Technologies). For the isolation of stable α -LA transfectants, Lec8-T cells were gene-transfected with cDNA encoding α -LA cloned in pCDNA6 vector, and stable cells were selected with both G418 (1 mg/ml) and blasticidin (5 μ g/ml) antibiotics.

RT-PCR and Western blot analyses

Total RNA and mRNA was isolated from α -LA stable transfectants and bovine mammary gland tissues. RT-PCR was performed with these RNA species as a template to amplify the α -LA message, in accordance with the manufacturer's protocols (Promega). The serum-free media obtained from stable cell cultures were freeze-dried and subjected to SDS/PAGE under reducing conditions, followed by electrophoretic transfer to nitrocellulose membrane with the use of standard protocols. Western blot analysis was performed with rabbit anti-(bovine α -LA) IgG (1 μ g/ml) and mouse monoclonal anti-rabbit IgG antibody conjugated with peroxidase (1:4000 dilution; Sigma). Protein signal for α -LA was detected with enhanced chemiluminescence reagents (ECL $^{\circ}$; Amersham) followed by exposure to BioMax-MR-1 film (Kodak Co.).

Figure 2 Analysis of lacdiNAc sequence in D-[6- 3 H]GlcNH $_2$ -labelled glycopeptides by descending paper chromatography

Total D-[6- 3 H]GlcNH $_2$ -labelled glycopeptides prepared from mock cells (A) and Lec8-L5 stable cells (B) were treated with and without β -N-acetylhexosaminidase (buffer only). The monosaccharides GlcNAc/GalNAc released were analysed by descending paper chromatography after treatment with buffer only (left panels) and with β -N-acetylhexosaminidase (right panels).

Figure 3 Analysis of lacdiNAc sequence in D-[6- 3 H]GlcNH $_2$ -labelled N-linked oligosaccharides from Lec8-L5 stable cells

(A) D-[6- 3 H]GlcNH $_2$ -labelled total glycoproteins were treated with buffer only (left panel) and with N-glycanase F (right panel) and the released N-linked oligosaccharides were separated on Sephadex G-50. Abbreviations: Vo, void volume; Ve, eluted volume. (B) The presences of GlcNAc and GalNAc in pure N-linked oligosaccharides were analysed by descending paper chromatography after treatment with buffer only (left panel) and with β -N-acetylhexosaminidase (right panel).

Preparation of glycopeptides and analyses of N-linked oligosaccharides

The total glycoproteins were prepared by the precipitation of metabolically radiolabelled cell lysates with 10% (w/v) trichloroacetic acid. To prepare glycopeptides, protein pellets were treated with Pronase for 24 h under a toluene atmosphere, as described [24]. The N-linked oligosaccharides were prepared by the treatment of total glycoproteins with N-glycanase F in 50 mM sodium phosphate buffer, pH 7.5, containing 0.5% SDS and 50 mM 2-mercaptoethanol for 48 h in accordance with the manufacturer's instructions (Roche Molecular Biochemicals). The N-linked oligosaccharide released was separated on a column of Sephadex G-50. The lacdiNAc sequence was analysed by descending paper chromatography [borate-impregnated Whatman no. 1 in butan-1-ol/pyridine/water (6:4:3, by vol.)] for the separation of terminal GalNAc and GlcNAc residues released by treatment with β -N-acetylhexosaminidase as described [24].

Glycosyltransferase assay and immunodepletion assay of the α -LA- β 1,4-galT complex

The confluent Lec8-*m* (mock transfectant) and Lec8-L5 cells (α -LA transfectants) were washed three times with cold PBS and cells were resuspended in 20 mM cacodylate buffer, pH 7.0, containing 1 mM CaCl $_2$. Cell suspensions were sonicated three times for 10 s each and centrifuged for 5 min at 600 *g* to remove nuclei. The postnuclear supernatant was diluted with 5 ml of

20 mM sodium cacodylate buffer, pH 7.0, and ultracentrifuged for 1 h at 100 000 *g* and 4 °C. Microsomal membrane pellets were separated and resuspended in 100 mM sodium cacodylate buffer, pH 7.0, containing 0.1% (v/v) Triton X-100, 2 mM MnCl $_2$, 20 mM GlcNAc and protease inhibitors. With microsomal membranes, the radioactive enzyme assays for β 1,4-galT and β 1,4-GalNAcT were conducted as described [24]. For immunodepletion assay of the α -LA- β 1,4-galT complex, the anti-(α -LA) IgG antibody (1:10 dilution) was added to microsomal membranes with dose increments and the immunocomplex was formed after incubation with rotation for 2 h at 4 °C followed by absorption overnight at 4 °C with 10 μ l of Protein A beads (Santa Cruz). Finally, the immunocomplex was removed and the supernatant was used for assaying β 1,4-GalNAcT activity, as described previously [23].

RESULTS AND DISCUSSION

Analysis of α -LA expression in stable transfectants by RT-PCR and immunoblotting

To investigate whether α -LA could modulate β 1,4-galT to generate lacdiNAc-R expression inside the cell, we transected bovine α -LA cDNA into Lec8 cells because these cells contain a truncated structure of N-linked oligosaccharides with a terminal GlcNAc [25] and isolated five stable transfectants that we designated Lec8-L1 to Lec8-L5. The stable cells were analysed by RT-PCR and immunoblotting to detect the expression of α -LA at the levels of RNA message and protein respectively. Significant

Figure 4 Comparative analysis of β 1,4-galT and β 1,4-GalNAcT enzyme activities and the immunodepletion of β 1,4-galT- α -LA complex-induced β 1,4-GalNAcT activity by the addition of polyclonal anti-(α -LA) IgG antibody in Lec8-*L5* microsomes

(A) The β 1,4-galT (black bars) and β 1,4-GalNAcT (hatched and grey bars) activities of Lec8-*L5* microsomes were measured in the presence (grey bars) or absence (hatched bars) of exogenous α -LA. (B) β 1,4-GalNAcT activity induced by the β 1,4-galT- α -LA complex was immunodepleted by the dose-dependent addition of polyclonal anti-(α -LA) IgG antibody followed by absorption on Protein A beads in Lec8-*L5* microsomes in the presence of 2 mM MnCl_2 and 20 mM GlcNAc. 'Excess' indicates that 30 μ l of anti-(α -LA) IgG was added to microsomes. Grey columns, Lec8-*m*; hatched columns, Lec8-*L5*. Results are representative of three independent experiments.

amounts of α -LA transcript were found in Lec8-*L2*, Lec8-*L4* and Lec8-*L5*, whereas α -LA transcripts were either very faintly or not detected in Lec8-*L1*, Lec8-*L3* and mock-treated cells (Lec8-*m*) (Figure 1A). The expression of α -LA at the protein level was also examined, showing that α -LA protein was clearly detected in the culture medium of Lec8-*L2*, Lec8-*L4* and Lec8-*L5* cells and was correlated quantitatively with the level of RNA transcripts (Figure 1B). As expected, no signal for α -LA was detected in the culture medium of Lec8-*m* and in the medium itself (Figure 1B). On the basis of α -LA expression, Lec8-*L5* cells were used for the further presentation of data because of their highest α -LA expression of the five stable cell lines. It should be mentioned that the laciNAc analyses of N-linked oligosaccharides from Lec8-*L2* and Lec8-*L4* cells gave similar results to Lec8-*L5* cells (results not shown).

Metabolic radiolabelling of cells with D-[6- ^3H]glucosamine and analysis of total glycopeptides

To investigate the formation of the laciNAc-R structure, Lec8-*m* and Lec8-*L5* cells were metabolically radiolabelled with D-[6- ^3H]glucosamine; total glycopeptides were prepared and analysed to identify whether they were modified with the laciNAc-R

sequence *in vivo*. As shown in Figure 2, the treatment of Lec8-*L5* glycopeptides with β -N-acetylhexosaminidase generated both GalNAc and GlcNAc as monosaccharides, whereas the same treatment of Lec8-*m* glycopeptides released only terminal GlcNAc. These results demonstrate that total glycopeptides from Lec8-*L5* cells clearly contain laciNAc structure, whereas those from Lec8-*m* cells do not.

Analysis of N-linked oligosaccharides released by treatment with N-glycanase F

To determine further whether the laciNAc-R structure was present on N-glycosylation, the total glycoproteins from D-[6- ^3H]glucosamine-radiolabelled Lec8-*L5* cells were treated with peptide N-glycanase F. The released N-linked oligosaccharides were separated on Sephadex G-50 and analysed directly by treatment with β -N-acetylhexosaminidase. As shown in Figure 3, the pure N-linked oligosaccharides synthesized from Lec8-*L5* cells were clearly modified with the laciNAc-R structure. The level of laciNAc-R modification on N-glycosylation turned out to be approx. 10% of the saturation level (Figure 3).

Association of endogenous β 1,4-galT with α -LA and immunodepletion of α -LA- β 1,4-galT complex

To elucidate further the mechanism of laciNAc-R modification *in vivo*, we measured the enzyme activities of β 1,4-galT and β 1,4-GalNAcT in the absence or presence of exogenously added α -LA. It was shown consistently that parental Lec8 cells contained endogenous β 1,4-GalNAcT activity, although this enzyme was not capable of acting on any endogenous acceptor glycoproteins [24]. In the absence of α -LA, the β 1,4-galT activity of mock-treated cells was 10–15% higher than that of Lec8-*L5* cells, whereas the β 1,4-GalNAcT activity of mock-treated cells was 15–20% lower than that of Lec8-*L5* cells (Figure 4A). These results indicate that approx. 10–15% of endogenous β 1,4-galT was likely to exist as an α -LA-associated complex and that this association might induce additional β 1,4-GalNAcT activity up to 15–20% of the endogenous β 1,4-GalNAcT activity in Lec8-*L5* cells. As shown in Figure 4(A), the addition of exogenous α -LA induced additional β 1,4-GalNAcT activity in both mock-treated cells and Lec8-*L5* cells with a small increase, suggesting that endogenous β 1,4-GalNAcT activity might be suppressed by exogenously added α -LA under these conditions. The addition of anti- α -LA antibody to Lec8-*L5* cell lysates followed by absorption on Protein A beads resulted in a dose-dependent immunodepletion of the induced β 1,4-GalNAcT activity to the level of endogenous β 1,4-GalNAcT activity, whereas the same treatment of Lec8-*m* cell lysates had no effect on endogenous β 1,4-GalNAcT activity (Figure 4B). As shown in Figure 4(B), the recovery of β 1,4-GalNAcT activity to the initial level was observed with the excessive addition of anti- α -LA antibody, indicating that an extra amount of antibody might lead to oversaturation of the Protein A beads. Again, these results show that a certain portion of cellular β 1,4-galT exists as an α -LA-associated complex in Lec8-*L5* cells and that this association induces not only additional β 1,4-GalNAcT activity but also the synthesis of laciNAc-R *in vivo*. The complete scheme of α -LA-mediated laciNAc-R synthesis in Lec8-*L5* cells is summarized in Figure 5. Thus our results demonstrate that stably expressed α -LA in Lec8 cells can associate with endogenous β 1,4-galT and that this α -LA- β 1,4-galT association induces the nucleotide sugar switching of endogenous β 1,4-galT to initiate laciNAc-R synthesis *in vivo*. In the present study we have identified for the first time the α -LA-mediated nucleotide sugar switching of β 1,4-

Figure 5 Diagram of α -LA-mediated cellular lacdiNAc-R expression in the lumen of trans-Golgi membranes

In parental Lec8 cells, the active β 1,4-galT (GT) stays in the trans-Golgi without a catalysing function because donor nucleotide sugar, UDP-Gal, is deficient inside Golgi, although the substrate glycoprotein (SG, in which G indicates a terminal GlcNAc residue) exists. However, in α -LA-expressing Lec8-L5 cells, the β 1,4-galT can associate with α -LA (filled circles) and consequently the β 1,4-galT can be modulated to utilize UDP-GalNAc instead of UDP-Gal. Finally, α -LA-bound β 1,4-galT catalyses the specific transfer of a GalNAc residue from UDP-GalNAc to the terminal GlcNAc moiety (G) on SG to synthesize the lacdiNAc-R structure.

galT in Lec8 cells *in vivo*, which causes these cells to synthesize the lacdiNAc structure as a neo-glycosylation. Our findings also provide a novel development of non-enzyme-induced oligosaccharide remodelling of lacdiNAc-R expression at the cellular level. In general, the synthesis of the lacdiNAc-R sequence could be directly controlled by UDP-GalNAc:GlcNAc-R β 1,4-GalNAcT. Several studies have presented some evidence to suggest that there might be at least two different types of enzyme, of which one is specific for the tri-amino acid Pro-Xaa-Arg/Lys motif and the other is non-specific [28–32]. However, little is understood about the nature of the β 1,4-GalNAcT and its regulatory role in lacdiNAc-R biosynthesis. In this context we have previously investigated cell-line-based β 1,4-GalNAcT activity and lacdiNAc-R sequence, showing that the lacdiNAc-R structure was synthesized only in human 293 cells but not in CHO or CHO-derived Lec8 cells [24]. These results confirmed previous results that recombinant bovine lutropin produced in CHO cells does not contain the lacdiNAc-R sequence, whereas recombinant protein C and tissue factor pathway inhibitor ('TFPI') expressed in human 293 cells acquire lacdiNAc-R structure [33,34]. Alternatively, it is possible that cellular lacdiNAc-R synthesis could be achieved with the aid of β 1,4-galT instead of β 1,4-GalNAcT. In fact, α -LA has long been known as a modifier protein that acts specifically on β 1,4-galT to change acceptor substrate specificity in the mammary gland during lactose synthesis [35]. Taken together with our previous study [23], our present results demonstrate that this modifier protein clearly has another function of β 1,4-galT modulation to alter the donor substrate specificity of the nucleotide sugar from UDP-Gal to UDP-GalNAc. Interestingly, recent studies have shown that various secreted proteins in bovine colostrum contained a lacdiNAc-R structure [36,37]. It would be worth determining whether the physiological environment *in vivo* might allow endogenous β 1,4-galT to initiate α -LA-mediated lacdiNAc-R synthesis on bovine milk glycoproteins during lactation. Furthermore, it would be useful to use this α -LA stable trans-

fectant as a recombinant host for the production of lacdiNAc-containing glycoproteins, such as cytokines and hormones [38]. We are currently investigating the interaction of α -LA with β 1,4-galT *in vivo* to maximize the expression of lacdiNAc-R; future studies should be aimed at identifying endogenous acceptor glycoproteins for β 1,4-GalNAcT induced in stable transfectants of α -LA.

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