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Generation of novel chimeric LacdiNAcS by gene fusion of α-lactalbumin and β1,4-galactosyltransferase 1

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Abstract Novel chimeric lacdiNAc (GalNAc(β1-4) GlcNAc) synthase (c-LacdiNAcS) was generated by gene fusion of α-lactalbumin (α-LA) and β1,4-galactosyltransferase 1 (β1,4-GalT1). c-LacdiNAcS was expressed in Lec8 Chinese hamster ovary (Lec8 CHO) cells and exhibited *N*-acetylgalactosaminyltransferase (GalNAcT) activity in the absence of exogenous α-LA as well as other glycosyltransferase activities including lactose synthase (LacS), and β1,4-GalT. These glycosyltransferase activities of c-LacdiNAcS were compared to those activities induced in LacS system under the co-presence of bovine β1, 4-GalT1 and α-LA, indicating that each domain of α-LA and β1,4-GalT1 on c-LacdiNAcS is not only folding correctly, but also interacting together. Furthermore, c-LacdiNAcS was found to be auto-lacdiNAcylated and can synthesize lacdiNAc structures on cellular glycoproteins, demonstrating that GalNAcT activity of c-LacdiNAcS is functional in Lec8 CHO cells.

Keywords β1,4-GalT1 · α-LA · LacdiNAc · c-LacdiNAcS · Lec8 CHO cells

Abbreviations

β1,4-GalT1	β1,4-galactosyltransferase 1
LacS	lactose synthase
GalNAcT	*N*-acetylgalactosaminyltransferase
c-LacdiNAcS	chimeric lacdiNAc synthase
lacNAc	Gal-β1,4-GlcNAc
lacdiNAc	GalNAc-β1,4-GlcNAc
α-LA	α-lactalbumin
GlcNAc	*N*-acetylglucosamine
Lec8 CHO	Lec8 Chinese hamster ovary

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In lactating mammary gland where high concentration of α-LA is present, β1,4-GalT1 changes its acceptor substrate specificity from *N*-acetylglucosamine (GlcNAc) to glucose and subsequently, produces lactose through the induced lactose synthase (LacS) activity [1]. Recent animal studies *in vivo* show that β1,4-GalT1 is indispensable to produce lactose in mammary gland [2]. Among mammalian β1,4-GalTs [3], β1,4-GalT1 shows to induce LacS activity in presence of α-LA [4]. Previous study has shown that β1,4-GalT1 can be modulated by α-LA to induce nucleotide sugar switching activity from UDP-gal to UDP-GalNAc toward GlcNAc to produce lacdiNAc sequences [5]. Further studies have shown that lacdiNAc structures can be synthesized on cellular glycoproteins by α-LA expression *in vivo* [6]. Crystal structure of β1,4-GalT1 together with UDP-gal [7], and another crystal structure of LacS have revealed the molecular interactions of β1,4-GalT1 and α-LA [8].

LacdiNAc structure exists in various organisms [9] and can be modified by sulfation, fucosylation and sialylation [10, 11]. Sulfated lacdiNAc structure of leutropin hormone functions as a ligand of hepatic lectin for a rapid clearance [10]. LacdiNAc structure of glycodelin A executes immunosuppressive effects and contraceptive properties [12]. Fucosylated lacdiNAc sequence of recombinant protein C contains anti-inflammatory properties [13]. Particularly,

lacdiNAc structures of schistosomes appears to be involved in immunopathogenesis during schistosomiasis [14].

Although glycosyltransferase activities responsible for synthesizing lacdiNAc structures have not been fully identified yet, two different enzyme activities appear to be present. One is specific for pituitary glycoprotein hormone, which recognizes specific peptide motifs containing PXR/K sequences [15] and the other have non-restricted peptide specificity [16, 17]. Recently, several cDNAs encoding the latter type of enzymes containing GalNAcT activity have been cloned from *C. elegans* and mammalian cells [18, 19].

In the present study, a novel chimeric lacdiNAc (GalNAc (β1-4)GlcNAc) synthase (c-LacdiNAcS) was generated by gene fusion of α-LA and β1,4-GalT1. These results show that c-LacdiNAcS contains LacS activity and GalNAcT activity without aid of exogenous α-LA. Furthermore, the present data demonstrate that c-LacdiNAcS is auto-lacdiNAcylated and catalytically active for lacdiNAc biosynthesis in Lec8 CHO cells.

Materials and methods

Construction of cDNAs

Total RNAs from A431 cells (ATCC) were prepared by TripureTM reagents (Roche Biochemicals, Germany) and cDNA was prepared by AccessTM RT-PCR (Promega) amplification according to manufacture's protocols. Reaction mixtures contain 0.2 mM dNTPs mix, 50 pmol of each gene-specific sense and antisense primers, 1 mM $MgSO_4$, avian myeloblastosis virus-reverse transcriptase (5 U/μl), and Tfl DNA polymerase (5 U/μl) in a final volume of 50 μl. Reaction mixtures were incubated at 48°C for 45 min to synthesize the first strand cDNA, denatured at 94°C for 2 min, and subjected to 30 cycles of main PCR amplification. Soluble form of β1,4-GalT1 was obtained using gene-specific primers (sense: 5′-GTTCCTCGAGCGCCTGCCC CAACTGGTC-3′, and antisense: 5′-GACATTCGAAC TAATGATGATGATGATGATGGCTCGGTGTCCC GATGTCCAC-3′) (20) and PCR product of β1,4-GalT1 (1.0 Kb) was subcloned into pcDNA6 (Invitrogen). cDNA for bovine α-LA was obtained by PCR using gene-specific primers (sense: 5′-GTGTGCTAGCATGATGTCCT TTGTCTC-3′, antisense: 5′-CTGTCTCGAGCAACTTCT CACAGAGCCA-3′) as described previously [6] and subcloned into pcDNA6 vector (Invitrogen) to form pcDNA6-V5-His/α-LA. Soluble β1,4-GalT1 was produced by treatment of pcDNA6/β1,4-GalT1 with XhoI and BstBI and was ligated to pcDNA6-V5-His/α-LA digested with XhoI and BstBI to finally produce pcDNA6-V5-His/α-LA:β1,4-GalT1.

Stable expression and purification of chimeric LacdiNAcS (c-LacdiNAcS)

Lec8 Chinese hamster ovary (Lec8 CHO cells, ATCC) were transfected with the chimeric fusion construct (pcDNA6-V5-His/α-LA:β1,4-GalT1) using LipofectAmine^{TM} (Invitrogen) according to manufacturer's instructions. Stable cells were screened under 10 μg/ml blasticidin. Stable clones were cultured in VP-SFM (GibcoBRL) for three days and the chimeric enzyme (c-LacdiNAcS) was purified from culture media using Ni^{2+}-agarose (Qiagen) according to manufacturer's procedures. Briefly, culture media were centrifuged at 1,500×*g* for 15 min to remove cellular debris and incubated with Ni^{2+}-agarose beads on a rotating platform at 4°C for overnight in cold room. Beads were collected and washed with 50 column volumes of wash buffer containing 20 mM Tris–HCl, pH 7.2, 150 mM NaCl, and 0.05% Tween 20. Bound materials were eluted with 200 mM imidazole and resolved by SDS-PAGE for analyzing the purity of c-LacdiNAcS. Eluted fractions containing c-LacdiNAcS were dialyzed against 20 mM Tris–HCl (pH 7.2) buffer and purified c-LacdiNAcS was re-absorbed on Ni^{2+}-agarose beads for glycosyltransferase activity assays including GalT, LacS, and GalNAcT.

SDS-PAGE and Western blot analysis

Cell lysates and microsomal membranes prepared as described previously [6], and purified c-LacdiNAcS were subjected to 10% SDS-PAGE and electro-transferred for Western blotting with α-LA antibody (rabbit anti-bovine IgG, 500x dilution, Bethyl Laboratories Inc.) and SMLDN1.1 (anti-lacdiNAc monoclonal IgM, 1,000× dilution) [23]. In general, transferred NC membrane was blocked with 5% bovine serum albumin and probed with specific primary antibody followed by secondary antibody conjugated with horseradish peroxidase as described previously [6]. Immuno-signals were visualized on Super RX film (Fuji) using SuperSignal West Pico (Pierce). Treatment of β-*N*-acetylhexosaminidase (Sigma) was performed in 0.1 M sodium citrate buffer (pH 4.6) and treatment of *N*-glycanase F (Calbiochem) was carried out in 20 mM sodium phosphate buffer, pH 7.5 containing 0.5% SDS and 50 mM β-mercaptoethanol according to manufacturer's protocols.

Assays of glycosyltransferase activities

All glycosyltransferase activities were indicated in picomoles of sugar transferred per hour per 20 μl of Ni^{+2}-agarose beads on which purified c-LacdiNAcS was re-absorbed. For control assay, Ni^{+2}-agarose beads absorbed with culture media from mock cells (vector only transfected stable clones)

were used. As described previously [5, 9], reaction mixtures were contained by either 20 μl of purified c-LacdiNAcS re-absorbed on Ni^{2+}-agarose beads or 10 mU of bovine β1,4-galT1, 5 mM ATP, 20 mM GlcNAc or 10 mM glucose, 100 μM UDP-[^{3}H]galactose or UDP-[^{3}H]GalNAc (1 x 10^5 cpm, 50,000 cpm/nmol, Amersham). 10 mM $MnCl_2$, and 100 mM sodium cacodylate buffer (pH 7.5) in the absence or presence of α-LA in a final concentration of 10 mg/ml. Glycosyltransferase activities in LacS system were assayed under the co-presence of 10 mU of bovine β1,4-GalT1 and 10 mg/ml of α-LA (Sigma). For glycosyltransferase assays of acceptor substrate specificity for GalT and GalNAcT activities, GlcNAc oligomers (chito-oligomers obtained from Sigma, and GlcNAc monomers to pentamers prepared in this laboratory) were used in 1 mM final concentrations, and di-, tri-, and tetra-antennary structure of either agalacto- or asialo-*N*-linked oligosaccharides (Calbiochem & Takara) were used in 0.1–1 mM concentrations. Reaction mixtures were incubated for 2 h at 37°C and reaction products were isolated by a passage over Dowex AG 1-X8 column (Cl^- form, Sigma) and further separated by Bio-Gel P-10 as described [5]. Each fraction containing radioactive products was analysed by liquid scintillation counter (Beckman).

Metabolic sugar-radiolabeling and lacdiNAc analyses

Stable cells were metabolically radiolabeled in DMEM containing 1 mCi of 6-[^{3}H]GlcNH$_2$ for 24 h. Cell pellets were washed three times with cold PBS, delipidated by acetone washing, and treated with pronase (Calbiochem) for 24 h at 60°C in 0.1 M Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ [5]. Resulting glycopeptides were desalted on Sephadex G-25, V_o fractions were applied to ConA-Sepharose on a 2 ml column (0.7×5 cm) (Pharmarcia), and fractionated into ConA-I, -II, and -III glycopeptides at room temperature as described previously [6]. Glycopeptides not bound by ConA-Sepharose were designated ConA-I. Glycopeptides bound by the column were eluted first with 10 mM α-methyl glucoside (designated ConA-II) followed by 100 mM α-methyl mannoside (designated ConA-III). Resulting ConA-I and ConA-II glycopeptides were pooled, dried in a vacuum shaker, and desalted on a Sephadex G-10 [21]. ConA-I and ConA-II glycopeptides were treated with 50 mU of β-*N*-acetylhexosaminidase (Sigma) in 0.1 M sodium citrate buffer (pH 4.6) at 37°C overnight. Released GlcNAc and GalNAc were analyzed by descending paper chromatography for 65 h on Whatman No. 1 borate-impregnated filter paper in 6:4:3 solvent system (*n*-butyl alcohol/pyridine/water, 6:4:3) [6]. The radioactivity on the paper chromatograms was measured by liquid scintillation counter (Beckman).

Results

Construction of fusion cDNA and stable transfection to produce recombinant c-LacdiNAcS

As schematically illustrated in Fig. 1, bovine α-LA was constructed to be linked to partial human β1,4-GalT1 sequence, in which fusion construct was comprised of 1–

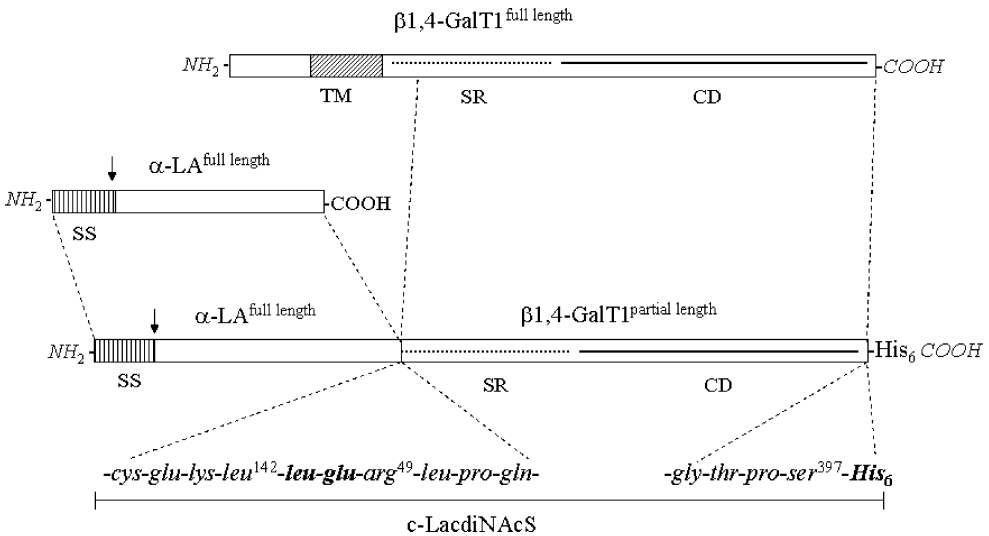

Fig. 1 Schematic representation of the fusion construct for c-LacdiNAcS. Human β1,4-GalT1 containing 1–397th amino acid residues and bovine α-LA containing 1–142nd amino acid residues was shaped in *box*, respectively. Partial sequence of β1,4-GalT1 comprising 49–397th amino acid residues was linked to *C*-terminus of α-LA, which resulted in a fusion construct encoding c-LacdiNAcS. Additional amino acids at the junction and His_6-tag sequence at the *C*-terminus of c-LacdiNAcS are *bold* treated. Cleavage of signal sequence was indicated by an *arrow*. *SS* signal sequence, *TM* transmembrane domain, *SR* stem region, *CD* catalytic domain

142nd amino acid residues from α-LA [22] and 49–397th amino acid residues from β1,4-GalT1 [20]. For stable transfection, Lec8 CHO cells were chosen as a host since these cells contained genetic defects on nucleotide sugar transporters, such as UDP-galactose and CMP-sialic acid [23] and could provide suitable acceptor substrates for GalNAcT activity [6]. Many stable clones were primarily screened by SDS-PAGE of serum-free culture medium (VP-SFM) and immunoblotting using anti-α-LA antibody (Fig. 2a). Three positive clones were selected and designated as Lg10, Lg35, and Lg60. As shown in Fig. 2a, three stable clones showed to strongly secrete the c-LacdiNAcS which corresponded to the expected molecular mass of 64 kDa protein (lane 3, 6, 9). Culture medium containing secreted c-LacdiNAcS from three stable clones was primarily absorbed with Ni^{2+}-agarose beads. c-LacdiNAcS bound on Ni^{2+}-agarose beads was used to assay glycosyltransferase activities, demonstrating that c-LacdiNAcS was found to contain multiple glycosyltransferase activities of GalT, LacS, and GalNAcT, respectively (Fig. 2b). These results suggest that each domain of α-LA and β1,4-GalT1 in c-LacdiNAcS appears to be correctly folding and completely interacting together. Secreted c-LacdiNAcS were mainly purified using Ni^{2+}-agarose affinity column from serum-free culture media of stable clones of either Lg10 or Lg60, and analyzed by SDS-PAGE followed by Coomassie staining and immunoblotting with anti-α-LA antibody, demonstrating that eluted fractions contain c-LacdiNAcS with more than 90% purity (Fig. 2c). This purified c-LacdiNAcS was re-absorbed on Ni^{2+}-agarose beads by which glycosyltransferase activities were directly assayed. As a control, Ni^{2+}-agarose beads absorbed with culture media from either wild type Lec8 CHO cells or mock cells (vector only transfected stable clones) were used in glycosyltransferase assays (results not shown).

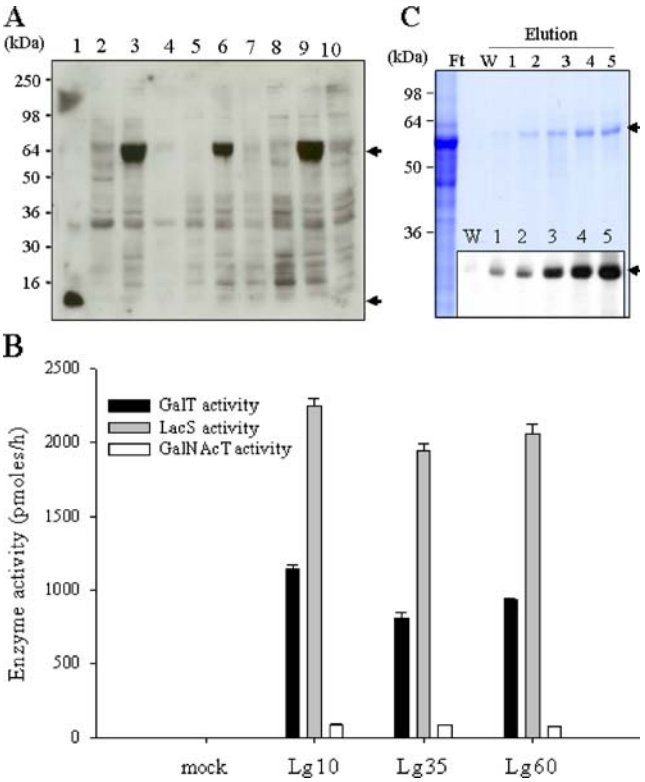

Fig. 2 Screening of stable cells and analyses of glycosyltransferase activities of purified c-LacdiNAcS. **a** Stable clone candidates including negative and positive clones were analyzed by SDS-PAGE and immunoblotting with anti-α-LA antibody. Immuno-signals for c-LacdiNAcS were detected in three positive clones (*lanes 3*, *6*, *9*) which were designated Lg10, 35, and 60, respectively, and indicated by an *upper arrow*. As a control, α-LA was loaded (*lane 1*) and indicated by a *lower arrow*. **b** Culture medium (VP-SFM) from three positive stable clones (*lanes 3*, *6*, *9*, designated Lg10, 35, and 60) was primarily absorbed with Ni^{2+}-agarose beads, and c-LacdiNAcS bound on Ni^{2+}-agarose beads was directly assayed for GalT, LacS, and GalNAcT activities as described in "Materials and methods." Ni^{+2}-agarose beads absorbed with culture media from mock cells (vector only transfected stable clones) were used in control assays of GalT, LacS, and GalNAcT activities (mock). All the assays were triplicated. **c** c-LacdiNAcS from serum-free culture media (VP-SFM) of Lg60 was purified with Ni^{+2}-agarose affinity column chromatography. Flow-through (*Ft*), washed (*W*), and eluted fractions (*lanes 1–5*) were analyzed by 10% SDS-PAGE with Coomassie staining and immunoblotting (*inset*) with anti-α-LA antibody. Expected molecular mass of Coomassie stained and immunoblotted c-LacdiNAcS were indicated by *arrows*

Comparison of glycosyltransferase activities of c-LacdiNAcS to those activities induced in LacS system under the co-presence of bovine β1,4-GalT1 and α-LA

GalT, LacS, and GalNAcT activities of c-LacdiNAcS were determined and compared to those activities induced in LacS system under the co-presence of bovine β1,4-GalT1 and α-LA (Fig. 3). Without α-LA, bovine β1,4-GalT1 showed only a GalT activity, and in LacS system, bovine β1,4-GalT1 was modulated to induce LacS and GalNAcT activities by exogenous addition of α-LA (Fig. 3a). c-LacdiNAcS possessed a significant amount of LacS and GalNAcT activities in the absence of α-LA (Fig. 3b). It should be noted that during the induction by α-LA, β1,4-GalT1 activity was reduced to more than about 30% of the original β1,4-GalT1 activity in the absence of α-LA. Also, the addition of α-LA to c-LacdiNAcS showed a similar inhibitory effect on its GalT activity but not on LacS and GalNAcT activities. Therefore, this inhibitory effect might be caused by some unexpected contaminants in α-LA preparation since no such inhibition occurred in case of c-LacdiNAcS itself. Comparisons of relative glycosyltransferase activities between pure c-LacdiNAcS and LacS system under the co-presence of bovine β1,4-GalT1 and α-LA are summarized in Table 1.

Next, biochemical properties of GalNAcT activity of c-LacdiNAcS were further analyzed. Synthesis of lacdiNAc

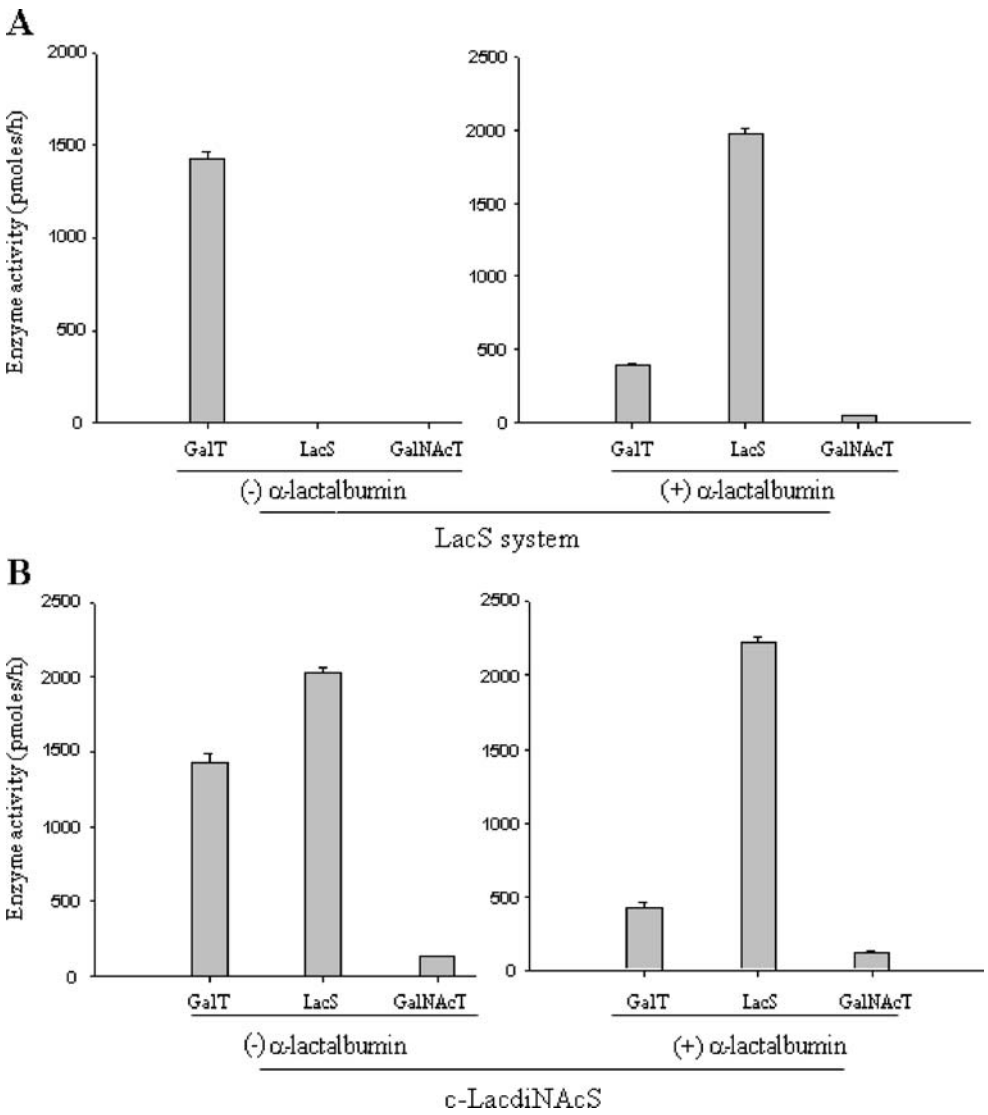

Fig. 3 Comparison of glycosyltransferase activities between c-LacdiNAcS and the LacS system. **a** GalT, LacS, and GalNAcT activities of the LacS system were assayed in the absence and presence of α-LA, respectively, using 10 mU of bovine β1,4-GalT1 as described in "Materials and methods." **b** GalT, LacS, and GalNAcT activities of the c-LacdiNAcS which purified from Lg60 stable clone and re-absorbed on Ni^{2+}-agarose beads were assayed in the absence and presence of α-LA, respectively, and compared to those activities induced in LacS system under the co-presence of bovine β1,4-GalT1 and α-LA. All the assays were triplicated

sequence was strongly enhanced in the presence of manganese divalent cations (Fig. 4a). Substrate preference between GalT and GalNAcT activity of c-LacdiNAcS showed that GalNAcT activity was highly sensitive to length of GlcNAc oilgomer, whereas GalT activity was much more tolerant (Fig. 4b–d). Further comparison of GalNAcT and GalT activities using agalacto-*N*-glycan substrates showed that GalNAcT activity of c-LacdiNAcS, and that activity induced in LacS system under the co-presence of bovine β1,4-GalT1 and α-LA preferred tri-antennary to di- and tetra-antennary structure (Fig. 4e). But GalT activity of c-LacdiNAcS, and that activity induced in the LacS system did not discriminate structural differences of agalacto-*N*-glycan substrates (Fig. 4f).

Table 1 Properties of glycosyltranferase activities between c-LacdiNAcS and LacS system under the co-presence of β1, 4-Ga1T1 and α-LA

Enzyme activity	LacS system		c-LacdiNAcS	
	(−)α-LA pmol/h (%)	(+)α-LA	(−)α-LA pmol/h (%)	(+)α-LA
GalT[a]	1,400 (100)	400 (28)	1,400 (100)	400 (28)
GalNAcT	1.4 (0.1)	47 (3.3)	122 (8.7)	120 (8.5)
LacS	0 (0)	2,000 (142)	2,000 (142)	2,200 (157)

[a] GalT activity was determined using 20 mM GlcNAc and 100 μM UDP-gal as described under "Materials and methods" and pre-adjusted to 100% to relatively evaluate GalNAcT and LacS activities. GlsNAc (20 mM) and 10 mM glucose was used as acceptor substrates for GalNAcT, and LacS activity assay, respectively. In the LacS system, 10 mU of bovine β1, 4-Ga1T was used in the presence of 10 mg/ml α-LA

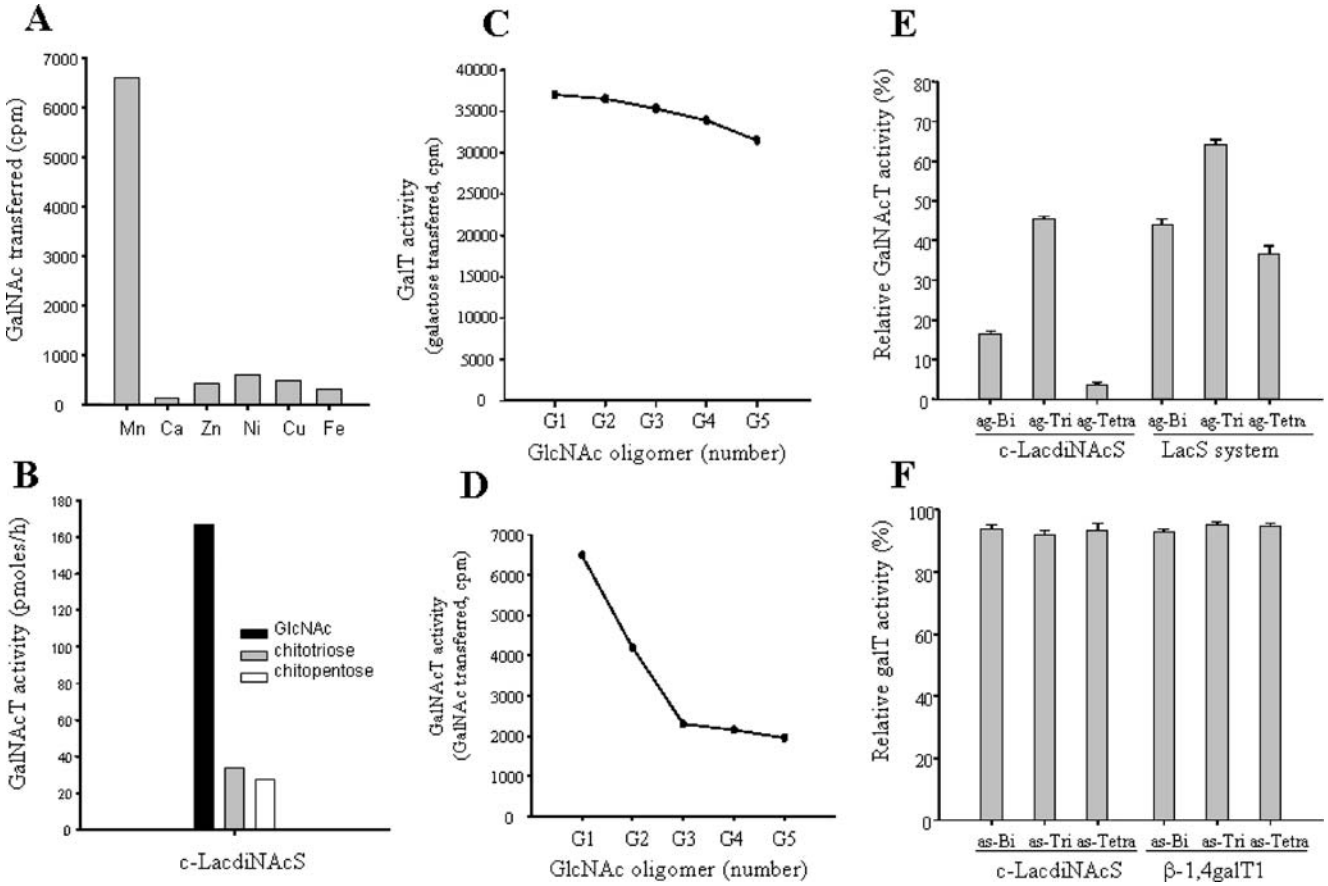

Fig. 4 Substrate preference between c-LacdiNAcS and the LacS system. **a**, **b** GalNAcT activity of c-LacdiNAcS, which purified c-LacdiNAcS from Lg60 stable clone and re-absorbed on Ni^{2+}-agarose beads was analyzed in the presence of various divalent cations, and using GlcNAc oligomers (purified products) as acceptor substrates. **c**, **d** Substrate preferences for GalT and GalNAcT activities of c-LacdiNAcS were analyzed using GlcNAc oligomers (monomer to pentamer, commercial products), respectively. **e**, **f** Substrate preferences for GalNAcT and GalT activities of c-LacdiNAcS, and LacS system under the co-presence of bovine β1,4-galT1 and α-LA were analyzed using agalacto-bi-/-tri-/-tetra-antennary *N*-glycans as acceptor substrates, respectively. GalT and GalNAcT activities were indicated as relative activities (%) compared to those activities (100%) when GlcNAc was used as an acceptor substrate, and UDP-gal or UDP-GalNAc used as a donor substrate, respectively. GalT and GalNAcT activities were assayed as described in "Materials and methods" and all the assays were triplicated

Expression of lacdiNAc structures *in vivo*

To further examine whether lacdiNAc structures could be synthesized *in vivo* by c-LacdiNAcS, cellular glycoproteins from Lg10, 35, and 60 stable clones were analyzed by immunoblotting using a SMLDN1.1 (monoclonal antibody against lacdiNAc glyco-epitope) [24]. Expression of lacdiNAc structures was positively detected on several subsets of protein bands from all stable clones, but not from control cells. (Fig. 5a). These results suggest that GalNAcT activity of c-LacdiNAcS is functionally active to produce lacdiNAc structures in Lec8 CHO cells. Purified c-LacdiNAcS were analyzed by immunoblotting using SMLDN1.1 before and after *N*-glycanase or β-*N*-acetylhexosaminidase treatment, respectively. As shown in Fig. 5b, c-LacdiNAcS showed strong reactivity to SMLDN1.1, suggesting auto-lacdiNAcylation on its *N*-linked oligosaccharides. Indeed, *N*-glycanase treatment totally abolished SMLDN1.1 reactivity (Fig. 5b, lane 4). Also, c-LacdiNAcS completely lost lacdiNAc determinants after β-*N*-acetylhexosaminidase treatment, further demonstrating that lacdiNAc sequence was neither elongated nor modified (Fig. 5b, lane 2).

To directly confirm lacdiNAc biosynthesis by c-LacdiNAcS, stable clones were metabolically radiolabeled with 6-[^{3}H]$GlcNH_2$ and total glycopeptides were applied on ConA-Sepharose (Fig. 5c). Previous studies have shown that ConA-Sepharose does not interact with high affinity to *O*-glycans, and *N*-glycans containing complex-type tri- and tetra-antennary oligosaccharide structures, whereas it interacts with high affinity to high mannose-type and hybrid-type *N*-glycans [21]. ConA-I glycopeptides were treated with β-*N*-acetylhexosaminidase to release terminal GalNAc

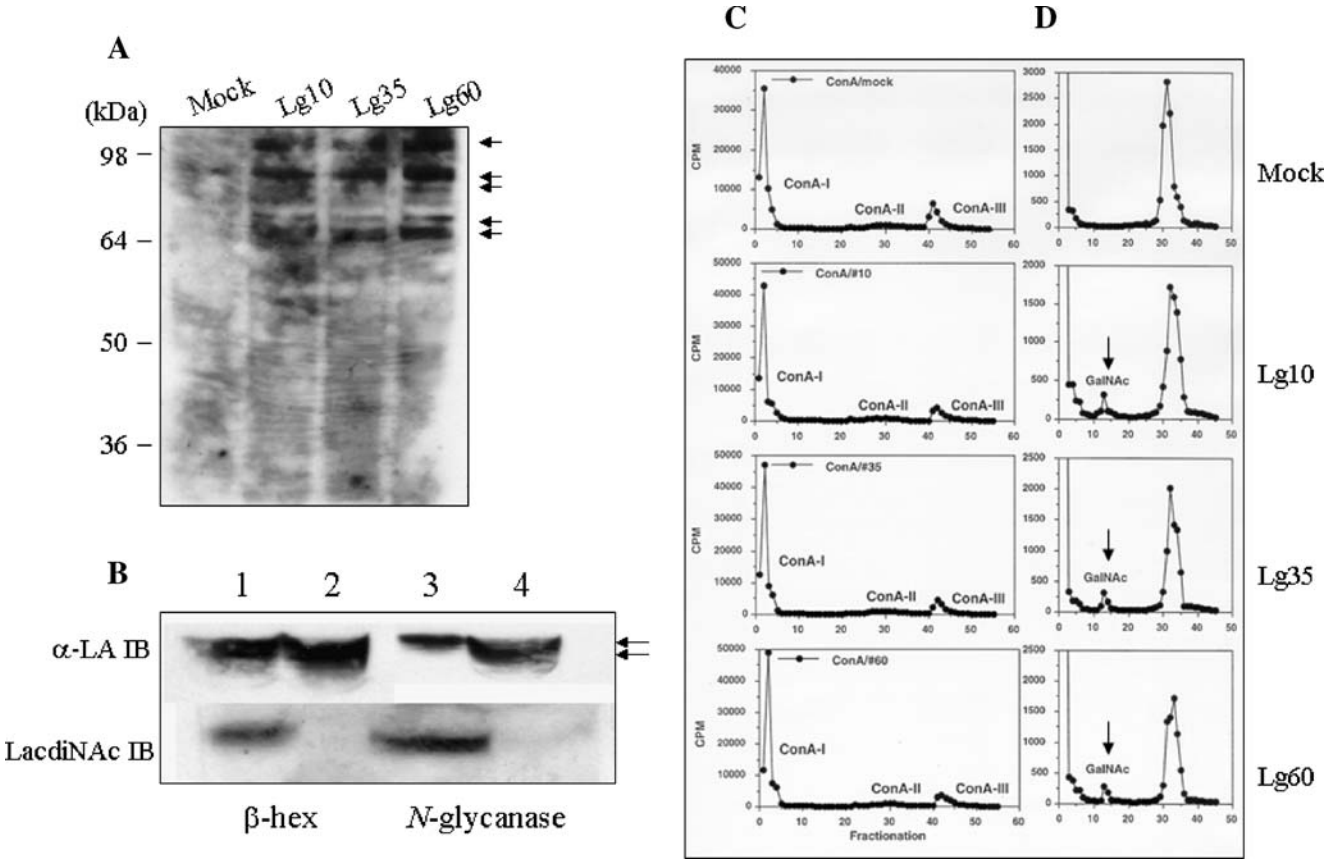

Fig. 5 Immunoblotting and radiolabeling analyses of lacdiNAc biosynthesis in stable cells. **a** LacdiNAc biosynthesis in stable clones was analyzed by immunoblotting using SMLDN1.1 mAb. Several subsets of glycoproteins positively probed by SMLDN1.1 were indicated by *arrows*. **b** c-LacdiNAcS from Lg60 was analyzed by immunoblotting using α-LA and SMLDN antibody, respectively, before (−) and after (+) treatment with β-*N*-acetylhexosaminidase (*lanes 1*, *2*) or *N*-glycanase (*lanes 3*, *4*). Glycosylated and de-glycosylated form of c-LacdiNAcS was indicated by *upper and lower arrows*, respectively. **c** Glycopeptides from 6-[^{3}H]GlcNH$_2$-radiolabeled Lg60 stable clone were fractionated into ConA-I (not bound by ConA-Sepharose), -II (eluted with 10 mM α-methy-glucoside), and -III (eluted with 100 mM α-methyl-mannoside) by ConA-Sepharose column chromatography. **d** ConA-I radiolabeled glycopeptides containing tri-/tetra-antennary *N*-glycans and *O*-glycans were pooled and treated with β-*N*-acetylhexosaminidase to release β-linked terminal sugar residues. Released GlcNAc and GalNAc were analyzed by descending paper chromatography in 6:4:3 solvent system as described in "Materials and methods." Under this system, fast and slow migrating peak from the origin is GlcNAc and GalNAc, respectively. Released GalNAc is indicated by *arrows* in stable clones of Lg10, 35, and 60

and GlcNAc residues and analyzed by descending paper chromatography in 6/4/3 solvent system. It should be noted that GalNAc presents in *O*-linked oligosaccharides will not be cleaved by this enzyme, because of its α-glycosidic linkage. Therefore, all the GalNAc residues released by β-*N*-acetylhexosaminidase treatment are originating from *N*-linked oligosaccharides. As expected, GalNAc was released from ConA-I glycopeptides of all stable clones, but not from control cells, while GlcNAc was released from both stable and control cells (Fig. 5d). ConA-II glycopeptides were also analyzed by the same treatment of β-*N*-acetylhexosaminidase resulting in similar patterns as in the case of ConA-I glycopeptides (results not shown). Together, these results indicate that lacdiNAc structures can be formed on both di-antennary and tri-/tetra-antennary *N*-linked oligosaccharides in Lec8 CHO cells.

Discussion

It has long been known that β1,4-GalT1 is modulated to induce LacS activity by an interaction with α-LA in mammary gland [1]. Previously, it has been shown that bovine β1,4-GalT1 can be switched to induce GalNAcT activity *in vitro* in the presence of α-LA [5]. Further studies have reported that stable expression of α-LA in Lec8 CHO cells can lead to *in vivo* formation of lacdiNAc structures [6]. In the present study, a novel c-LacdiNAcS was generated by chimeric fusion of α-LA and β1,4-GalT1. c-LacdiNAcS was found to possess intrinsically LacS and GalNAcT activities without exogenously added α-LA.

Interestingly, c-LacdiNAcS showed about three fold higher GalNAcT activity than that activity induced in LacS system under the co-presence of bovine β1,4-GalT1 and α-

LA (Table 1). It should be noted that GalNAcT activity was measured under the condition that GalT activity was equally adjusted in c-LacdiNAcS and the LacS system (Fig. 3a, b). Particularly, GalNAcT activity of c-LacdiNAcS prefers tri-antennary *N*-glycans rather di- and tetra-antennary *N*-glycans as acceptor substrates although GalT activity of c-LacdiNAcS does not significantly discriminate these structural differences (Fig. 4e, f). These observations were repeatedly observed in the LacS system, indicating that GalNAcT activity of c-lacdiNAcS may reflect a similar mode of action to that activity induced in lacS system under the co-presence of bovine β1,4-GalT1 and α-LA. This c-LacdiNAcS, to my knowledge, is the first engineered glycosyltransferase that exhibits multiple catalytic activities including GalT, LacS, and GalNAcT.

Recent studies for lacdiNAc synthesis in mammalian cells have shown that the pathway for lacdiNAc biosynthesis is likely regulated by several cell-specific GalNAcT activities and also, lacdiNAc biosynthesis competes with GalT-dependent Gal(β1-4)GlcNAc (lacNAc) biosynthesis [25]. Interestingly, analyses of *N*-glycosylation of secreted c-LacdiNAcS from Lg60 cells showed that c-LacdiNAcS was auto-lacdiNAcylated (Fig. 5b). Since α-LA stably expressed in Lec8 CHO cells appears not to be *N*-glycosylated although it has three potential *N*-glycosylation sites [6], it is very likely that lacdiNAcylation of c-LacdiNAcS must occur at the *N*-glycosylation site of β1,4-GalT1. Furthermore, lacdiNAc structure of c-LacdiNAcS was shown to be rarely elongated by sialylation or sulfation since recognition of lacdiNAc epitope by SMLDN1.1 is completely eliminated after β-*N*-acetylhexosaminidase treatment (Fig. 5b). This is consistent with previous studies showing the lack of α2,6-sialyltransferase and GalNAc-specific sulfotransferase in CHO and Lec8 CHO cells, respectively [26]. Furthermore, previous studies have confirmed the lack of lacdiNAc biosynthesis in wild type Lec8 CHO cells [9]. Although the precise location of lacdiNAc modification on *N*-linked oligosaccharides in stable clones is not defined, biochemical analyses of metabolically radiolabeled stable clones suggest that lacdiNAc structures can be synthesized on both di- and tri-/tetra-antennary *N*-linked oligosaccharides (Fig. 5c, d). A recent report has shown that Lec8 CHO cells transfected with cDNA encoding GalNAcT activity from *C. elegans* initiate to synthesize lacdiNAc sequence as well as its polymeric form, poly-lacdiNAc sequence [25]. In the present data, based on no significant decrease of molecular mass after β-*N*-acetylhexosaminidase treatment of c-LacdiNAcS, it seems to be unlikely that *N*-linked oligosaccharides of c-LacdiNAcS contains poly-lacdiNAc structure. However, it will be of interest to perform more detailed analyses whether Lg60 cells can synthesize poly-lacdiNAc structures.

At present, mode of action of c-LacdiNAcS is expected to be achieved through the correct folding of each domain of α-LA and GalT1 and interacting together. Here, I propose possible explanations for molecular interactions of c-LacdiNAcS as either *cis*- (interaction within a monomer) or *trans*-interaction (interaction between monomers). Based on previous observations that considerable amount of α-LA in a level of several mg quantities is required for modulating bovine β1,4-GalT1 in the LacS system [5], it is very possible that c-LacdiNAcS acts in *cis*- rather *trans*-interaction.

Although lacdiNAc structures have been widely recognized in a growing number of species including parasites and mammals, its biological significance is just now emerging [27, 28]. Thus, the present c-LacdiNAcS and stable cell lines producing recombinant c-LacdiNAcS can provide valuable tools for identifying roles of lacdiNAc structures and producing lacdiNAc-containing glycoproteins in mammalian cells.

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