

Developmental Regulation of a Pregnancy-specific Oligosaccharide Structure, NeuAca α 2,6GalNAc β 1,4GlcNAc, on Select Members of the Rat Placental Prolactin Family*

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Successful pregnancy is dependent upon an array of signaling proteins secreted by the trophoblast cells of the placenta. Among these is a group of proteins related to pituitary prolactin, known as the prolactin/growth hormone family. These proteins are expressed at specific times during gestation and synthesized in distinct trophoblast cell types in the rat placenta. We report here that select members of this family, prolactin-like protein (PLP-A), PLP-B, PLP-C, decidual/trophoblast PRP, and placental lactogen I variant, only which are expressed in the spongiotrophoblast, late in rat placental development bear Asn-linked oligosaccharides terminating with NeuAca α 2,6GalNAc β 1,4GlcNAc β -R. This reflects the concurrent expression of these prolactin/growth hormone family members with the peptide-specific β 1,4GalNAc-transferase and an α 2,6-sialyltransferase, which can add sialic acid to terminal β 1,4-linked GalNAc. We have determined that at least one of the prolactin-like proteins, PLP-A, is recognized by the protein-specific GalNAc-transferase. The presence of NeuAca α 2,6GalNAc β 1,4GlcNAc β -R on only a limited number of glycoproteins synthesized by the spongiotrophoblasts between mid gestation and birth reflects the need for both the GalNAc-transferase and the peptide recognition determinant for efficient addition of GalNAc. Thus, expression of the GalNAc-transferase and specific members of the prolactin/growth hormone family is developmentally regulated in the rat placenta, suggesting a physiological role for the terminal NeuAca α 2,6GalNAc β 1,4GlcNAc β -R sequence on Asn-linked oligosaccharides of these proteins.

The placenta is a complex organ that is essential for successful growth and maturation of the fetus throughout pregnancy (1). In addition to bringing oxygen and nutrients to the fetus, the placenta produces a number of hormones, cytokines, and growth factors which influence the endocrine, immune, and metabolic functions of the mother. Among these is a group of closely related proteins synthesized by the rat placenta, the prolactin/growth hormone family. We demonstrate here that specific members of this family bear asparagine (Asn)-linked oligosaccharides with NeuAca α 2,6GalNAc β 1,4GlcNAc β . This is

the result of high expression levels of a protein-specific GalNAc-transferase in the rat placenta. Levels of the transferase increase from undetectable at mid gestation to maximal at day 18 of gestation. This corresponds precisely with the expression of the prolactin/growth hormone family members PLP¹-A, PLP-B, PLP-C, d/t PRP, and PL-Iv by spongiotrophoblasts from the junctional zone of the placenta. At least one of these family members, PLP-A, contains a recognition determinant that can be utilized by this protein-specific GalNAc-transferase, which we have previously shown (2–5) to mediate β 1,4-linked GalNAc addition to Asn-linked oligosaccharides on the pituitary glycoprotein hormones lutropin and thyrotropin.

We have shown previously that unique Asn-linked oligosaccharides terminating with the sequence SO₄-4-GalNAc β 1,4GlcNAc β 1,2Man, which is closely analogous to the oligosaccharide structure on the placental prolactin family members, are present on the pituitary glycoprotein hormones lutropin and thyrotropin and are critical for the expression of full biological activity *in vivo* (6–8). The sulfated oligosaccharides determine the circulatory half-life of the glycoproteins bearing them because they are recognized by a receptor, the GalNAc-4-SO₄ receptor, in hepatic endothelial cells, which rapidly removes these glycoproteins from circulation (9–12). In the case of lutropin, the short circulatory half-life is essential for producing the episodic rise and fall of hormone in the blood throughout the ovulatory cycle (13–15). The presence of terminal GalNAc-4-SO₄ on lutropin and thyrotropin reflects the action of a protein-specific GalNAc-transferase and a GalNAc-4-sulfotransferase expressed in the gonadotrophs and thyrotrophs of the pituitary (16). The peptide sequences recognized by the GalNAc-transferase consist of a cluster of basic amino acids in the α subunit and a similar cluster of basic residues in the β subunit of lutropin (4, 5). The recognition determinant on the α subunit, as well as GalNAc-4-SO₄, is found on pituitary glycoprotein hormones of all classes of vertebrates, indicating that these are highly conserved and biologically important structures (17). In addition, the levels of the GalNAc-transferase in the pituitary gonadotroph rise and fall in parallel to the expression of lutropin in response to changing estrogen levels thus assuring that the oligosaccharides on lutropin are always fully modified with GalNAc-4-SO₄ (18).

A rapidly increasing number of glycoproteins that bear Asn-linked oligosaccharides with β 1,4-linked GalNAc, but which

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¹ The abbreviations used are: PLP, prolactin-like protein C; d/t PRP, decidual/trophoblast prolactin-related protein; PL-Iv, placental lactogen I variant; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; WGA, wheat germ agglutinin; WFA, *Wisteria floribunda* agglutinin; hCG, human chorionic gonadotropin; Trf, transferrin; ConA, concanavalin A; PAGE, polyacrylamide gel electrophoresis; TGC, trophoblast giant cell(s); r, recombinant; h, human; -R, core Asn-linked oligosaccharide.

are unrelated to the glycoprotein hormones, have subsequently been described. Like the glycoprotein hormones, the β 1,4-linked GalNAc on a number of these glycoproteins has been shown to be modified with sulfate at the 4-hydroxyl (19–25). In other instances oligosaccharides have been shown to bear terminal GalNAc (23, 25, 26), NeuAc α 2,6GalNAc (25, 27–32), and/or GalNAc β 1,4(Fuc α 1,3)GlcNAc (25, 29, 32, 33). Each of these structures is confined to a limited number of glycoproteins because of the peptide specificity of the GalNAc-transferase. In contrast to the sulfated oligosaccharides on lutropin and thyrotropin, biological significance of these structures remains to be established; however, the presence of such unique structures on limited numbers of glycoproteins makes it highly probable that they too will be recognized by specific receptors and play critical biological roles.

The concurrent expression in the rat placenta of the protein-specific GalNAc-transferase and specific members of the prolactin/growth hormone family which contain a peptide recognition determinant results in nearly quantitative modification of their Asn-linked oligosaccharides with the termini NeuAc α 2,6GalNAc β 1,4GlcNAc β . Taken together this suggests that these structures will play a role in the expression of the biological activity of prolactin/growth hormone family members during pregnancy.

EXPERIMENTAL PROCEDURES

Materials— ^{35}S PAPS was enzymatically synthesized as described previously (34) using $^{35}\text{SO}_4$ from ICN. UDP- ^3H GalNAc and CMP- ^3H NeuAc were purchased from American Radiolabeled Chemicals, Inc., St. Louis. Wheat germ agglutinin (WGA), *Wisteria floribunda* agglutinin (WFA), and *Clostridium perfringens* neuraminidase were obtained from Sigma.

Animals, Tissue Preparation, and Tissue Culture—Timed pregnant female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis). Animals were sacrificed by CO_2 inhalation on days 9–21 of pregnancy (day 0 being the day rats were found to be sperm-positive). The placentas were removed and washed with ice-cold sterile 20 mM phosphate buffer containing 0.15 M NaCl (pH 7.4). Placentas were homogenized and used for glycosyltransferase assays, or tissue slices were made for isolation of secreted glycoproteins. Undifferentiated Rcho-1 trophoblast cells were obtained from subconfluent cultures in fetal bovine serum, and differentiated Rcho-1 trophoblast cells were obtained from confluent cultures maintained in donor horse serum as described in (35, 36).

Glycosyltransferase Assays—Extracts of whole rat placentas, isolated junctional and labyrinth zones, or Rcho-1 trophoblast cells were made as described previously (16). The protein concentration of the combined postnuclear supernatants was determined by the Bradford dye binding assay (Bio-Rad) using bovine serum albumin as a standard.

Transfer of GalNAc or Gal by the glycoprotein hormone β 1,4GalNAc-transferase or the β 1,4Gal-transferase, respectively, to Asn-linked oligosaccharides acceptors on hCG was compared using the assay described previously (18).

^3H GalNAc transfer to Asn-linked oligosaccharides of different protein acceptors was assessed as described previously (4). Recombinant prolactin-like protein A (PLP-A) and recombinant prolactin-like protein B (PLP-B) were isolated from Chinese hamster ovary culture medium (37, 38). Day 18 rat placenta postnuclear supernatant (100 μg) or partially purified bovine pituitary GalNAc-transferase (2.4 microunits) was incubated with 0.25 mM UDP- ^3H GalNAc (1×10^7 cpm) and either agal-Trf, agal-hCG, agal-rPLP-A, or agal-rPLP-B at either 4 μM or 7.5 μM concentrations under conditions used previously for the GalNAc-transferase assay (4). The enzyme reaction was terminated by the addition of 450 μl of 0.1 M Tris-HCl (pH 8.0), 0.02 M CaCl_2 containing 1 mg of Pronase (Calbiochem), and ^3H GalNAc incorporation was determined as described previously (4).

Rat placenta postnuclear supernatants were assayed for α 2,6-sialyltransferase activity using GalNAc-Trf (39), a modification of a method described previously (40). Reactions consisted of 100 μg of postnuclear supernatant protein, 50 mM sodium cacodylate (pH 6.0), 0.5% (w/v) Triton X-100, 50 μg of bovine serum albumin, protease inhibitors described above for the GalNAc-transferase assay, 2 μM CMP- ^3H NeuAc (2×10^5 cpm), and 20 μM GalNAc-Trf in a total volume of 60 μl . The enzyme reaction was terminated by the addition of 40 μl of 5 mg/ml

bovine serum albumin and 100 μl of ice-cold 10% (w/v) trichloroacetic acid, 4% (w/v) phosphotungstic acid. The precipitated protein was pelleted by brief centrifugation, the supernatant containing unincorporated CMP- ^3H NeuAc was discarded, and the pellet was resuspended and washed three times with 5% trichloroacetic acid. The final precipitated protein was solubilized with 250 μl of 1 N NaOH, neutralized with 250 μl of 1 N HCl, and incorporated ^3H NeuAc was determined by liquid scintillation counting.

Isolation of β 1,4GalNAc-containing Secreted Placental Glycoproteins by Lectin Affinity Chromatography—Individual rat chorioallantoic placentas from mid to late pregnancy were sliced using a Stadie-Riggs slicer (A. H. Thomas Co., Philadelphia) and incubated in a 35-mm dish containing 2 ml of minimum essential medium/Earle's medium with penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). Alternatively, sliced placentas were incubated with sulfate-, cysteine-, and methionine-free minimum essential medium/Earle's containing 100 $\mu\text{Ci}/\text{ml}$ Trans- ^{35}S -label (ICN Inc., Irvine, CA; 70% ^{35}S methionine, 1,173 Ci/mmol). In either case, medium was collected after 16 h of incubation at 37 $^\circ\text{C}$ under an atmosphere of 95% air, 5% CO_2 . Medium was separated from tissue debris by centrifugation at 10,000 $\times g$ for 20 min. Saturated ammonium sulfate was added to give a final concentration of 70% (v/v) and stirring for 1 h at 4 $^\circ\text{C}$. Precipitated proteins were isolated by centrifugation at 10,000 $\times g$ for 20 min.

^{35}S Cysteine/methionine-labeled ammonium sulfate pellets were resuspended in TBS (20 mM Tris-HCl (pH 7.5), 150 mM NaCl), and unincorporated ^{35}S label was separated from the protein in 2-ml aliquots by gel filtration on a 45-ml bed volume column of Sephadex G-25 (Pharmacia Biotech Inc.) equilibrated in water. Protein-containing fractions were pooled and lyophilized. The proteins were resuspended in 200 μl of 20 mM sodium cacodylate (pH 6.0) and incubated with either *C. perfringens* or Newcastle disease virus neuraminidase as described below. The protein mixture was diluted to 1 ml with TBS and applied to a 1.5-ml bed volume column of WFA-agarose (EY-laboratories, Inc., San Mateo, CA) equilibrated in TBS. Following adsorption, the column was washed with TBS (~50-column volumes) until radioactivity was no longer detectable, and bound proteins were specifically eluted with TBS containing 50 mM GalNAc.

Nonradiolabeled ammonium sulfate precipitates were resuspended in TBS and dialyzed against 3 \times 2 liters of TBS and applied to 5-ml columns of WGA-Sepharose equilibrated in TBS. The unbound fraction of proteins was brought to 1 mM MnCl_2 and 1 mM CaCl_2 and adsorbed to 5-ml columns of ConA-Sepharose (Pharmacia) equilibrated in TBS containing 1 mM MnCl_2 and 1 mM CaCl_2 . After washing with 25 column volumes of the equilibration buffer, bound proteins were specifically eluted with TBS containing 0.5 M α -methyl mannoside.

WGA-Sepharose columns were washed with 25 column volumes of TBS, and bound proteins were specifically eluted with TBS containing 0.5 M GlcNAc. Following removal of GlcNAc by dialysis and lyophilization, the bound glycoproteins were resuspended in 200 μl of 20 mM sodium cacodylate (pH 6.0), treated with neuraminidase, and subjected to WFA affinity chromatography as described above.

Glycosidase Digestions—Peptide-N-glycanase F digestions were carried out as described previously (10). Digestions with *C. perfringens* neuraminidase, Newcastle disease virus neuraminidase, or diplococcal β -galactosidase were performed in 20 mM sodium cacodylate (pH 6.0). Digestion with jack bean β -hexosaminidase was carried out in 50 mM sodium citrate (pH 4.5). All buffers contained protease inhibitors described above in the GalNAc-transferase assay.

Western Blots—Glycoprotein fractions purified from rat placenta tissue slice medium (7.5 $\mu\text{g}/\text{well}$) were subjected to 12.5% SDS-PAGE and electroblotted on to Immobilon-P membranes (Millipore). Immobilized proteins were probed with either biotinylated WFA (20 ng/ml) (23) or with antisera directed against PLP-A (1:2,000), PLP-B (1:1,000), PLP-C (1:500), d/t PRP (1:2,000), or PL-IV (1:1,000) (37, 38, 41–43).

In Vitro Incorporation of ^{35}S SO $_4$ —Partially purified bovine pituitary GalNAc-4-sulfotransferase was incubated with 5 \times 10⁶ cpm of ^{35}S PAPS and 25 μg of potential glycoprotein substrates under conditions described previously for the GalNAc-4-sulfotransferase (17, 23). Enzymatic reactions were terminated by the addition of an equal volume of sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.003% bromphenol blue, and 62.5 mM Tris-HCl (pH 6.8)). ^{35}S -Labeled proteins were separated by 10% SDS-PAGE and detected by autoradiography.

RESULTS

Expression of the Glycoprotein Hormone-specific GalNAc-transferase, but Not β 1,4Gal-transferase, Increases during Late

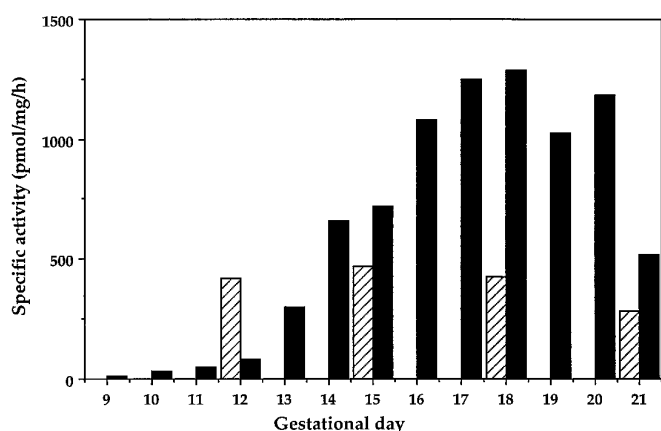


FIG. 1. Glycoprotein hormone GalNAc-transferase specific activity increases during late gestational development of the rat placenta, whereas β 1,4 Gal-transferase specific activity remains relatively constant. Postnuclear supernatants from rat placentas obtained on the gestational days indicated were assayed for GalNAc-transferase and Gal-transferase as described under "Experimental Procedures." Filled bars denote GalNAc-transferase specific activity, and stippled bars indicate Gal-transferase specific activity.

Gestational Development of the Rat Placenta—Extracts of placenta were prepared at various times during gestation and tested for the presence of the glycoprotein hormone-specific GalNAc-transferase and GalNAc-4-sulfotransferase. GalNAc-transferase activity with the expected properties was detected in placental extracts (Fig. 1), commencing at day 9 of gestation and increased 150-fold by day 18 before falling to lower levels just before parturition at day 21. The specific activity of the GalNAc-transferase on day 18 is 8-fold higher than that typical of rat pituitary extracts, making late gestational rat placenta the richest source of glycoprotein hormone GalNAc-transferase found to date. The placental GalNAc-transferase displays the same protein specificity as the pituitary enzyme transferring 78 pmol of GalNAc to the oligosaccharide acceptor on agal-hCG compared with 1 pmol to the oligosaccharides on agal-Trf at an acceptor concentration of 4 μ M. GalNAc-4-sulfotransferase was not detected in placental extracts at any point during gestation (not shown). Furthermore, the specific activity for β 1,4Gal-transferase, which is not protein-specific (2), does not change over the same time frame (Fig. 1). Thus, a GalNAc-transferase with the same peptide and oligosaccharide specificity as the pituitary GalNAc-transferase is expressed at high levels in the rat placenta during pregnancy between days 9 and 21 of gestation.

The absence of detectable GalNAc-4-sulfotransferase activity suggested that the oligosaccharides produced would either terminate with β 1,4-linked GalNAc or be capped by a moiety other than sulfate. Oligosaccharides terminating with GalNAc β 1,4GlcNAc (23, 25, 26, 32), GalNAc β 1,4(Fuca1,3)GlcNAc (25, 29, 32, 33), or NeuAca2,6GalNAc β 1,4GlcNAc (25, 27–32) have been described on native and recombinant glycoproteins. Even though the high levels of GalNAc-transferase activity suggested that large amounts of a glycoprotein or glycoproteins bearing β 1,4-linked GalNAc are produced in the placenta during the late phases of gestation, affinity chromatography on WFA-agarose did not reveal large amounts of glycoprotein(s) with terminal β 1,4-linked GalNAc following metabolic labeling. This suggested that terminal GalNAc moieties were efficiently capped with another group like sialic acid. Since the α 2,6-sialyltransferase (EC 2.4.99.1) will transfer sialic acid to either terminal β 1,4-linked Gal or GalNAc (44), we tested for the presence of α 2,6-sialyltransferase in rat placenta membrane preparations from mid to late pregnancy using

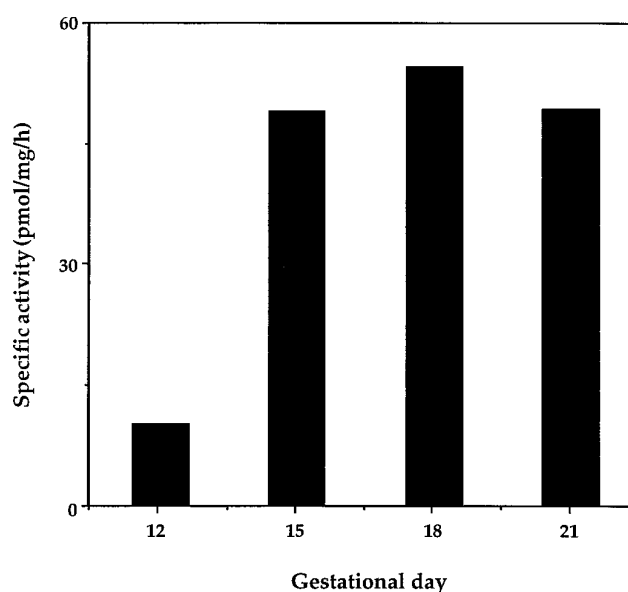


FIG. 2. α 2,6-Sialyltransferase activity, which uses acceptors containing terminal β 1,4GalNAc, is present in late gestational rat placenta. Rat placenta postnuclear supernatants from gestational days indicated were assayed for α 2,6-sialyltransferase activity as described under "Experimental Procedures."

GalNAc-Trf, transferrin synthetically modified to contain Asn-linked oligosaccharides terminating with β 1,4-linked GalNAc, as an acceptor substrate (Fig. 2). Sialyltransferase activity was detected beginning at day 12 and increased 5-fold by day 18 of pregnancy. Day 18 placental extracts were also able to transfer sialic acid to the hydrophobic substrate GalNAc β 1,4GlcNAc- β 1,2Man-(CH₂)₈COOCH₃ (not shown). The incorporated [³H] sialic acid was resistant to release by Newcastle disease virus neuraminidase, which is specific for α 2,3-linked sialic acid, but was sensitive to release by *C. perfringens* neuraminidase, which removes both α 2,3- and α 2,6-linked sialic acid (not shown). The concurrent increase in α 2,6-sialyltransferase and the GalNAc-transferase activity suggested that the absence of oligosaccharides containing terminal β 1,4-linked GalNAc might reflect efficient capping with α 2,6-linked sialic acid to produce NeuAca2,6GalNAc β 1,4GlcNAc β -R.

A Limited Number of Late Gestational Rat Placenta Glycoproteins Bear Asn-linked Oligosaccharides Terminating with NeuAca2,6GalNAc β 1,4GlcNAc β -R—To identify glycoproteins bearing oligosaccharides with β 1,4-linked GalNAc, tissue slices from day 18 rat placenta were incubated with [³⁵S]cysteine/methionine for 16 h so as to incorporate ³⁵S label into the peptide backbone of newly made glycoproteins. The medium containing secreted glycoproteins was collected, gel filtered over Sephadex G-25 to remove unincorporated ³⁵S label, and then incubated overnight in the presence or absence of neuraminidase. These samples were then fractionated by lectin affinity chromatography using immobilized WFA, which is specific for terminal β 1,4-linked GalNAc (45). The bound ³⁵S-labeled proteins were eluted and detected by fluorography following separation by 10% SDS-PAGE (Fig. 3). Secreted placenta glycoproteins were not bound by WFA prior to treatment with *C. perfringens* neuraminidase (Fig. 3, lane 3), whereas large amounts of a limited number of glycoproteins were bound following digestion (Fig. 3, lane 1). Digestion with Newcastle disease virus neuraminidase did not result in binding to the WFA column (Fig. 3, lane 2), indicating that a major fraction of β 1,4-linked GalNAc is substituted with α 2,6-linked sialic acid.

The secreted placenta glycoproteins recognized by WFA after neuraminidase digestion are bound quantitatively by immobi-

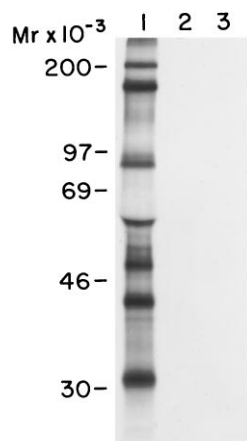


FIG. 3. Specific glycoproteins synthesized and secreted by the rat placenta on day 18 of gestation are bound by immobilized WFA only after treatment with *C. perfringens* neuraminidase. Secreted glycoproteins from gestational day 18 rat placenta were metabolically labeled with [^{35}S]cysteine/methionine as described. The ^{35}S -labeled glycoproteins were incubated with *C. perfringens* neuraminidase (lane 1), Newcastle disease virus neuraminidase (lane 2), or in the absence of neuraminidase (lane 3). Following application to WFA-agarose, the bound glycoproteins were eluted and examined by 10% SDS-PAGE autoradiography.

lized WGA. Secreted, placental glycoproteins that are bound by immobilized WGA represent 15% of the protein applied. The glycoproteins bound by WGA were digested with neuraminidase, applied to WFA-agarose, and equal amounts of protein from the unbound fraction (lane 1) and bound fraction (lane 2) were separated by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride (Fig. 4A). Glycoproteins bearing terminal β 1,4-linked GalNAc were visualized using biotinylated WFA. The absence of glycoproteins reactive with biotinylated WFA in the unbound fraction (Fig. 4A, lane 1) and their presence in the bound fraction indicated quantitative binding by WFA-agarose (Fig. 4A, lane 2). Digestion of glycoproteins eluted from WFA-agarose with diplococcal β -galactosidase (Fig. 4A, lane 3) did not reduce their reactivity with biotinylated WFA, whereas digestion with jack bean β -hexosaminidase (Fig. 4A, lane 4), reduced the reactivity with biotinylated WFA. Taken together these results indicate that glycoproteins bearing terminal β 1,4-linked GalNAc are efficiently bound by immobilized WFA and that virtually all of the β 1,4-linked GalNAc is capped by α 2,6-sialic acid.

The GalNAc-4-sulfotransferase is highly specific for the terminal sequence GalNAc β 1,4GlcNAc β -R (46). Incubation of the neuraminidase-digested, WFA-bound fraction of glycoproteins from day 18 placenta with [^{35}S]PAPS does not result in $^{35}\text{SO}_4$ incorporation (not shown), confirming that no endogenous GalNAc-4-sulfotransferase is present. The addition of exogenous partially purified bovine pituitary GalNAc-4-sulfotransferase results in $^{35}\text{SO}_4$ incorporation into the oligosaccharides of multiple glycoproteins (Fig. 4B, lane 1) with mobilities similar to those identified above by blotting with WFA (Fig. 4A, lane 2) and to those identified by affinity chromatography of metabolically labeled glycoproteins on immobilized WFA (Fig. 3). The labeled glycoproteins are of placental origin since only two bands are radiolabeled if the partially purified GalNAc-4-sulfotransferase is incubated with [^{35}S]PAPS in the absence of added placental proteins (Fig. 4B, lane 3). The incorporated $^{35}\text{SO}_4$ is quantitatively released by digestion with peptide:N-glycanase F (lane 2), indicating that terminal β 1,4-linked GalNAc is present exclusively on Asn-linked oligosaccharides of these placental glycoproteins.

Members of the Rat Placental Prolactin Family Which Are

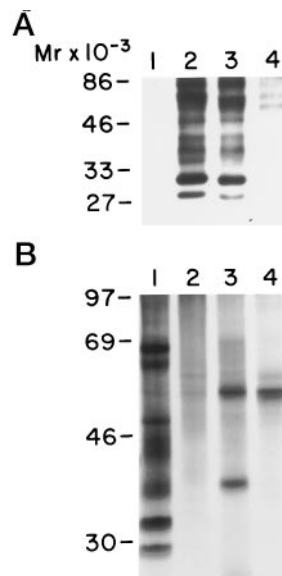


FIG. 4. Specific glycoproteins synthesized and secreted by gestational day 18 rat placenta contain β 1,4-linked GalNAc on their Asn-linked oligosaccharides. Secreted glycoproteins from day 18 rat placenta were fractionated by sequential lectin affinity chromatography using WGA-Sepharose and WFA-agarose as described under "Experimental Procedures." *Panel A*, the WFA-unbound fraction (lane 1), WFA-bound fraction (lane 2), WFA-bound fraction digested with diplococcal β -galactosidase (lane 3), and WFA-bound fraction digested with jack bean β -hexosaminidase (lane 4) were analyzed by 12.5% SDS-PAGE and Western blotting with biotinylated WFA (20 ng/ml). *Panel B*, the WFA-agarose-bound fraction was examined for incorporation of $^{35}\text{SO}_4$ into terminal β 1,4-linked GalNAc by bovine pituitary GalNAc-4-sulfotransferase. Lane 1, WFA-bound fraction and GalNAc-4-sulfotransferase; lane 2, WFA-bound fraction and GalNAc-4-sulfotransferase after digest with peptide:N-glycanase F; lane 3, bovine pituitary GalNAc-4-sulfotransferase alone; lane 4, pituitary GalNAc-4-sulfotransferase alone digested with peptide:N-glycanase F. $^{35}\text{SO}_4$ incorporation was monitored by autoradiography after 10% SDS-PAGE.

Concomitantly Expressed with the Glycoprotein Hormone GalNAc-transferase Bear Asn-linked Oligosaccharides Terminating with NeuAca2,6GalNAc β 1,4GlcNAc β —The results presented above indicated that only a select group of rat placenta glycoproteins bears Asn-linked oligosaccharides modified with NeuAca2,6GalNAc β 1,4GlcNAc β . The time during which GalNAc-transferase activity increases in the rat placenta coincides with the expression of a subset of members of the prolactin/growth hormone family: PLP-A, PLP-B, PLP-C, d/t PRP, and PL-Iv (Fig. 5). d/t PRP and PLP-B are expressed in either the decidua or the placenta from early pregnancy until term (38, 43, 47), whereas PLP-A, PLP-C, and PL-Iv are expressed in the placenta beginning around gestational day 12–13 and increasing until parturition at day 21 (48). PLP-A and PL-Iv each has two electrophoretic forms of 33 and 29 kDa, whereas PLP-B has a single electrophoretic form of 30–31 kDa. PLP-C and d/t PRP also each has a single electrophoretic form of 29 kDa. All are expressed in the junctional zone of the placenta which is made up of trophoblast giant cells (TGC) and spongiotrophoblasts at mid gestation (49). PLP-A, PLP-C, d/t PRP, and PL-Iv are expressed in both these cell types, whereas PLP-B is expressed exclusively in spongiotrophoblasts (49). Since a number of the rat placental glycoproteins that are specifically bound by WFA-agarose have the same apparent molecular weights as members of the prolactin/growth hormone family and are expressed over the same time frame as the protein-specific GalNAc-transferase, we examined this family of glycoproteins for the presence of β 1,4-linked GalNAc on their Asn-linked oligosaccharides.

Secreted glycoproteins were isolated from gestational day 12,

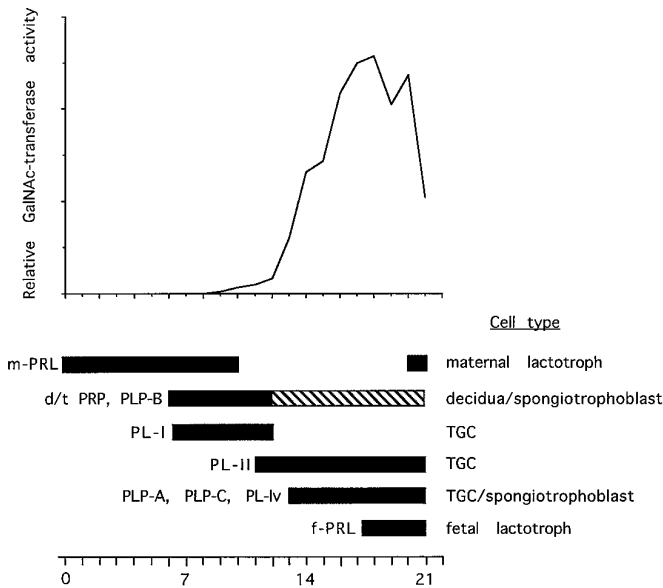


FIG. 5. Concomitant expression of prolactin/growth hormone family members and the GalNAc-transferase in the rat placenta. The time during which various prolactin family members are expressed as well as their cell(s) of origin are shown schematically compared with GalNAc-transferase levels. *mPRL*, maternal pituitary prolactin; *PL-I* and *PL-II*, placental lactogen I and II; *fPRL*, fetal pituitary prolactin. This figure was adapted with permission from Ref. 49.

15, 18, and 21 rat placental tissue slices by sequential lectin affinity chromatography as described in Fig. 4. Glycoproteins bound by WGA-Sepharose were digested with neuraminidase and separated into fractions that were not bound (–) or bound (+) by WFA. These fractions were separated by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride, and probed with biotinylated WFA to determine their relative levels of β 1,4GalNAc-containing oligosaccharides (Fig. 6). At day 12, when the transferase levels are low, no glycoprotein binding to WFA-agarose is detected even though d/t PRP and PLP-B are expressed at this time. At day 15, there is a 10-fold increase in GalNAc-transferase activity (see Fig. 1) over day 12, and there is a corresponding increase in binding of glycoproteins to WFA-agarose (Fig. 6). Maximal levels of β 1,4GalNAc-bearing glycoproteins migrating with apparent molecular masses of 29 and 33 kDa occurs on day 18 when the GalNAc-transferase levels are also maximal (see Fig. 1). As the transferase levels decline on day 21, so does the amount of WFA reactive material. Thus, the addition of β 1,4-linked GalNAc to rat placenta glycoproteins in the range of 29 and 33 kDa is proportionate to the levels of GalNAc-transferase during late gestation.

The WFA bound (+) and unbound (–) fractions were probed with antibodies specific for PLP-A, PLP-B, PLP-C, d/t PRP, and PL-IV (Fig. 7, WFA-agarose) to determine which members of this family were modified with β 1,4-linked GalNAc. PLP-A, PLP-B, PLP-C, d/t PRP, and PL-IV bound to WFA-agarose (Fig. 6), indicating that they are all modified with β 1,4-linked GalNAc.

The forms of PLP-A, PLP-B, PLP-C, d/t PRP, and PL-IV which bear NeuAca α 2,6GalNAc β 1,4GlcNAc β are bound quantitatively by WGA and WFA. In contrast, those glycoproteins not bound to WGA-Sepharose in the isolation procedure do not bear β 1,4-linked GalNAc since digestion with neuraminidase does not result in binding to WFA-agarose or incorporation of $^{35}\text{SO}_4$ by exogenous GalNAc-4-sulfotransferase (not shown).

The forms of PLP-A, PLP-B, PLP-C, d/t PRP, and PL-IV present in the unbound fraction from WGA-Sepharose were bound by ConA-Sepharose, indicating they are glycosylated (Fig. 7, ConA-Sepharose). The amount of each placental pro-

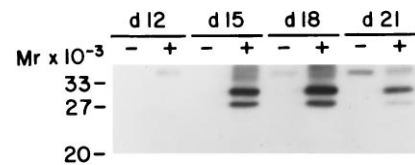


FIG. 6. The amount of rat placental glycoproteins modified with β 1,4-linked GalNAc is proportionate to the level of GalNAc-transferase being expressed. Secreted glycoproteins containing β 1,4-linked GalNAc were isolated from rat placenta tissue slices at gestational days 12, 15, 18, and 21 by sequential lectin chromatography with WGA-Sepharose and WFA-agarose. Equal amounts of the WFA-agarose bound (+) and unbound (–) glycoproteins (7.5 μg each) were separated on 12.5% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with biotinylated WFA as in Fig. 4A.

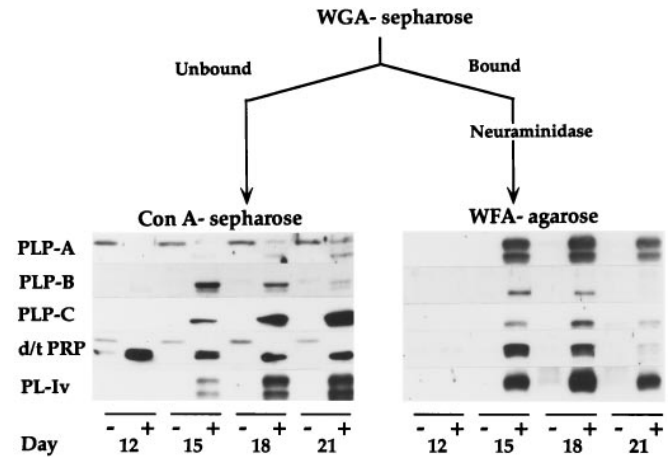


FIG. 7. Glycoproteins bearing terminal NeuAca α 2,6GalNAc β 1,4GlcNAc β late in gestation include members of the rat placental prolactin family. Secreted glycoproteins were isolated from rat placenta tissue slices at gestational days 12, 15, 18, and 21. Equal amounts of protein (7.5 μg) from ConA-Sepharose bound (+) and unbound (–) or WFA-agarose bound (+) and unbound (–) fractions were separated by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antibodies directed against PLP-A, PLP-B, PLP-C, d/t PRP, and PL-IV. The figure is a composite of several Western blots depicting only the region of the blots bordered by 33 and 29 kDa.

lactin family member in the WGA(–)/ConA(+) fraction was compared with that in the WGA(+)/WFA(+) fraction (Fig. 7) to determine the proportion of each glycoprotein which is modified with NeuAca α 2,6GalNAc β 1,4GlcNAc β . PLP-A is fully modified with GalNAc as it is found almost exclusively in the WFA-bound fraction on days 15, 18, and 21 of gestation. In contrast, PLP-B, PLP-C, d/t PRP, and PL-IV are not fully modified with β 1,4-linked GalNAc as they are present in both the WGA(–)/ConA(+) and the WGA(+)/WFA(+) fractions. Only the 33 kDa form of PL-IV is present in the WGA(+)/WFA(+) fraction, whereas both the 33- and 29-kDa forms are present in the WGA(–)/ConA(+) fraction. PL-IV has two potential Asn glycosylation sites, and the 29- and 33-kDa forms are thought to arise from differential glycosylation at one of these two sites. The presence of GalNAc exclusively on the 33-kDa form suggests that the less efficiently utilized glycosylation site on the 33-kDa form is a significantly better substrate for GalNAc addition than the more efficiently modified glycosylation site found on both the 33- and 29-kDa forms.

The results presented in Figs. 6 and 7 demonstrate that the proportion of modification with terminal β 1,4-linked GalNAc reflects the level of GalNAc-transferase expressed; however, not all of the placental prolactin family members are modified with the same efficiency (see Fig. 7). Addition of β 1,4-linked GalNAc to glycoproteins by the peptide-specific GalNAc-transferase requires that the transferase and target protein be expressed in the same cell type and that the target protein con-

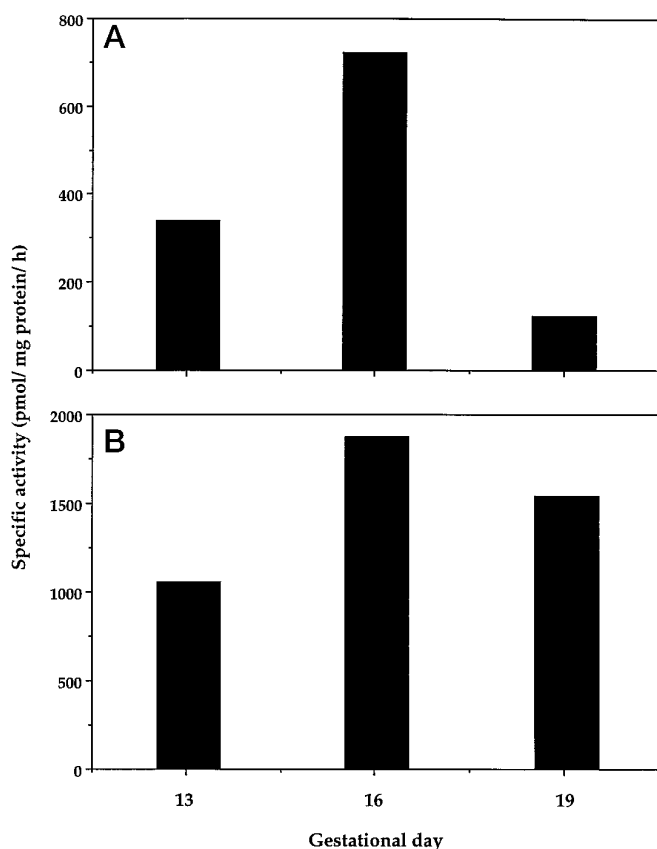


FIG. 8. Isolated junctional and labyrinth zones from the rat placenta display the same developmentally regulated expression of GalNAc-transferase activity as whole placenta. The junctional zone (panel A) and labyrinth zone (panel B) were isolated from rat placentas by dissection at the gestational days indicated. Postnuclear supernatants from each were assayed for GalNAc-transferase activity as described under "Experimental Procedures."

tains a peptide determinant that is recognized by the transferase. Differences in either the site of glycosylation and/or the recognition determinant could account for this.

The Peptide-specific GalNAc-transferase Is Expressed in Spongiotrophoblasts during Late Development of the Rat Placenta—At mid gestation, dramatic changes occur in cell types that make up the rat uteroplacental unit (49). At day 9, decidualized uterus is abundant, and the developing placenta is made up of stem cells and TGC. By day 12 the decidua begins to regress, and spongiotrophoblasts develop in the junctional zone of the placenta. Expression of d/t PRP and PLP-B switches from anti-mesometrial cells of the decidua to spongiotrophoblasts of the placenta.² Spongiotrophoblasts account for the bulk of PLP-A, PLP-C, and PL-IV expression. The increase in number of spongiotrophoblasts that express placental prolactin family members coincides with the increase in expression of GalNAc-transferase activity, suggesting that GalNAc-transferase is expressed selectively by spongiotrophoblasts.

Homogenates were made from isolated day 10 decidua and either isolated junctional or labyrinth zones from day 13, 16, and 19 of gestation. β 1,4Gal-transferase activity, but no GalNAc-transferase activity, was detected in decidua (not shown). In contrast, GalNAc-transferase was present in both junctional (Fig. 8A) and labyrinth (Fig. 8B) zones and showed the same pattern of increasing and decreasing expression with time as whole placenta homogenates. This is consistent with the absence of β 1,4-linked GalNAc on glycoproteins of decidual origin

TABLE I

Comparison of acceptor substrates for the bovine pituitary glycoprotein hormone GalNAc-transferase

The amount of [³H]GalNAc incorporated by partially purified bovine pituitary GalNAc-transferase into Asn-linked oligosaccharides on agal-transferrin (Agal-Trf), agal-recombinant PLP-A (Agal-rPLP-A), agal-recombinant PLP-B (Agal-rPLP-B), and agal-human chorionic gonadotropin (Agal-hCG) was determined at a substrate concentration of 7.5 μ M as described under "Experimental Procedures."

Substrate	GalNAc transferred
	pmol
Agal-hCG	188
Agal-rPLP-A	50
Agal-rPLP-B	2
Agal-hTrf	0.3

and their presence on glycoproteins from the junctional zone. It is not clear which cell types in the junctional zone express GalNAc-transferase, since at mid gestation the junctional zone is made up of both spongiotrophoblasts and TGC.

Although TGC cannot be separated from spongiotrophoblasts, the Rcho-1 trophoblast cell line can be induced to differentiate into TGC (35, 36). When cultured in the presence of fetal bovine serum under subconfluent conditions, Rcho-1 trophoblast cells have the properties of trophoblast progenitor cells, whereas Rcho-1 trophoblast cells allowed to grow to confluence in the presence of horse serum differentiate into TGC over a period of 12 days. Undifferentiated Rcho-1 trophoblast cells contained 215 pmol/mg/h GalNAc-transferase activity. Following induction to TGC, no GalNAc-transferase activity was detected even though β 1,4Gal-transferase activity was readily detectable (not shown). These results suggest that GalNAc-transferase is expressed exclusively in spongiotrophoblasts of the junctional zone along with PLP-A, PLP-B, PLP-C, d/t PRP, and PL-IV. Since these placental prolactin family members are all expressed in spongiotrophoblasts, the more efficient modification of PLP-A with β 1,4-linked GalNAc than the other family members may reflect features of the glycoproteins themselves, most likely recognition of the peptide determinant.

PLP-A Serves as an in Vitro Target for the Glycoprotein Hormone GalNAc-transferase—We have shown that the glycoprotein hormone GalNAc-transferase recognizes a peptide determinant as well as the oligosaccharide acceptor (2–5). In the presence of this determinant the catalytic efficiency for GalNAc addition to the same acceptor oligosaccharide is increased by as much as 500-fold. The high levels of transferase seen in near term rat placenta extracts could result in modification of glycoproteins devoid of the recognition determinant. This seems unlikely since even among the placental prolactin family members examined (Fig. 7), significant amounts of PLP-B, PLP-C, d/t PRP, and PL-IV are not modified. Transfer of GalNAc to oligosaccharide acceptors on PLP-A and PLP-B were compared with transferrin, which does not have a recognition determinant, and hCG, which has recognition determinants on its α and β subunits (Table I). PLP-A and hCG are both modified efficiently with GalNAc. PLP-B is modified more efficiently than transferrin, 10-fold more GalNAc incorporated, but is a poor substrate when compared with PLP-A (Table I). PLP-A appears to have a recognition determinant that is recognized with an efficiency similar to those on hCG. The recognition determinant on PLP-B, if present at all, is poorly recognized. The difference in recognition of PLP-A and PLP-B by the GalNAc-transferase most likely accounts for the lower amount of β 1,4-linked GalNAc found on the Asn-linked oligosaccharides of PLP-B even though PLP-A and PLP-B are both synthesized in spongiotrophoblasts late in gestation.

² K. E. Orwig, C. A. Rasmussen, and M. J. Soares, unpublished data.

DISCUSSION

The mammalian placenta is a complex tissue that performs a number of essential functions, among them: 1) nutrient and waste exchange between the fetal and maternal circulations; 2) modulation of the maternal immune response to prevent rejection of the embryo; and 3) transduction of fetal and maternal signals (1). The rat has proved to be a useful model to study placental development (48, 49). Between implantation and mid gestation the choriovitelline placenta, which contains a single differentiated trophoblast cell type, the TGC, plays the dominant role. From mid gestation onward the chorioallantoic placenta, which consists of a junctional zone and labyrinth zone, predominates. Of four differentiated trophoblastic cell types, TGC, glycogen cells, and spongiotrophoblasts are present in the junctional zone, whereas TGC and syncytial trophoblasts are present in the labyrinth zone. Individual PLPs that are structurally and functionally related to the prolactin and growth hormone produced by the pituitary are expressed at characteristic times during gestation by cells within the decidualized uterus, choriovitelline placenta, and chorioallantoic placenta (see Fig. 5). The initial appearance and rapid increase in glycoprotein hormone-specific GalNAc-transferase after day 13 of gestation in extracts from both the junctional and labyrinth zones of the chorioallantoic placenta suggested that specific trophoblast lineages of the chorioallantoic placenta are induced to express high levels of the GalNAc-transferase as they differentiate. The absence of either GalNAc-transferase or glycoproteins bearing β 1,4-linked GalNAc on Asn-linked oligosaccharides in extracts from either decidualized uterus or the placenta prior to day 13 and the direct correlation of late developmental increases in GalNAc-transferase activity with spongiotrophoblast development support this conclusion.

PLP-A, PLP-B, PLP-C, d/t PRP, and PL-IV are among the glycoproteins that we have shown bear Asn-linked oligosaccharides terminating with NeuAca α 2,6GalNAc β GlcNAc β . PLP-A, PLP-C, and PL-IV are expressed predominantly in spongiotrophoblasts from mid gestation to term. The glycoforms of PLP-A, PLP-C, PL-IV bearing β 1,4-linked GalNAc are virtually quantitatively modified with sialic acid since little if any of these glycoproteins is bound by WFA prior to neuraminidase digestion, whereas each is essentially quantitatively bound following enzymatic removal of sialic acid. Thus, it is the concurrent expression of both the α 2,6-sialyltransferase and the GalNAc-transferase in spongiotrophoblasts over the same time frame as their glycoprotein substrates which accounts for the highly efficient modification of their Asn-linked oligosaccharides with both GalNAc and sialic acid.

PLP-B and d/t PRP are also expressed in spongiotrophoblasts; however, prior to day 13 of gestation they are expressed in cells of the decidualized uterus (47). PLP-B and d/t PRP synthesized prior to mid gestation are devoid of NeuAca α 2,6GalNAc β GlcNAc β , whereas after day 12 significant amounts of PLP-B and d/t PRP contain NeuAca α 2,6GalNAc β GlcNAc β , consistent with their synthesis in spongiotrophoblasts. As a result of the change in the cell type synthesizing these hormones, their glycoforms are altered.

We have shown previously that the glycoprotein-specific GalNAc-transferase recognizes peptide as well as oligosaccharide determinants (2–5). In the case of the glycoprotein hormone α subunit, the peptide determinant consists of a cluster of basic amino acids present in two turns of an α -helix. The crystal structure of hCG led us to the conclusion that it is the proximity of these residues in three-dimensional space to the oligosaccharides rather than their relationship within the linear amino acid sequence which is critical for efficient transfer of GalNAc. PLP-A also contains a peptide recognition determinant that is

recognized by the GalNAc-transferase from bovine pituitary and is modified with nearly the same efficiency as hCG at a concentration of 7.5 μ M. PLP-B, in contrast, is modified almost 10-fold more efficiently than transferrin but at 1% of the rate of hCG. Thus, even though PLP-B is expressed in spongiotrophoblasts after day 12 of gestation, it is not modified to the same extent as PLP-A. PLP-C and PL-IV are also not completely modified with GalNAc, suggesting that their peptide recognition determinants are not as effective as that on PLP-A.

The rodent prolactin/growth hormone family members are homologous with 30–80% similarity in their amino acid residues (48). The crystal structure of growth hormone is known; and based on similarities to the placental prolactin family members, it has been predicted that each prolactin family member consists of four tightly packed α -helical domains (51). There is little homology in the first three domains of these proteins, but the fourth domain at the carboxyl terminus is highly conserved. This domain contains a high number of basic amino acids that may take on an α -helical conformation. Even though this region is carboxyl-terminal to the Asn-linked oligosaccharides on each of the placental prolactin family members, it is a good candidate region for the peptide determinant recognized by the GalNAc-transferase.

The terminal sequence NeuAca α 2,6GalNAc β 1,4GlcNAc β has to date been described on a number of glycoproteins. However, only a limited number of the glycoproteins synthesized by the rat placenta between mid gestation and birth contain this structure. Efficient modification of these glycoproteins with β 1,4-linked GalNAc requires that they contain a peptide recognition determinant and be synthesized in cells expressing the GalNAc-transferase. In the case of PLP-B and d/t PRP, the mid gestational cell type switch from anti-mesometrial cells of the decidua to spongiotrophoblasts of the placenta is accompanied by a major change in their glycoforms.

What is the biological significance of oligosaccharides terminating with NeuAca α 2,6GalNAc β 1,4GlcNAc β rather than the more commonly encountered NeuAca α 2,6Gal β 1,4GlcNAc β ? Our results demonstrate that the synthesis of the former structure is developmentally regulated and that it is confined to a limited number of rat placenta glycoproteins. We are able to find PLP-A and other glycoproteins with the NeuAca α 2,6GalNAc β 1,4GlcNAc β structure in the maternal circulation between mid gestation and birth, but not at other times. Glycodelin (placental protein 14), a human decidual and placental protein expressed in high levels early in pregnancy, is reported to have immunomodulatory effects (52, 53). Glycodelin has recently been shown to bear Asn-linked oligosaccharides terminating with NeuAca α 2,6GalNAc β 1,4GlcNAc β (32). The authors suggest that this oligosaccharide structure may have a role in immunomodulation; however, direct evidence for this remains to be obtained.

The oligosaccharide structure terminating with NeuAca α 2,6GalNAc β 1,4GlcNAc β may be highly characteristic of pregnancy in a number of species including humans. There are a number of potentially important roles for this structure. Receptors specific for NeuAca α 2,6GalNAc β 1,4GlcNAc β could regulate circulating half-life or direct glycoproteins bearing it to specific sites in the mother or fetus. This structure could have an immunomodulatory role by interacting with carbohydrate-specific receptors related to CD22 or the selectins (50, 54). Receptors for many of the placental prolactin family members are likely to be present in both the fetus and mother. Specific glycoforms could direct the different members of this family to specific sites and/or modulate the activation of their receptors.

Clearly many potential biological roles for NeuAca α 2,6GalNAc β 1,4GlcNAc β are possible. The highly regulated ex-

pression of the GalNAc-transferase and α 2,6-sialyltransferase at the same time as select members of the placental prolactin family strongly supports the view that this structure will prove biologically important for these hormones.

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