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Chinese Hamster Ovary (CHO) Cells May Express Six β4-Galactosyltransferases (β4GalTs)

CONSEQUENCES OF THE LOSS OF FUNCTIONAL β4GalT-1, β4GalT-6, OR BOTH IN CHO GLYCOSYLATION MUTANTS*

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Six β4-galactosyltransferase (β4GalT) genes have been cloned from mammalian sources. We show that all six genes are expressed in the Gat⁻2 line of Chinese hamster ovary cells (Gat⁻2 CHO). Two independent mutants termed Pro⁻5Lec20 and Gat⁻2Lec20, previously selected for lectin resistance, were found to have a galactosylation defect. Radiolabeled biantennary *N*-glycans synthesized by Pro⁻5Lec20 were proportionately less ricin-bound than similar species from parental CHO cells, and Lec20 cell extracts had a markedly reduced ability to transfer Gal to GlcNAc-terminating acceptors. Northern blot analysis revealed a severe reduction in β4GalT-1 transcripts in Pro⁻5Lec20 cells. The Gat⁻2Lec20 mutant expressed β4GalT-1 transcripts of reduced size due to a 311-base pair deletion in the β4GalT-1 gene coding region. Northern analysis with probes from the remaining five β4GalT genes revealed that Gat⁻2 CHO and Gat⁻2Lec20 cells express all six β4GalT genes. Unexpectedly, the β4GalT-6 gene is not expressed in either Pro⁻5 or Pro⁻5Lec20 cells. Thus, in addition to a deficiency in β4GalT-1, Pro⁻5Lec20 cells lack β4GalT-6. Nevertheless, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry data of *N*-glycans released from cellular glycoproteins showed that both the β4GalT-1⁻ (Gat⁻2Lec20) and β4GalT-1⁻/β4GalT-6⁻ (Pro⁻5Lec20) mutants have a similar Gal deficiency, affecting neutral and sialylated bi-, tri-, and tetraantennary *N*-glycans. By contrast, glycolipid synthesis was normal in both mutants. Therefore, β4GalT-1 is a key enzyme in the galactosylation of *N*-glycans, but is not involved in glycolipid synthesis in CHO cells. β4GalT-6 contributes only slightly to the galactosylation of *N*-glycans and is also not involved in CHO cell glycolipid synthesis. These CHO glycosylation mutants provide insight into the variety of *in vivo* substrates of different β4GalTs. They may be used in glycosylation engineering and in investigating roles for β4GalT-1 and β4GalT-6 in generating specific glycan ligands.

β4-Galactosyltransferase (β4GalT)[1] is a Golgi-localized, type II transmembrane glycoprotein that catalyzes the transfer of galactose to GlcNAc, forming Galβ1,4GlcNAc (lactosamine) units in glycoconjugates (1, 2). Lactosamine sequences are important components of carbohydrate ligands that mediate cell recognition events such as fertilization (3) and lymphocyte trafficking (4). Also, terminal galactose residues function as ligands for galactose-binding proteins such as galectins (5), contactinhibin (6), and the hepatic binding protein (7).

A gene that encodes β4GalT-1 was initially cloned from bovine kidney (1) and mammary gland (2) cDNA libraries. Further cloning and data base analyses identified five additional human genes that have 30–55% amino acid identity to the original β4GalT-1 (8). Each encodes a galactosyltransferase that utilizes the donor substrate UDP-Gal and transfers Gal in a β1,4-linkage to GlcNAc or Glc (8–11). β4GalT-1 and β4GalT-2 show similar activity in the presence of α-lactalbumin in that their acceptor specificity is changed from GlcNAc to Glc (9). β4GalT-3, -4, and -5 do not synthesize lactose (9–11). Interestingly, the ability of β4GalT-4 to transfer Gal to GlcNAc is activated by α-lactalbumin (11), and the activity of both β4GalT-1 and β4GalT-2 with GlcNAc is inhibited by α-lactalbumin (9). It was reported that β4GalT-4 is most efficient in galactosylating mucin-type, core 2 branch oligosaccharides (12), whereas β4GalT-1 is most efficient in galactosylating i/I antigens (13). It was also suggested that β4GalT-5 may function best in transferring Gal to *O*-glycans (14).

For glycolipid acceptors, all β4GalTs show a different activity. Thus, β4GalT-1 has high activity for GlcCer, Lc_3, and nLc_5 in *in vitro* assays (9). β4GalT-3, -4, and -5 have little activity with GlcCer (9, 11, 14). β4GalT-6 is a lactosylceramide synthase predicted to be important for glycolipid biosynthesis (15). β4GalT-3 utilizes Lc_3 efficiently, but not nLc_5 (11); and nLc_5 is a poor substrate for β4GalT-4 and β4GalT-5 (10, 11).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF318896.

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[1] The abbreviation used are: β4GalT, β4-galactosyltransferase; GlcCer, glucosylceramide; LacCer, lactosylceramide; Lc_3, GlcNAcβ1,3Galβ1,4GlcCer; nLc_5, GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4GlcCer; GM3, NeuAcα2,3Galβ1,4GlcCer; CHO, Chinese hamster ovary; ConA, concanavalin A; PHA-E, *Phaseolus vulgaris* erythroagglutinin; PHA-L, *P. vulgaris* leukoagglutinin; RCA, *Ricinus communis* agglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; VSV, vesicular stomatitis virus; GnGn, GlcNAc-terminating biantennary *N*-glycan, GnGnβ4Gn, GlcNAc-terminating triantennary *N*-glycan with a β1,4-linked branch; GnGnGnGn, GlcNAc-terminating tetraantennary *N*-glycan; PNGase F, peptide *N*-glycosidase F; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; kb, kilobase(s); bp, base pair(s).

To identify *in vivo* functions of each β4GalT, it is important to consider the tissue expression pattern as well as acceptor specificity. For example, β4GalT-1 is up-regulated in lactating mammary glands (16), whereas β4GalT-2 is not.[2] Furthermore, mice deficient in β4GalT-1 do not produce lactose in milk (17, 18). The β4GalT-1, -3, -4, and -5 genes are ubiquitously expressed, whereas the β4GalT-2 and β4GalT-6 genes exhibit a more restricted expression pattern (8, 10, 11). Although ~80% mice lacking β4GalT-1 die soon after birth, the remainder are viable and fertile (17, 18). Serum glycoproteins from β4GalT-$1^{-/-}$ mice were found to be galactosylated to ~10% compared with those from wild-type mice (19), providing evidence for the existence of other functional β4GalTs. However, almost nothing is known of the biological roles of these β4GalTs, and their acceptor specificity has not been defined for *in vivo* substrates.

To identify glycosyltransferases and other factors that regulate glycosylation in mammals, we have isolated a number of lectin-resistant CHO glycosylation mutants (20). Two independent CHO mutants that were selected for resistance to PHA-E belong to the Lec20 complementation group and behave as loss-of-function mutants in somatic cell hybrids (21). In this study, we show that they are both defective in the β4-galactosylation of *N*-glycans due to independent mutations in the β4GalT-1 gene. We also report that Pro$^-$5 CHO cells lack β4GalT-6 transcripts; and therefore, Pro$^-$5Lec20 mutants derived from Pro$^-$5 CHO cells lack both β4GalT-1 and β4GalT-6 activities. Analyses of *N*-glycans and glycolipids synthesized by these four CHO cell lines identified *in vivo* substrates for several β4GalTs.

EXPERIMENTAL PROCEDURES

Materials—D-[6-^{3}H]Gal (31.5 Ci/mmol), D-[6-^{3}H]GlcN hydrochloride (31.5 Ci/mmol), UDP-[6-^{3}H]GlcNAc (41.60 Ci/nmol), UDP-[6-^{3}H]Gal (10 Ci/mmol), and ConA-Sepharose were from Amersham Pharmacia Biotech. PHA-E lectin, PHA-L-agarose, and RCA_{II}-agarose were from Vector Laboratories, Inc. Bio-Gel P-2 (45–95 mesh), the detergent compatible protein assay reagent, and AG 1-X4 resin (200–400 mesh, Cl$^-$ form) were from Bio-Rad. Pronase (*Streptomyces griseus*), EDTA-free protease inhibitor tablets, and β-galactosidase (*Diplococcus pneumoniae*) were from Roche Molecular Biochemicals. Triton X-100, Nonidet P-40, Triton CF-54, CHAPS, sodium deoxycholate, β-galactosidase (bovine testis and jack bean), neuraminidase (*Clostridium perfringens*), GlcNAc, UDP-GlcNAc, UDP-Gal, human fibrinogen, fetuin, human α_1-acid glycoprotein, GlcCer, LacCer, and GM3 were from Sigma. Tween 20, Brij 35, and Lubrol-PX were from Pierce. G418, fetal bovine serum, and α-medium were from Life Technologies, Inc. Ecolume was from ICN Biomedicals. The detergent G3634A was a gift of Dr. Subashu Basu (Notre Dame University).

Cell Lines and Cell Cultures—Pro$^-$5, Gat$^-$2, Pro$^-$5Lec20 (clone 15C), and Gat$^-$2Lec20 (clone 6A) CHO cells were isolated as previously described (21). Cells were grown in suspension at 37 °C in complete α-medium containing 10% fetal bovine serum.

Preparation of Radiolabeled VSV Glycopeptides—Cells growing in suspension were infected with VSV and subsequently cultured in α-medium containing reduced glucose (0.5 mg/ml), 2% Nuserum (Collaborative Research), and 83 μCi of [^{3}H]GlcN/10 ml as described previously (22). Virus was purified by gradient centrifugation and exhaustively digested with Pronase to generate Pronase glycopeptides.

Lectin Affinity Chromatography of Radiolabeled Glycopeptides—VSV glycopeptides desalted on Bio-Gel P-2 were fractionated on a 5-ml column of ConA-Sepharose as described previously (22) into branched and biantennary *N*-glycans. The ConA-bound fraction was desalted, treated with neuraminidase, and fractionated on a 5-ml column of RCA_{II}-agarose. Lectin chromatography was performed at room temperature or at 4 °C. Phosphate-buffered saline containing 100 mM GalNAc or 200 mM lactose was used to elute bound glycopeptides. Samples of 0.5–1.0 ml were mixed with Ecolume at a ratio of ~1:10 and counted in a scintillation counter.

Preparation of Cell Extracts—Post-nuclear supernatant from Lec20 and parental CHO cells was prepared as described (23). Briefly, cells (~6×10^7) were washed two times with saline, followed by one wash with homogenizing buffer (10 mM Tris-HCl, pH 7.4, and 250 mM sucrose), and incubated in 1 ml of homogenizing buffer containing an EDTA-free protease inhibitor tablet on ice. After 20 min, the swollen cells were homogenized using a Balch homogenizer (Industrial Tectonic Inc.) at 4 °C. The lysate was centrifuged at 3000 rpm for 30 min at 4 °C. Glycerol was added to the supernatant to a final concentration of 20% before storage at −80 °C. For preparation of microsomal membranes, the post-nuclear supernatant was centrifuged at 100,000 × *g* for 1 h at 4 °C. Also, cell-free extract was prepared in 1.5% Triton X-100 after cell washing, to which glycerol was added to 20% by volume before storage at −80 °C as described (23).

Preparation of GlcNAc-terminating Glycopeptides—Biantennary *N*-linked glycopeptides with no fucose and terminating with GlcNAc (GnGn) were isolated from human fibrinogen as described (24). Triantennary *N*-linked glycopeptides (GnGnβ4Gn) were prepared from fetuin, and tetraantennary *N*-linked glycopeptides (GnGnGnGn) were prepared from α_1-acid glycoprotein. Briefly, desialylated glycopeptides (3.2 mg of phenol-sulfuric acid-positive material) prepared from fetuin by exhaustive Pronase digestion and mild acid treatment (0.01 M HCl, 80 °C, 2 h) were digested with β-galactosidases (jack bean, *D. pneumoniae*, and bovine testis) separately. Jack bean β-galactosidase digestion was carried out in 50 mM acetate buffer, pH 3.5, at room temperature for 48 h with a total of 200 milliunits of enzyme. The desalted glycopeptides were digested with *D. pneumoniae* β-galactosidase (a total of 20 milliunits) in 20 mM cacodylate buffer, pH 6.5, at 37 °C for 48 h. After desalting, glycopeptides were digested with bovine testis β-galactosidase in 50 mM sodium citrate/phosphate buffer, pH 4.3, at 37 °C for 48 h with a total of 40 milliunits enzyme, desalted, and freeze-dried (~1.8 mg of phenol-sulfuric acid-positive material based on the standard curve for mannose).

Desialylated glycopeptides from α_1-acid glycoprotein (32.8 mg of phenol-sulfuric acid-positive material) were fractionated on ConA-Sepharose (15 × 43 cm), and the desalted ConA-unbound glycopeptides (~14 mg) were fractionated on PHA-L-agarose (1 × 30 cm) in 1-mg aliquots. The PHA-L-retarded glycopeptides (1.5 mg) were digested with β-galactosidase as described above, desalted, and freeze-dried (0.55 mg of phenol-sulfuric acid-positive material). The glycopeptides were checked by monosaccharide analysis and high-performance anion-exchange chromatography with pulsed amperometric detection. Acid hydrolysis (2.5 M trifluoroacetic acid, 100 °C, 4 h) was followed by fractionation on PA-10 eluted with 18 mM NaOH. When the mannose content was normalized to 3 residues, glycopeptides from fetuin had 0.1 Gal and 4.7 GlcNAc residues, and glycopeptides from α_1-acid glycoprotein had 0.4 Gal and 6.3 GlcNAc residues.

Enzyme Assays—Enzyme assays with cell extracts or microsomal membranes were carried out in 1.5-ml Eppendorf tubes in a 50-μl total volume. For cell extracts, the reaction contained 5 μmol of MES, pH 6.5, 3 μmol of $MnCl_2$, 1.2% Triton X-100, 25 nmol of UDP-[6-^{3}H]Gal (~10,000 cpm/nmol), and ~100 μg of protein. For microsomal membranes, the reaction contained 25 nmol of UDP-[6-^{3}H]Gal (~10,000 cpm/nmol), 1.5 μmol of $MnCl_2$, 1.2% Triton X-100, 5 μmol of sodium cacodylate buffer, pH 6.5, and 20–25 μg of protein. Acceptors were 0.5–1 μmol of GlcNAc, 2 μmol of Glc (and 0.4 mg/ml α-lactalbumin), and 2 μmol of Galβ3(GlcNAcβ6)GalNAcα-*O*-paranitrophenol (Toronto Research Chemicals) or 0.11 μmol of GnGn, 0.1 μmol of GnGnβ4Gn, 0.05 μmol of GnGnGnGn, and 0.05 μmol of GlcCer. Reactions lacking acceptor were used to determine incorporation into endogenous acceptors and degradation of donor sugar. After incubation at 37 °C for 2 h, the reaction was stopped by adding 1 ml of cold water. Reactions containing simple sugar or glycopeptides were passed through a 1-ml column of AG 1-X4 (Cl$^-$ form), which was subsequently washed with 2 ml of water to obtain unbound product. Reactions with Galβ3(GlcNAcβ6)GalNAcα-*O*-paranitrophenol or GlcCer were passed through a Sep-Pak C_{18} (Waters), and radiolabeled product was eluted with 50% aqueous methanol. Radioactivity was measured in a liquid scintillation counter.

Release of N-Linked Oligosaccharides by PNGase F—Cells were harvested and washed three times with phosphate-buffered saline. The cell pellet was resuspended in 20 mM Tris-HCl, pH 7.4, to obtain ~1×10^{10} cells/ml, to which an equal volume of 3% Triton X-100 was added. The suspension was mixed well and incubated on ice for 10 min and at room temperature for 10 min. The suspension was vortexed for ~2 min and then centrifuged at 5000 rpm for 30 min. The supernatant was removed and stored at −80 °C until further use. The protein concentration of the supernatant was ~10 mg/ml. *N*-Linked oligosaccharides were released by PNGase F treatment of glycoproteins bound to polyvinylidene difluoride membranes using a high-throughput microscale method as described by Papac *et al*. (25). Released oligosaccharides were passed

[2] J. H. Shaper, personal communication.

through a 0.6-ml cation-exchange resin (AG-50W-X8 resin, H^+ form, 100–200 mesh, Bio-Rad) to remove salt and protein contaminants prior to analysis by mass spectrometry.

Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)—MALDI-TOF-MS was performed on a Voyager DE Biospectrometry workstation (PerSeptive Biosystems) equipped with delayed extraction. A nitrogen laser was used to irradiate samples with ultraviolet light (337 nm), and an average of 240 scans were taken. The instrument was operated in linear configuration (1.2-m flight path), and an acceleration voltage of 20 kV was used to propel ions down the flight tube after a 60-ns delay. Samples (0.5 μl) were applied to a polished stainless steel target to which 0.3 μl of matrix was added and dried under vacuum (50×10^{-3} torr). Oligosaccharide standards were used to achieve a two-point external calibration for mass assignment of ions (26, 27). 2,5-Dihydroxybenzoic acid/5-methoxysalicylic acid and 2,4,6-trihydroxyacetophenone matrices were used in the analysis of neutral and acidic oligosaccharides, respectively.

Generation of β4GalT-1 Transfectants—Different amounts of plasmid pSVL DNA containing a bovine β4GalT-1 cDNA (a generous gift of Dr. Joel H. Shaper) were mixed with pSV2neo DNA (5 μg) separately and transfected into Pro$^-$5Lec20 cells using the Polybrene method described previously (28). Transfectants were selected for resistance to G418 (1.5 mg/ml active weight). The transfectants were expanded and tested for lectin resistance to PHA-E and for β4GalT activity with GlcNAc as acceptor.

Northern Blot Analysis—Total RNA from CHO or mutant cells was prepared using 1 ml of TRIzol Reagent (Life Technologies, Inc.) for 10^7 cells to obtain ~100 μg of total RNA, and poly(A)$^+$ RNA was prepared using an oligo(dT) column. RNA was electrophoresed on a formaldehyde-agarose gel, transferred to a Nytran membrane, and cross-linked using a Spectrolinker UV cross-linker. Blots were hybridized using ULTRAhyb (Ambion Inc.) according to the manufacturer's instructions. The probe for each β4GalT family member was generated by PCR using forward and reverse primers corresponding to the beginning and end of the corresponding full-length murine coding sequence (~1 kb). Probes were labeled with [^{32}P]dCTP to similar specific activities, and the blots were hybridized overnight at 42 °C. After washing and exposure to film for ~3 days, a PhosphorImager (Molecular Dynamics, Inc.) was used to quantitate band intensity. The primer pairs were as follows: for β4GalT-1, 5′-GATGAGGTTTCGTGAGCAGT-3′ (forward) and 5′-TATCTCGGTGTCCCGATGTC-3′ (reverse); for β4GalT-2, 5′-ACGTCTATGCCCAGCACCTG-3′ (forward) and 5′-TGGGCTGTCCAATGTCCACT-3′ (reverse); for β4GalT-3, 5′-TGGAGAGACCCTGTACATTG-3′ (forward) and 5′-TGTGGTTGGCAGTGGGCA-3′ (reverse); for β4GalT-4, 5′-CCTTATCACCTCTCCTACAG-3′ (forward) and 5′-GCAGTCCAGAAATCCACTGT-3′ (reverse); for β4GalT-5, 5′-GGCATAGTGAACACCTACCT-3′ (forward) and 5′-GCATCTCAGTACTCAGTCAC-3′ (reverse); and for β4GalT-6, 5′-ACGTACCTCTTTATGGTACAAGCT-3′ (forward) and 5′-AACCAGTATTTTGGGTGTGT-3′ (reverse). The glyceraldehyde-3-phosphate dehydrogenase probe was generated by PCR of a mouse cDNA clone. The fragment generated was 250 bp. The forward primer was 5′-CCATGGAGAAGGCTGGGG-3′, and the reverse primer was 5′-CAAAGTTGTCATGGATGACC-3′.

Reverse Transcription (RT)-PCR—For reverse transcription, 2 μg of poly(A)$^+$ RNA, 0.5 μg of oligo(dT)$_{12-18}$ primer, and 0.05 μg of random hexamer were heated to 70 °C for 10 min and slowly cooled to room temperature before adding 200–400 units of Superscript II reverse transcriptase together with first strand buffer (Life Technologies, Inc.), 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 10 mM dithiothreitol, and 1 unit/μl RNasin (Promega). Reactions were incubated for 50 min at 42 °C, heated at 70 °C for 15 min, and stored at −20 °C.

PCR for β4GalT-1 gene sequencing was performed using the forward primer 5′-GTAGCCCACMCCCYTCTTAAAGC-3′ and the reverse primer 5′-AATGAGAGGGACCAGCCCAG-3′. The primers were designed on the basis of proximal 5′- and 3′-untranslated region sequences of the human, bovine, and mouse β4GalT-1 genes. The PCR mixture contained 15 pmol of primers, 2 μl of reverse transcription product, 1 μl of 10 mM dNTPs, 0.5 μl of *Taq* DNA polymerase, 5 μl of 10× PCR buffer, and 3 μl of 25 mM $MgCl_2$ in a total volume of 50 μl. The mixtures were heated at 94 °C for 2 min, followed by 94 °C for 1 min, annealing at 65 °C for 1 min, and elongation at 72 °C for 2 min through 40 cycles. PCR products were purified using a QIAquick gel extraction kit (QIAGEN Inc.) and sequenced, either directly or after subcloning into the pCR2.1 vector using the Original TA cloning kit from Invitrogen.

For RT-PCR in Fig. 5*B*, the forward primer for CHO β4GalT-1 was 5′-TCACAGCCCCGGCACATTTCT-3′ from exon III. The reverse primer in exon VI was 5′-TATCTTGGTGTCCCGATGTC-3′. For β4GalT-6, the forward primer 5′-ATGTCTGCGCTCAAGCGGAT-3′ corresponded to the 5′-end of the coding sequence of CHO β4GalT-6, and the reverse primer 5′-GTCTTCGATTGGAGCTAACTC-3′ corresponded to the 3′-end of the coding sequence. Each sample was subjected to one cycle at 94 °C for 1 min, followed by 30 cycles at 94 °C for 1 min, 57 °C for 2 min, and 72 °C for 3 min.

Glycolipid Extraction and Purification—About 10^9 exponentially growing cells were washed twice with cold phosphate-buffered saline. Glycolipids were extracted with chloroform/methanol (2:1) as described (29). The extract was evaporated to dryness, redissolved in chloroform/methanol (1:1) at 10^8 cell eq/ml, and stored at −20 °C. For purification, 0.9 ml of this preparation were evaporated to dryness and saponified with 1 M sodium hydroxide in methanol at 40 °C for 1 h. After being neutralized with 1 M acetic acid, the solution was evaporated to dryness, and the residue was dissolved in 2 ml of methanol and 1.6 M aqueous sodium acetate (1:1). The solution was applied to a Sep-Pack C_{18} cartridge, and the flow-through was collected and reapplied twice. The cartridge was washed with 40 ml of water, and glycolipids were eluted with 2 ml of methanol, followed by 10 ml of chloroform/methanol (2:1). The eluant was evaporated to dryness, dissolved in 1 ml of chloroform/methanol (2:1), and stored at −20 °C.

Thin-layer Chromatography—Purified glycolipids (1 ml) were dried under nitrogen gas and resuspended in 25 μl of chloroform/methanol (2:1) and 25 μl of chloroform/methanol (1:2). Twenty-five μl were spotted on a Silica Gel 60 high-performance TLC plate (EM Science) with standard GlcCer (20 μg), LacCer (27 μg), and GM3 (4 μg). The plate was developed by ascending chromatography in chloroform, methanol, and 0.02% $CaCl_2$ (60:40:9). The dried plate was stained by resorcinol/H_2SO_4 reagent and scanned.

RESULTS

Reduced Galactosylation of N-Glycans in Pro$^-$5Lec20 CHO Cells—Independently isolated Lec20 CHO mutants are resistant to the Gal-binding lectins PHA-E, PHA-L, and ricin (21), consistent with a reduction in cell-surface Gal residues. To rapidly determine if *N*-glycans synthesized in Lec20 cells lack Gal residues, uniformly labeled Pronase glycopeptides of the G-glycoprotein of VSV grown in parental (Pro$^-$5 CHO) or Pro$^-$5Lec20 cells were subjected to serial lectin affinity chromatography. ConA-Sepharose chromatography showed no difference in the proportion of branched (~20%) and biantennary (~80%) complex *N*-glycans between parental and mutant-derived VSV glycopeptides. However, when the ConA-bound, biantennary population of *N*-glycans was fractionated on RCA_{II}-agarose at room temperature, a marked difference between parental and mutant glycopeptides was revealed. Whereas 46% of the Pro$^-$5 CHO/VSV biantennary *N*-glycans bound to RCA_{II}-agarose, consistent with the presence of 2 Gal residues/*N*-glycan (30), there were no RCA_{II}-bound glycopeptides among the Pro$^-$5Lec20/VSV biantennary species (Fig. 1*A*). This could be due to increased sialylation or decreased galactosylation. After neuraminidase treatment, ~71% of the Pro$^-$5Lec20/VSV biantennary glycopeptides bound to RCA_{II}-agarose at 4 °C (Fig. 1*B*) and were eluted with GalNAc, consistent with the presence of only 1 Gal residue/biantennary *N*-glycan (30). Proof that this binding was due to terminal Gal was obtained by β-galactosidase treatment, after which no Lec20/VSV glycopeptides bound to RCA_{II}-agarose (Fig. 1*C*). Reduced RCA_{II}-agarose binding of desialylated biantennary *N*-glycans was also found with [^{3}H]Gal-labeled glycopeptides from Pro$^-$5Lec20 cellular glycoproteins (data not shown). The combined data suggest that Pro$^-$5Lec20 cells have a defect in the addition of Gal residues to complex *N*-glycans.

Lec20 Mutants Have Reduced β4GalT Activity—β4GalT enzyme assays were performed with detergent cell extracts and GlcNAc or biantennary GlcNAc-terminating glycopeptide (GnGn) as acceptor. Pro$^-$5Lec20 and Gat$^-$2Lec20 cell extracts had ≤10% galactosyltransferase activity compared with parental cells (Table I). Mixing equal amounts of parental and mutant cell extracts yielded one-half the level of β4GalT activity

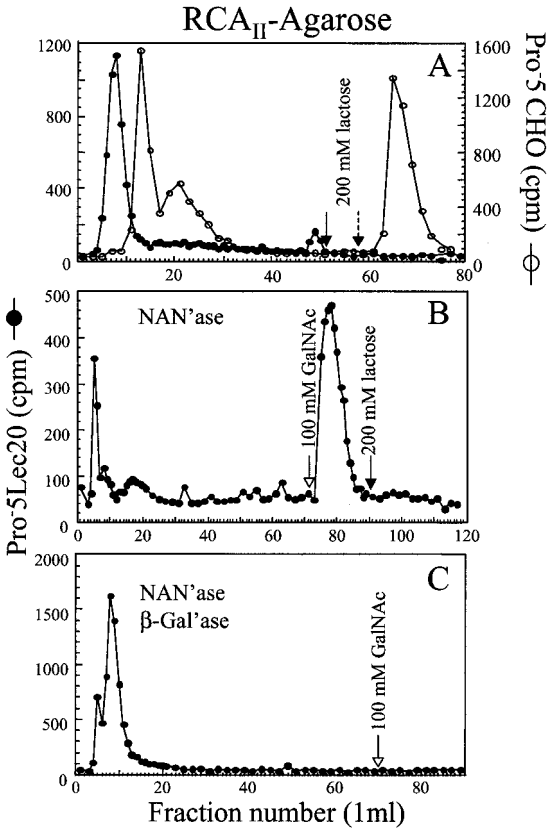

FIG. 1. **RCA$_{II}$-agarose affinity chromatography.** [^{3}H]Glucosamine-labeled Pro$^-$5 CHO/VSV (○) and Pro$^-$5Lec20/VSV (●) Pronase glycopeptides that bound to and were eluted from ConA-Sepharose were desalted and fractionated on RCA$_{II}$-agarose at room temperature (*A*). These biantennary *N*-glycans were also treated with neuraminidase (*NAN'ase*) (*B*) or neuraminidase and β-galactosidase (*β-Gal'ase*) (*C*) and fractionated on RCA$_{II}$-agarose at 4 °C. Bound glycopeptides were eluted with 100 mM GalNAc (*open arrow*), followed by 200 mM lactose (*closed arrow*). In *A*, lactose was added at slightly different fractions for Pro$^-$5Lec20 (*solid arrow*) and Pro$^-$5 CHO (*dashed arrow*).

(Table I), showing that the reduced activity in Lec20 cells is not due to the presence of an inhibitor.

β4GalT-1 Transcripts Are Altered in Lec20 Mutants—When a Northern blot was probed with a mouse β4GalT-1 probe, the CHO β4GalT-1 signal was observed at ~4.1 kb, and it was apparent that β4GalT-1 transcripts were almost absent in Pro$^-$5Lec20 cells (Fig. 2). β4GalT-1 gene transcripts in Gat$^-$2Lec20 cells were somewhat reduced and were notably smaller in size (Fig. 2).

The coding region of β4GalT-1 cDNAs from Gat$^-$2 CHO cells was sequenced (Fig. 3*A*). The sequence predicts a polypeptide of 393 amino acids, and hydropathy analysis (31) revealed a single hydrophobic membrane-spanning domain of 20 amino acids near the N terminus, which predicts the type II transmembrane topology typical of Golgi glycosyltransferases (32). The sequence also predicts one putative *N*-glycosylation site. ClustalW analysis showed that Gat$^-$2 CHO β4GalT-1 is 90.2% identical to mouse, 83.2% to human, 76.7% to bovine, and 61.9% to chicken β4GalT-1 at the amino acid level. The number and positions of all 7 Cys residues are conserved in Gat$^-$2 CHO β4GalT-1 (Fig. 3*A*).

Comparison of the Gat$^-$2 CHO and Gat$^-$2Lec20 β4GalT-1 sequences revealed that the Lec20 mutant was identical except for a 311-bp deletion that results in the production of a truncated protein of 214 amino acids derived from exons I, II, and V (Fig. 3*B*). This deletion includes a significant portion of the catalytic domain and therefore appears to be responsible for the lectin resistance phenotype and reduced β4GalT activity of

TABLE I
β4GalT activity of Lec20 CHO mutants

Cell extracts containing ~100 μg of protein were incubated with GlcNAc (1 μmol) or GnGn (0.2 μmol) as described under "Experimental Procedures." For mixing experiments, ~50 μg of protein from two detergent extracts were mixed together. The reaction contained 5 μmol of MES, pH 5.7, 3 μmol of $MnCl_2$, 1.2% Triton X-100, and 25 nmol of UDP-[^{3}H]Gal (~10,000 cpm/nmol).

Cell extract	Specific activity			
	GlcNAc		GnGn	
	Actual	Expected	Actual	Expected
	nmol/h/mg			
Pro$^-$5 CHO	15.5		5.6	
Pro$^-$5Lec20	1.9		1.7	
Pro$^-$5 CHO + Pro$^-$5Lec20	7.4	8.7	4.0	3.7
Gat$^-$2Lec20	2.1		1.1	
Pro$^-$5 CHO + Gat$^-$2Lec20	8.7	8.8	4.2	3.4

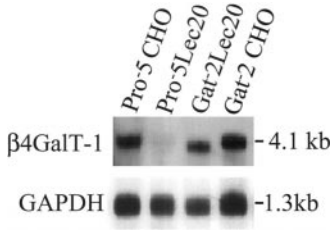

FIG. 2. **β4GalT-1 transcripts in CHO and Lec20 cells.** A Northern blot containing ~7 μg of poly(A)$^+$ RNA was hybridized to an ~1-kb murine β4GalT-1 probe and subsequently to a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe of a 250-bp PCR product.

the Gat$^-$2Lec20 mutant (Table I). The marked reduction of β4GalT-1 gene transcripts in Pro$^-$5Lec20 cells gives rise to an essentially identical galactosylation-defective phenotype.

Bovine β4GalT-1 Corrects the Phenotype of Lec20 Cells—To confirm that the reduced β4GalT activity and the lectin resistance phenotype of Lec20 cells result from an absence of β4GalT-1, bovine β4GalT-1 cDNA was transfected into Pro$^-$5Lec20 cells. Transfectants were obtained by selection with G418 and tested for their ability to bind the lectin PHA-E, for which the Lec20 mutant shows 7-fold resistance (21), and for their β4GalT activity. All transfectants were more sensitive to the toxicity of PHA-E and had increased β4GalT activity (Fig. 4). Two transfectants reverted almost to the parental phenotype. These results support the conclusion that the loss of β4GalT-1 is the cause of the Lec20 mutant phenotype.

Pro$^-$5 CHO Cells Lack β4GalT-6 Transcripts—The absence of functional β4GalT-1 in Lec20 CHO mutants clearly does not lead to a complete loss of β4GalT activity in cell extracts (Table I). Thus, it was important to determine which of the other five mammalian β4GalT genes are expressed in CHO and Lec20 cells. Two Northern blots were prepared with poly(A)$^+$ RNA from parental and mutant cells and hybridized with probes of ~1 kb derived by RT-PCR from the coding region of the corresponding murine β4GalT sequence. The results in Fig. 5*A* show that Gat$^-$2 CHO and Gat$^-$2Lec20 cells express the six β4GalT genes at similar levels. β4GalT-1 transcripts were the only ones altered in size in Gat$^-$2Lec20 cells. By contrast, Pro$^-$5 CHO and the Pro$^-$5Lec20 mutant were missing β4GalT-6 transcripts. Both also had a somewhat reduced level of β4GalT-3 transcripts (Fig. 5*A*). A complete lack of β4GalT-6 transcripts in Pro$^-$5 CHO and Pro$^-$5Lec20 cells was confirmed by RT-PCR (Fig. 5*B*). Thus, the Pro$^-$5 CHO cell, considered a "wild-type" CHO cell, is actually a "mutant" lacking β4GalT-6. Pro$^-$5Lec20 is a double mutant, essentially missing β4GalT-1 (transcripts were detected by the sensitive RT-PCR experiment in Fig. 5*B*, but not by Northern analysis in Fig. 2) and completely lacking

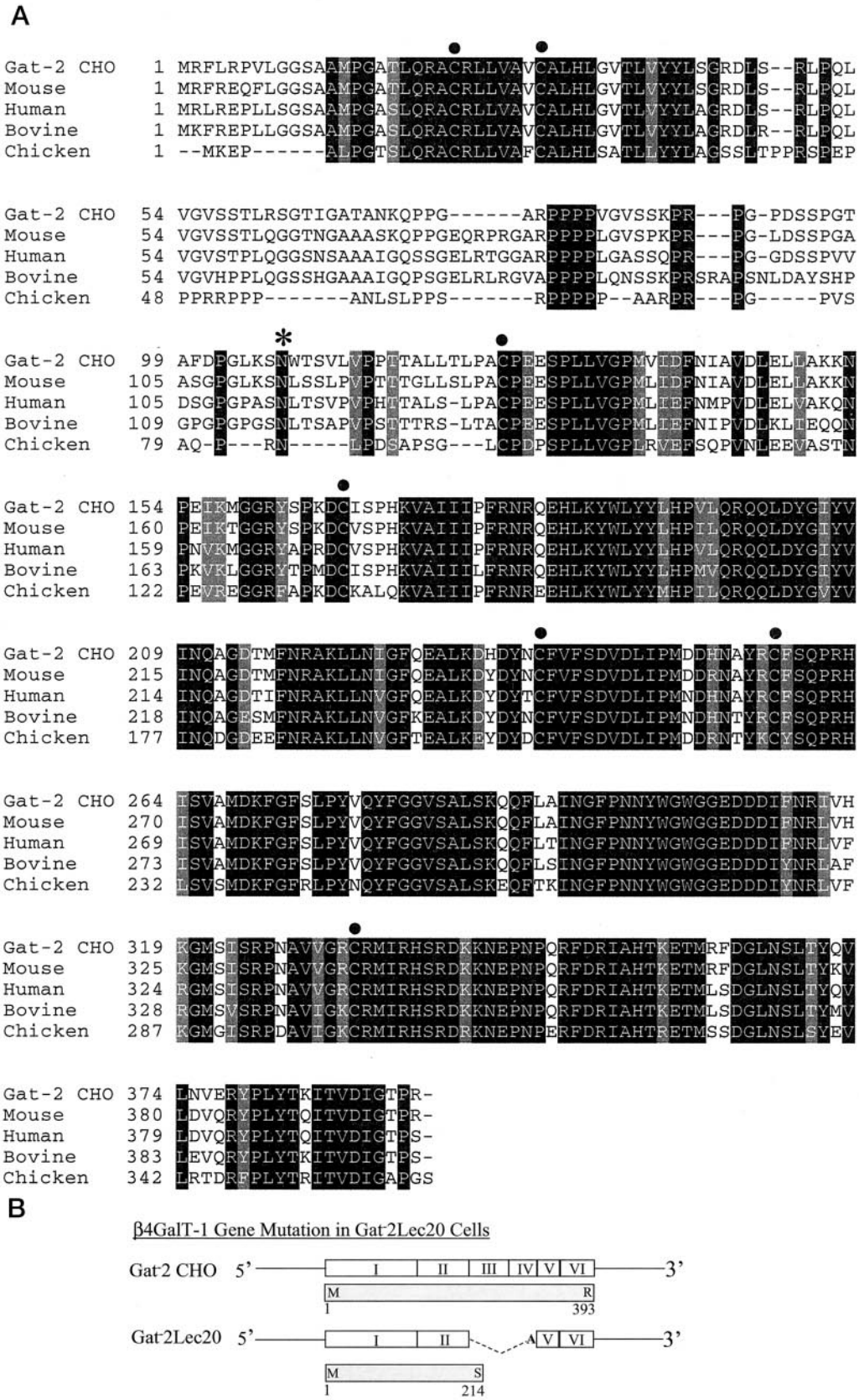

FIG. 3. *A*, ClustalW alignment of β4GalT-1 from CHO cells, mouse, human, bovine, and chicken. A potential *N*-glycosylation site (*) and conserved β4GalT-1 cysteine residues (●) are marked. *B*, the β4GalT-1 gene deletion in Gat^{-}2Lec20. Shown is a schematic diagram of a β4GalT-1 cDNA and protein product. Exons I–VI are based on the human β4GalT-1 gene (9). The Gat^{-}2Lec20 β4GalT-1 cDNA lacked nucleotides reflecting deletion of exon III and all of exon IV except for the last A residue. *Shaded bars* represent translated protein with the first and last amino acids.

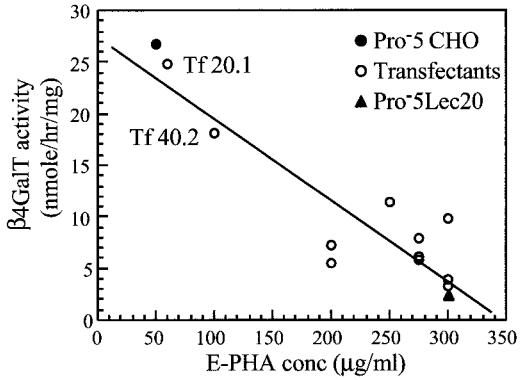

FIG. 4. **A bovine β4GalT-1 cDNA corrects the Lec20 mutant.** The β4GalT activity and PHA-E sensitivity of Pro⁻5 CHO, Pro⁻5Lec20, and bovine β4GalT-1 transfectants (*Tf*) of Pro⁻5Lec20 cells showed an inverse relationship. *Tf 20.1* had a phenotype very similar to that of parental cells.

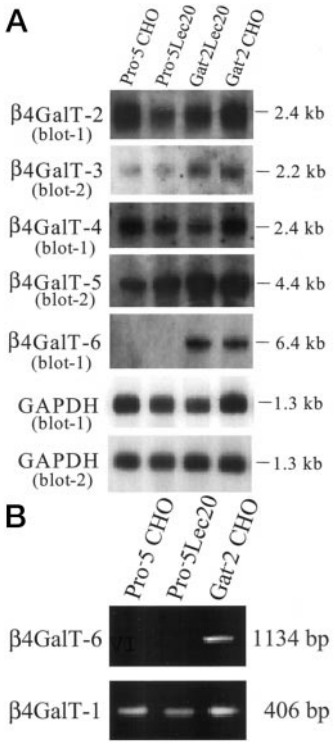

FIG. 5. *A*, shown is the expression of β4GalT-2, -3, -4, -5, and -6 in CHO and Lec20 cells. Two separate Northern blots (*blot-1* and *blot-2*) containing 7 μg of poly(A)$^+$ RNA from each cell line were hybridized to an ~1-kb murine probe and subsequently to a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe of a 250-bp PCR product as described under "Experimental Procedures." *B*, RT-PCR was performed with specific primers for CHO β4GalT-6 and CHO β4GalT-1 as described under "Experimental Procedures." The β4GalT-6 cDNA generated from Gat⁻2 CHO RNA was confirmed by digestion with *Nar*I. The predicted 656- and 478-bp products were generated.

β4GalT-6. Changes in galactosylation of glycoproteins and glycolipids in the three CHO β4GalT mutants must be interpreted on this basis.

MALDI-TOF-MS Analysis of N-Glycans in CHO Cells Lacking β4GalT-1, β4GalT-6, or Both—MALDI-TOF-MS has been used for both structural characterization (33) and relative quantitation (34) of neutral and sialylated *N*-glycans in a mixture. To examine *in vivo* galactosylation of the spectrum of *N*-glycans in CHO glycoproteins, *N*-glycans were released from parental and mutant CHO cellular glycoproteins by PNGase F and analyzed by MALDI-TOF-MS. Because most *N*-glycans derived from mammalian glycoproteins are composed of only a

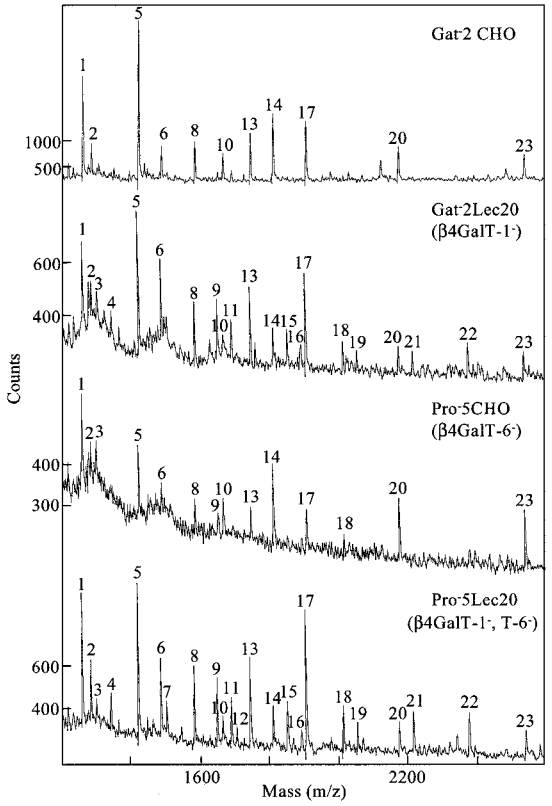

FIG. 6. **MALDI-TOF-MS of neutral *N*-glycans.** *N*-Glycans were prepared from cellular glycoproteins of the four cell lines using PNGase F as described under "Experimental Procedures" and subjected to MALDI-TOF-MS with the spectrometer in positive mode.

few monosaccharides and generate structures with unique masses that are of the oligomannosyl or bi-, tri-, or tetraantennary complex type, the nature of the species released by PNGase F may be deduced from their molecular mass in the context of known *N*-glycan structures (25–27).

The mass spectrometry of neutral *N*-glycans revealed markedly increased complexity for the β4GalT-1 mutants Gat⁻2Lec20 and Pro⁻5Lec20 compared with the β4GalT-6 mutant Pro⁻5 CHO and wild-type Gat⁻2 CHO cells (Fig. 6), consistent with the synthesis of a range of immature, undergalactosylated *N*-glycans in Lec20 cells. Analysis of these spectra is presented in Table II, in which the numbered peaks in Fig. 6 are identified based on the observed mass of $[M + Na]^+$ ions. It can be seen that each CHO cell line synthesizes a similar complement of oligomannosyl structures. Therefore, a lack of one or two β4GalTs did not significantly alter the proportion of these species, as expected. By contrast, both cell lines lacking a functional β4GalT-1 had an increased proportion of all the possible forms of undergalactosylated bi- (peaks 4, 6, 7, and 9), tri- (peaks 11, 12, 15, and 18), and tetraantennary (peaks 16, 19, 21, and 22) *N*-glycans. They also made significantly less fully galactosylated bi- (peaks 10 and 14) and triantennary (peak 20) *N*-glycans (Fig. 6), consistent with their reduced *in vitro* activities with exogenous acceptors (see Table IV). Nevertheless, fully galactosylated tetraantennary *N*-glycans (peak 23) were equivalently represented in wild-type and Lec20 cells (Fig. 6). Most striking was the fact that fully galactosylated *N*-glycans of each branched type, including biantennary, were synthesized in the absence of functional β4GalT-1. Although radiolabeled, desialylated VSV biantennary G-glycopeptides from Lec20 did not contain 2 Gal residues (see Fig. 1), high-performance anion-exchange chromatography with pulsed amperometric detection analysis of the reduced proportion of

TABLE II
Neutral N-glycans of CHO and CHO β4GalT mutants

Peak	Mass[a] ([M + Na]$^+$)		Predicted *N*-glycan	Gat$^-$2 CHO	Gat$^-$2Lec20 (β4GalT-1$^-$)	Pro$^-$5 CHO (β4GalT-6$^-$)	Pro$^-$5Lec20 (β4GalT-1$^-$/ β4GalT-6$^-$)
	Observed	Predicted[b]					
High mannose *N*-glycans							
1	1258.03	1258.10	M_5Gn_2	+	+	+	+
5	1420.17	1420.24	M_6Gn_2	+	+	+	+
8	1582.19	1582.38	M_7Gn_2	+	+	+	+
13	1744.30	1744.52	M_8Gn_2	+	+	+	+
17	1906.37	1906.66	M_9Gn_2	+	+	+	+
Hybrid *N*-glycans							
2	1282.97	1283.15	GnM_3Gn_2F	+	+	(+)[c]	++
3	1299.12	1299.15	$GnMM_3Gn_2$	−	+	(+)	(+)
Biantennary *N*-glycans							
4	1340.22	1340.20	$Gn_2M_3Gn_2$	−	+	−	+
6	1486.22	1486.34	$Gn_2M_3Gn_2F$	+	++	(+)	++
7	1502.21	1502.34	$GGn_2M_3Gn_2$	−	(+)	−	(+)
9	1648.29	1648.48	$GGn_2M_3Gn_2F$	−	++	(+)	++
10	1664.12	1664.48	$G_2Gn_2M_3Gn_2$	+	(+)	+	(+)
14	1810.34	1810.62	$G_2Gn_2M_3Gn_2F$	++	+	++	+
Triantennary *N*-glycans							
11	1689.51	1689.53	$Gn_3M_3Gn_2F$	−	+	−	+
12	1704.53	1705.53	$GGn_3M_3Gn_2$	−	−	−	(+)
15	1851.19	1851.67	$GGn_3M_3Gn_2F$	−	+	−	+
18	2013.52	2013.81	$G_2Gn_3M_3Gn_2F$	−	+	(+)	+
20	2175.69	2175.95	$G_3Gn_3M_3Gn_2F$	++	+	++	+
Tetraantennary *N*-glycans							
16	1892.19	1892.72	$Gn_4M_3Gn_2F$	−	(+)	−	(+)
19	2054.58	2054.86	$GGn_4M_3Gn_2F$	−	+	−	+
21	2216.48	2217.0	$G_2Gn_4M_3Gn_2F$	−	+	−	+
22	2378.90	2379.14	$G_3Gn_4M_3Gn_2F$	−	+	−	+
23	2541.12	2541.28	$G_4Gn_4M_3Gn_2F$	+	+	+	+

[a] Masses were taken from the spectra in Fig. 6 (observed). Among different spectra, species with the same peak number had a mass within 0.5 units of that given in the table.
[b] *N*-Glycans were predicted on the basis of the mass and composition of known neutral *N*-glycan structures (G, galactose; Gn, *N*-acetylglucosamine; M, mannose; F, fucose).
[c] (+), peaks were present, but in low amounts.

[^{3}H]Gal-labeled, desialylated biantennary glycopeptides from Lec20 cellular glycoproteins revealed biantennary glycopeptides that eluted in the position of a digalactosylated species (data not shown), consistent with the mass spectrometry data obtained from total *N*-glycanase-released species.

The lack of β4GalT-6 in Pro$^-$5 CHO cells had a very small effect on the spectrum of galactosylated neutral *N*-glycans (Fig. 6). The only truncated *N*-glycans that appeared to be slightly increased in cells lacking β4GalT-6 were peaks 3, 9, and 18 (Fig. 6 and Table II). There were almost no partially galactosylated tri- or tetraantennary *N*-glycans in the absence of β4GalT-6. Remarkably, although partially galactosylated biantennary *N*-glycans were found in wild-type Gat$^-$2 CHO and in the absence of β4GalT-6 (Pro$^-$5 CHO cells), there was no evidence of incomplete tri- or tetraantennary structures in glycoproteins from either of these cells. It appears that once a third or fourth GlcNAc is added to the trimannose core of an *N*-glycan, galactosylation goes to completion. By contrast, there were many partially galactosylated tri- and tetraantennary *N*-glycans in the absence of β4GalT-1. Most interestingly, fully galactosylated complex *N*-glycans (peaks 10, 14, 20, and 23 in Table II) are well represented in the absence of functional β4GalT-1 and β4GalT-6. Thus, it is clear that the combined activities of β4GalT-2, -3, -4, and -5 cause Gal to be added to all antennae. It can be concluded that β4GalT-6 plays an insignificant role in the galactosylation of neutral *N*-glycans and that β4GalT-1 plays an important role in the efficient completion of their galactosylation.

In the case of sialylated *N*-glycans, the picture is somewhat different (Fig. 7). First, there were significant differences between the MALDI-TOF-MS spectra from Gat$^-$2 CHO and Pro$^-$5 CHO *N*-glycans, indicating some clear consequences of the lack of β4GalT-6 in Pro$^-$5 CHO cells (Fig. 7). Pro$^-$5 CHO cells had a reduced proportion of sialylated bi-, tri-, and tetraantennary species (see particularly peaks 7, 8, 13, 15, 16, and 18 in Fig. 7), suggesting that β4GalT-6 is involved in the galactosylation of all sialylated *N*-glycans, including those with

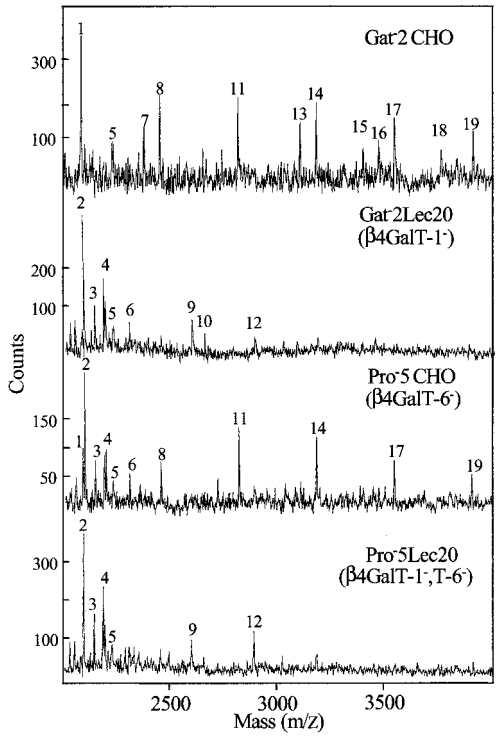

FIG. 7. **MALDI-TOF-MS of sialylated *N*-glycans.** *N*-Glycans were prepared from cellular glycoproteins of the four cell lines using PNGase F as described under "Experimental Procedures" and subjected to MALDI-TOF-MS with the spectrometer in negative mode.

TABLE III
Sialylated N-glycans of CHO and CHO β4GalT mutants

Peak	Mass[a] ([M − H]⁻)		Predicted *N*-glycan	Gat⁻2 CHO	Gat⁻2Lec20 (β4GalT-1⁻)	Pro⁻5 CHO (β4GalT-6⁻)	Pro⁻5Lec20 (β4GalT-1⁻/ β4GalT-6⁻)
	Observed	Predicted[b]					
Biantennary *N*-glycans							
1	2076.95	2077.88	$SG_2Gn_2M_3Gn_2F$	+++	−	+	−
2	2086.46[c]	2077.88	$SG_2Gn_2M_3Gn_2F$	−	+++	+++	+++
5	2221.87	2223.0	$S_2G_2Gn_2M_3Gn_2$	(+)[d]	(+)	(+)	(+)
7	2368.87	2369.14	$S_2G_2Gn_2M_3Gn_2F$	+	−	−	−
Triantennary *N*-glycans							
3	2136.66	2134.93	$SG_2Gn_3M_3Gn_2$ [e]	−	+	+	+
8	2442.24	2443.21	$SG_3Gn_3M_3Gn_2F$	++	−	+	−
6	2297.29	2297.07	$SG_3Gn_3M_3Gn_2$	−	(+)	(+)	(+)
9	2588.43	2588.33	$S_2G_3Gn_3M_3Gn_2$	−	+	−	+
12	2880.09	2879.59	$S_3G_3Gn_3M_3Gn_2$	−	(+)	(+)	+
Tetraantennary *N*-glycans							
4	2178.28	2175.98	$SG_1Gn_4M_3Gn_2$ [e]	−	+	+	+
10	2645.95	2646.40	$SG_3Gn_4M_3Gn_2F$	(+)	(+)	−	−
11	2808.23	2808.54	$SG_4Gn_4M_3Gn_2F$	++	−	++	−
13	3098.84	3099.80	$S_2G_4Gn_4M_3Gn_2F$	++	−	−	−
15	3390.11	3391.06	$S_3G_4Gn_4M_3Gn_2F$	(+)	−	−	−
Polylactosamine *N*-glycans							
14	3173.99	3173.87	$SG_5Gn_5M_3Gn_2F$	++	−	++	−
16	3463.81	3465.13	$S_2G_5Gn_5M_3Gn_2F$	(+)	−	−	−
17	3539.22	3539.2	$SG_6Gn_6M_3Gn_2F$	++	−	+	−
18	3756.10	3758.39	$SG_7Gn_7M_3Gn_2$	(+)	−	−	−
19	3904.69	3904.53	$SG_7Gn_7M_3Gn_2F$	+	−	+	−

[a] Masses were taken from the spectra in Fig. 7. Among different spectra, species with the same peak number had a mass within 0.5 units of that given in the table.
[b] *N*-Glycans were predicted on the basis of the mass and composition of known sialylated *N*-glycan structures (S, sialic acid; G, galactose; Gn, *N*-acetylglucosamine; M, mannose; F, fucose).
[c] This mass was assigned the previously predicted structure (26). The origin of the ~10-mass unit difference between observed and predicted m/z is not known.
[d] (+), peaks were present, but in low amounts.
[e] These are the only partially galactosylated *N*-glycans present in significant amounts in mutants and absent from Gat⁻2 CHO.

polylactosamine units such as peaks 16 and 18 (Table III). Second, it is clear that β4GalT-1 is involved in synthesizing all classes of sialylated *N*-glycans and to a much greater extent than β4GalT-6. Most of the sialylated complex *N*-glycans present in Gat⁻2 CHO cells (peaks 11 and 13–19 in Table III) were missing in Gat⁻2Lec20 and Pro⁻5Lec20 cells. However, most of the small population of sialylated *N*-glycans that were present in Lec20 cells contained a full complement of Gal (peaks 1/2, 6, 9, and 12 in Table III). In particular, it is notable that equivalent peaks of $SG_2Gn_2M_3Gn_2F$ (where S is sialic acid, G is galactose, Gn is *N*-acetylglucosamine, M is mannose, and F is fucose) (peaks 1/2) were present in the wild type and cells lacking β4GalT-1, β4GalT-6, or both (Fig. 7 and Table III). In addition, few of the many partially galactosylated neutral species generated in the absence of β4GalT-1 (Table II) were found as sialylated *N*-glycans (Fig. 7 and Table III). The only exceptions were peaks 3 and 4 in Fig. 7 ($SG_2Gn_3M_3Gn_2$ and $SG_1Gn_4M_3Gn_2$ in Table III). Interestingly, these species were equally represented in cells lacking functional β4GalT-1, β4GalT-6, or both. The combined data suggest that fully galactosylated *N*-glycans are sialylated more efficiently than *N*-glycans with a partial Gal complement.

CHO Mutants Lacking β4GalT-1, β4GalT-6, or Both Have a Normal Complement of Glycolipids—In *in vitro* assays, GlcCer is a good acceptor for β4GalT-1 (11), and β4GalT-6 is a lactosylceramide synthase (15). To identify *in vivo* acceptors for these β4GalTs, we isolated glycolipids from Gat⁻2 CHO, Gat⁻2Lec20, Pro⁻5 CHO, and Pro⁻5Lec20 mutants and performed high-performance TLC with the standards GlcCer, LacCer, and GM3. Gat⁻2Lec8 CHO cells were used as a control because they have an inactive UDP-Gal Golgi translocase (35) and do not add Gal to glycolipids (29). CHO cells synthesize GM3 and a small amount of GlcCer and LacCer, but no complex gangliosides (29), as shown in Fig. 8. As expected, the Gat⁻2Lec8 mutant possessed a very small amount of GM3 and LacCer and an increased amount of GlcCer compared with Gat⁻2 CHO parental cells. Interestingly, the glycolipid expression pattern of CHO cells that lack functional β4GalT-1 (Gat⁻2Lec20) or β4GalT-6 (Pro⁻5 CHO) or both (Pro⁻5Lec20) was very similar to that of parental Gat⁻2 CHO cells, which express all six β4GalTs. The major glycolipid was GM3 in all CHO cell lines, and there was no significant increase in GlcCer levels in cells lacking β4GalT-1, β4GalT-6, or β4GalT-1 and β4GalT-6. These results show that although β4GalT-1 and β4GalT-6 have activity for GlcCer *in vitro*, in CHO cells, neither is required for glycolipid synthesis. β4GalT-5 and/or β4GalT-4 seem likely to be responsible for glycolipid synthesis in CHO cells.

In Vitro β4GalT Acceptor Specificities of CHO Cells Lacking β4GalT-1, β4GalT-6, or Both—To correlate the activities of β4GalT-2, -3, -4, and -5 in cell extracts with the glycans they produce *in vivo*, β4GalT assays were performed under a range of conditions. The mixture of β4GalTs present in Lec20 mutants had little activity for the transfer of Gal to GlcNAc when Lec20 cell extracts were assayed under various conditions of substrate concentration and pH or in the presence of different nonionic detergents (Fig. 9). When microsomal membranes from Gat⁻2 CHO cells, which express the six β4GalT genes, were assayed under the optimized conditions determined in Fig. 9, a specific activity of ~24 nmol/h/mg of protein was obtained (Table IV). In Pro⁻5 CHO cells, which are missing β4GalT-6, the specific activity was slightly but significantly reduced, suggesting that recombinant β4GalT-6 can use GlcNAc as a substrate, although not efficiently. In Gat⁻2Lec20 cells lacking functional β4GalT-1, the activity with GlcNAc was reduced to ~34%. In Pro⁻5Lec20 cells, with defective β4GalT-1 and β4GalT-6, activity with GlcNAc was only 28% of that in Gat⁻2 CHO cells.

In the mammary gland, β4GalT-1 interacts with α-lactalbumin, resulting in a change of acceptor specificity from GlcNAc

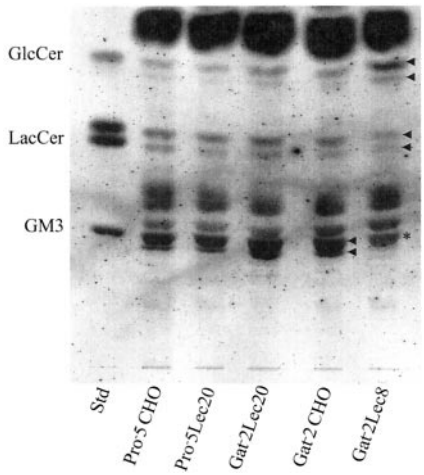

FIG. 8. **Thin-layer chromatography of glycolipids.** Purified glycolipids extracted from the four cell lines and Gat$^-$2Lec8 cells were spotted on a Silica Gel 60 high-performance TLC plate with standards (*Std*) of GlcCer (20 μg), LacCer (27 μg), and GM3 (4 μg). The plate was developed by ascending chromatography in chloroform, methanol, and 0.02% $CaCl_2$ (60:40:9) and stained with resorcinol/H_2SO_4 reagent. Glycolipids are marked with *arrowheads*. The band marked with an *asterisk* in the *Gat$^-$2Lec8 lane* is not GM3.

to Glc and the production of lactose. β4GalT-2 is also able to produce lactose efficiently (9). However, β4GalT-2 in CHO cells does not appear to associate with α-lactalbumin *in vitro* since Gat$^-$2Lec20 cells, which lack β4GalT-1 activity but have a normal complement of β4GalT-2 transcripts (Fig. 5*A*), had only 3% of the parental Gat$^-$2 CHO activity for transfer of Gal to Glc in the presence of α-lactalbumin (Table IV).

When more complex *N*-glycans were assayed as acceptors, Gat$^-$2 CHO extracts always had a higher specific activity than Pro$^-$5 CHO extracts, suggesting that β4GalT-6 is acting on complex acceptors *in vitro* (Table IV). For Gat$^-$2Lec20 cells lacking functional β4GalT-1, the most severe reduction in activity (~97%) was observed for the biantennary *N*-glycan acceptor GnGn. Therefore, under the assay conditions used, none of the five other β4GalTs efficiently galactosylated a biantennary complex glycopeptide *in vitro*. The tetraantennary *N*-glycan acceptor GnGnGnGn was more effectively galactosylated in the Gat$^-$2Lec20 β4GalT-1$^-$ mutant (~27% compared with Gat$^-$2 CHO cells). However, the triantennary acceptor was a significantly better acceptor for the mixture of β4GalT-2, -3, -4, -5, and -6 in Gat$^-$2Lec20 cell extract (~54% compared with wild-type Gat$^-$2 CHO cells). Therefore, whereas β4GalT-1 appears to be the major activity transferring Gal to complex *N*-glycans in CHO cell microsomes, other β4GalTs efficiently transfer Gal to the triantennary complex acceptor GnGnβ4Gn. β4GalT-6 is also able to use GnGnβ4Gn efficiently as an acceptor since the absence of β4GalT-6 in Pro$^-$5 CHO resulted in a reduction of 43% activity (Table IV).

Lactosylceramide synthase activity was equivalent in wild-type Gat$^-$2 CHO cells and each mutant line (Table IV), as would be predicted from the glycolipid analysis in Fig. 8. Since β4GalT-6 is known to be a lactosylceramide synthase (15), it was surprising that Pro$^-$5 CHO cells, which lack β4GalT-6, had equivalent *in vitro* activity for GlcCer. Similarly, Gat$^-$2Lec20 and Pro$^-$5Lec20, which lack functional β4GalT-1 or β4GalT-1 and β4GalT-6, respectively, showed no decrease in transfer to GlcCer. Thus, neither β4GalT-1 nor β4GalT-6 appears to contribute to glycolipid synthesis in CHO cells.

Finally, both β4GalT-1 and β4GalT-6 contributed to the transfer of Gal to the mucin core 2 acceptor (Table IV). Compared with Gat$^-$2 CHO cells, Pro$^-$5 CHO cells were reduced ~25%, suggesting that β4GalT-6 adds Gal to core 2; Gat$^-$2Lec20 cells were reduced ~40%, suggesting that β4GalT-1 also transfers Gal to core 2. Clearly, other β4GalTs such as β4GalT-4, which has been identified as having a high degree of specificity for a core 2 acceptor (12), contribute in CHO extracts to the transfer of Gal to the core 2 oligosaccharide.

DISCUSSION

The knowledge that mammals have six β4GalTs with overlapping *in vitro* acceptor specificities (8, 36, 37) presents the challenge of sorting out their unique biological functions. An important question is the degree of redundancy between different members of the β4GalT family in transferring Gal to complex glycoprotein and glycolipid acceptors. To begin to address this question, we have identified *N*-glycan and glycolipid structures synthesized in CHO cells that express the six β4GalTs compared with mutant cells that lack β4GalT-1, β4GalT-6, or both. We have shown that mutants of the Lec20 complementation group (21) lack β4GalT-1 activity. In Gat$^-$2Lec20 CHO cells, it is due to a deletion mutation that removes exons III and IV of the β4GalT-1 gene so that only the N-terminal 214 amino acids of β4GalT-1 can be synthesized. Pro$^-$5Lec20 CHO cells have a mutation that results in extremely low steady-state levels of β4GalT-1 transcripts. The overall phenotype of both Lec20 mutants is essentially identical (21); and thus, it was of interest to discover that Pro$^-$5Lec20 cells have no detectable β4GalT-6 transcripts. The β4GalT-6 deficiency in Pro$^-$5Lec20 cells originated from the parental Pro$^-$5 CHO cells, which are also completely devoid of β4GalT-6 gene transcripts. Therefore, we used the four cell lines to investigate the relative contributions of β4GalT-1, β4GalT-6, and the four remaining β4GalTs to *in vivo* Gal transfer to glycoproteins and glycolipids and to *in vitro* galactosyltransferase acceptor specificity for various acceptors.

A summary of mutants and their properties is given in Table V. The loss of β4GalT-1 had the most profound effect on *in vitro* lactose synthase activity and on the transfer of Gal to the biantennary GnGn glycopeptide (Table IV). One or more of the remaining five β4GalTs transferred Gal quite efficiently to GlcNAc, tri- and tetraantennary glycopeptides, and the core 2 oligosaccharide, although β4GalT-1 provided $\geq$50% of the activity with these acceptors. Interestingly, β4GalT-1 did not contribute to the transfer of Gal to GlcCer in CHO cell extracts, even though recombinant β4GalT-1 uses GlcCer as an acceptor (9). Thin-layer chromatography of glycolipids from both the Lec20 mutant lines confirmed that β4GalT-1 does not contribute to the synthesis of LacCer or GM3 in CHO cells (Fig. 8).

The *in vitro* results with microsomal membranes suggest that β4GalT-1 is the most important β4GalT in galactosylating biantennary *N*-glycans (Table IV). This conclusion is supported by MALDI-TOF-MS analysis of neutral and sialylated *N*-glycans (Tables II and III). Gat$^-$2 CHO cells, which express all six β4GalTs, synthesize fully galactosylated tetra- or triantennary neutral *N*-glycans, but have appreciable amounts of undergalactosylated biantennary *N*-glycans. In Lec20 mutants that lack functional β4GalT-1, almost every possible partially galactosylated *N*-glycan is synthesized, but the predominant species is undergalactosylated biantennary *N*-glycans. Thus, β4GalT-1 is required for efficiently generating fully galactosylated bi-, tri-, and tetraantennary neutral *N*-glycans. Most interestingly, only a very small proportion of the partially galactosylated structures that predominate in Lec20 mutants acquire sialic acid (Table III). In fact, no biantennary *N*-glycans containing 1 Gal residue capped with sialic acid were detected. This strongly suggests that the first sialic acid is not transferred by the α2,3 sialyltransferase in CHO cells until both Gal residues are present on a biantennary structure. In addition, it is apparent

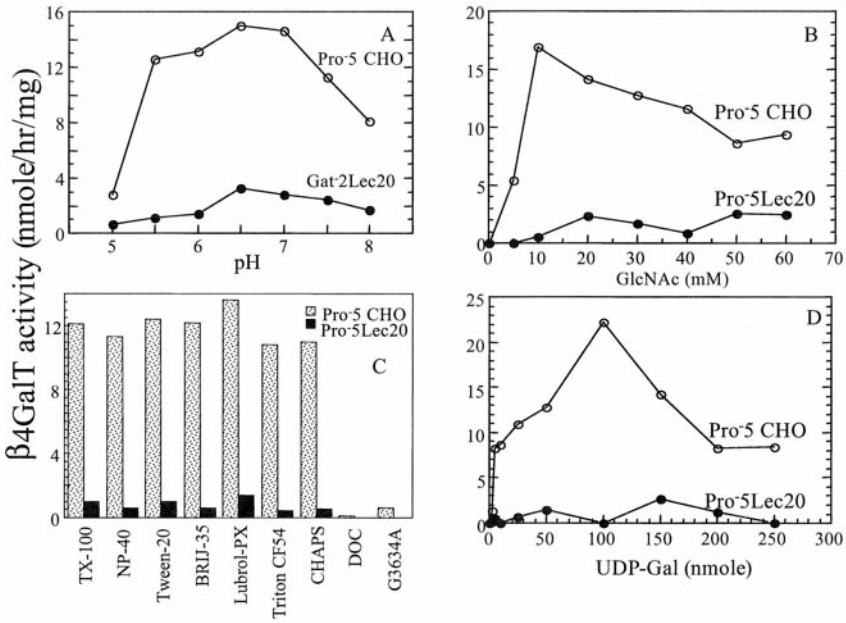

FIG. 9. **β4GalT activities of Pro⁻5 CHO and Pro⁻5Lec20 under different assay conditions.** β4GalT activities of Pro⁻5 CHO and Pro⁻5Lec20 cell extracts were assayed using GlcNAc as acceptor as described under "Experimental Procedures." The reaction contained 5 μmol of MES, pH 6.5 (*C*) or pH 5.7 (*B* and *D*), 3 μmol of $MnCl_2$, 1.2% Triton X-100, 25 nmol of UDP-[6-^{3}H]Gal (~10,000 cpm/nmol) (*A–C*), 0.5 μmol of GlcNAc (*A*, *C*, and *D*), and ~100 μg of protein. In *A*, MES was used for pH 5~6.5, and MOPS was used for pH 7–8. In *C*, all detergents were used at a final concentration of 1%. *TX-100*, Triton X-100; *NP-40*, Nonidet P-40; *DOC*, sodium deoxycholate.

TABLE IV
β4GalT activities of CHO cells lacking β4GalT-1, β4GalT-6, or both

β4GalT activities were measured under optimized conditions using microsomal membranes as described under "Experimental Procedures." *O*-pNP, *O*-paranitrophenol.

Acceptor	Specific activity[a]			
	Gat⁻2 CHO	Gat⁻2Lec20 (β4GalT-1⁻)	Pro⁻5 CHO (β4GalT-6⁻)	Pro⁻5Lec20 (β4GalT-1⁻/β4GalT-6⁻)
	nmol/h/mg protein			
GlcNAc	23.7 ± 1.0	8.08 ± 0.74 (34.1)[b]	19.34 ± 1.05 (81.6)[c]	6.7 ± 0.49 (28.3)
Glc (with α-lactalbumin)	10.0 ± 0.5	0.25 ± 0.12 (2.5)	9.06 ± 0.3 (90.6)[c]	0.36 ± 0.16 (3.6)
Biantennary (GnGn)	7.6 ± 0.6	0.23 ± 0.1 (3.0)	5.4 ± 0.55 (71.1)	0.84 ± 0.28 (11.1)
Triantennary (GnGnβ4Gn)	13.9 ± 0.9	7.47 ± 0.95 (53.7)	7.94 ± 0.63 (57.1)	4.89 ± 0.87 (35.2)
Tetraantennary (GnGnGnGn)	13.3 ± 0.9	3.6 ± 0.36 (27.1)	9.38 ± 0.72 (70.5)	3.05 ± 0.18 (22.9)
GlcNAcβ1,6GalNAcα-*O*-pNP Galβ1,3 (core 2)	7.3 ± 0.6	4.4 ± 0.14 (60.3)	5.43 ± 0.16 (74.4)[c]	4.35 ± 0.15 (59.6)
GlcCer	0.23 ± 0.02	0.3 ± 0.08 (130.4)	0.4 ± 0.11 (173.9)	0.43 ± 0.07 (186.9)

[a] The activity is the mean ± S.D. of three or four different experiments performed in duplicate.
[b] Percent compared with the activity of Gat⁻2 CHO.
[c] $p < 0.02$ by Student's *t* test compared with Gat⁻2 CHO.

TABLE V
Summary of CHO β4GalT mutants

	Gat⁻2 CHO	Gat⁻2Lec20	Pro⁻5 CHO	Pro⁻5Lec20
β4GalT transcripts				
β4GaT-1	+	Deletion	+	Few[a]
β4GaT-2	+	+	+	+
β4GaT-3	+	+	+	+
β4GaT-4	+	+	+	+
β4GaT-5	+	+	+	+
β4GaT-6	+	+	Absent	Absent
Neutral *N*-glycans (MALDI-TOF-MS)				
Undergalactosylated	(+)	++++	+	++++
Sialylated *N*-glycans (MALDI-TOF-MS)				
Undergalactosylated	(+)	++	+	++
Glycolipid synthesis (HPTLC[b])				
GM3 and LacCer	++++	++++	++++	++++

[a] Although Northern analysis did not detect β4GalT-1 transcripts, RT-PCR gave the expected product.
[b] High-performance TLC.

that only one partially galactosylated triantennary structure ($SG_2Gn_3M_3Gn_2$) and one tetraantennary structure ($SG_1Gn_4M_3Gn_2$) were present in Lec20 mutants. None of the other neutral *N*-glycans with 1, 2, or 3 Gal residues (see Table II) were sialylated (see Table III). Also of interest are the several *N*-glycans that appear to have polylactosamine sequences among the sialylated species.

The results of *in vitro* galactosyltransferase assays and glycan analyses reveal subtle but significant effects of the absence of β4GalT-6 in CHO cells. The Pro⁻5 CHO cell extract, which lacks β4GalT-6, had significantly, although slightly, reduced activity with all acceptors except GlcCer (Table IV). By far the

biggest effect of the loss of β4GalT-6 in Pro⁻5 CHO was in the ~44% reduced transfer of Gal to the triantennary acceptor from fetuin (GnGnβ4Gn). This was not reflected in an abundance of undergalactosylated triantennary *N*-glycans in Pro⁻5 CHO glycoproteins, however. In fact, there were only minor peaks of bi- and triantennary neutral *N*-glycans lacking Gal residues in Pro⁻5 CHO glycoproteins. By contrast, for the sialylated *N*-glycans, the absence of β4GalT-6 gave rise to the same species of undergalactosylated tri- and tetraantennary structures as did the absence of β4GalT-1. Thus, Pro⁻5 CHO cells lacking only β4GalT-6 contained only a subset of the fully galactosylated, sialylated *N*-glycans synthesized by the full complement of six β4GalTs in Gat⁻2 CHO cells. Finally, it can be seen from the spectrum of complex *N*-glycans synthesized in the double mutant Pro⁻5Lec20 that the effects of missing β4GalT-1 and β4GalT-6 are essentially additive.

Perhaps the most unexpected result with cells lacking β4GalT-6 was the fact that this was not reflected in an increased amount of GlcCer due to reduced synthesis of LacCer and GM3 (Fig. 8). β4GalT-6 was called LacCer synthase when first cloned (15) and has been proposed to be a major β4GalT responsible for LacCer synthesis. Although it is true that all the β4GalTs can synthesize LacCer *in vitro*, β4GalT-6 and β4GalT-5 are more closely related to each other than to the other β4GalTs at the amino acid level. Thus, it may be that β4GalT-5 is the β4GalT that synthesizes LacCer in CHO cells because it is clear that neither β4GalT-1 nor β4GalT-6 is responsible. In summary, the Gal transfer properties of Gat⁻2 CHO cells, which express all six β4GalTs, compared with those of glycosylation mutants lacking functional β4GalT-1 (Gat⁻2Lec20), β4GalT-6 (Pro⁻5 CHO), or both (Pro⁻5Lec20) show that β4GalT-1 is a key enzyme for the galactosylation of complex *N*-glycans and that neither β4GalT-1 nor β4GalT-6 is involved in glycolipid synthesis in CHO cells (Table V).

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