β1,4-N-Acetylgalactosaminytransferase (GM2 Synthase) Is Released from Golgi Membranes as a Neuraminidase-sensitive, Disulfide-bonded Dimer by a Cathepsin D-like Protease*

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Many Golgi membrane-bound glycosyltransferases are released from cells in a soluble form. To characterize this release process, we stably transfected Chinese hamster ovary cells with three myc epitope-tagged forms of cloned β1,4-N-acetylgalactosaminytransferase (GalNAcT); two of these forms resided in the Golgi, while the third was retained in the endoplasmic reticulum (ER). GalNAcT was released into the culture medium from cells transfected with the Golgi forms but not with the ER form of the enzyme. The medium from cells transfected with the Golgi forms contained disulfide-bonded dimers of GalNAcT, which carried neuraminidase-sensitive, complex N-linked carbohydrate chains. This soluble species represented the major degradation product of cellular GalNAcT, which turned over with a half-time of about 1.7 h. The soluble species consisted of a mixture of truncated GalNAcT molecules, the major form of which was produced by cleavage near the boundary between the transmembrane and luminal domains between Leu-23 and Tyr-24. This cleavage site fits the sequence pattern for sites cleaved by cathepsin D (van Noort, J.M., and van der Drift, A.C.M. (1989) J. Biol. Chem. 264, 14159–14164). These findings suggest that GalNAcT is converted from a membrane-bound to a soluble form as a result of cleavage by a cathepsin D-like protease in a compartment late in the Golgi secretory pathway.

The glycosyltransferases responsible for the synthesis of glycoprotein and glycosphingolipid (GSL)1 sugar chains are resident membrane proteins of the endoplasmic reticulum (ER) and Golgi apparatus. Many of these enzymes are type II proteins containing an NH2-terminal cytoplasmic region, a transmembrane domain, an extended, flexible stem or stalk region, and a large catalytic domain at the COOH terminus (1). Soluble forms of several endogenously expressed glycosyltransferases have been found in milk, serum, and other body fluids (reviewed in Ref. 1). Also, most cloned glycosyltransferases have been reported to be released into the culture medium from transfected cells (Refs. 2–5; see “Discussion”), but blood group H α1,2-fucosyltransferase (6) and O-linked GalNAcT (7) were not released when transfected into COS-1 cells and SF9 insect cells, respectively.

For three endogenously expressed glycosyltransferases, comparison of the NH2-terminal sequences of their soluble forms with the sequences of the respective intact forms revealed that the soluble forms were the result of proteolytic cleavage of the stem or stalk region that separated the catalytic domain from the membrane-spanning domain. Specifically, the cleavage sites of GlcNAc β1,4-GT (LR | TGGAR) (8, 9), O-linked GalNAcT (ER | GLPAG) (7), and Gal α2,6-ST (SN | SKQDP) (10) were located 62, 12, and 35 amino acid residues, respectively, from the transmembrane domain. The sequences of these cleavage sites suggest that the first two may have been the result of cleavage by a serine protease, while the third could have been cut by a cathepsin D-like protease (11). In fact, Jamieson and colleagues (12–14) have provided evidence that Gal α2,6-ST was released from rat liver Golgi membranes as a result of cleavage within an acidic compartment, presumably the trans-Golgi or trans-Golgi network, by a cathepsin D-like protease. In addition to cases in which soluble transferases could arise as a result of cleavage of membrane-bound enzymes, there is the possibility that some soluble glycosyltransferases may represent bona fide secretory proteins; e.g. some transcripts coding for kidney α2,6-ST would produce proteins lacking cytoplasmic and transmembrane domains (15). Therefore, additional studies are necessary to delineate the origin of soluble glycosyltransferases and to define the proteases responsible for their production where applicable.

Among the glycosyltransferases that have been cloned to date, only a few are specific for GSL synthesis. One of these cloned enzymes, β1,4-N-acetylgalactosaminyltransferase (GalNAcT), synthesizes the GSL GM2, GD2, and Gb2 (16–19). Recently, we described the generation of stable transfectants of Chinese hamster ovary (CHO) cells expressing each of three myc epitope-tagged forms of cloned GalNAcT; two of these

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1 The abbreviations used are: GSL, glycosphingolipid(s); CHO, Chinese hamster ovary; GM2, GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glc-ceramide; Gb2, GalNAcβ1,4Galβ1,4Glc-ceramide; GD2, GalNAcβ1,4Galα1,4Glc-ceramide; Gd2, GalNAcβ1,4Galβ1,4Glc-ceramide; Gd3, NeuAcα2,8NeuAcα2,3Galβ1,4Glc-ceramide; Gb3, NeuAcα2,6Galβ1,4Glc-ceramide; GD3, NeuAcα2,6Galβ1,4Glc-ceramide; endo-H, endo-β-N-acetylgalacosaminidase H; GalNAcT, β1,4 N-acetylgalactosaminyltransferase (GM2/GD2 synthase); GNT, N-acetylgalactosaminyltransferase I; GlcNAc β1,4,β-N-acetyltetragalactosaminidase, β1,4-galactosyltransferase; Gal α2,6-ST, Galβ1,4Glcα2,6-sialyltransferase; O-linked GalNAcT, UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase; Ip33, p33 form of human invariant chain; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; EMEM, methionine- and cysteine-free minimal essential medium.
forms resided in the Golgi, while the third was retained in the ER (20). In this study we report that GalNACT is released into the culture medium from cells transfected with the Golgi forms but not with the ER form of the enzyme. Furthermore, only an endo-H-resistant, neuraminidase-sensitive glycoform of GalNACT is released from cells transfected with the Golgi-targeted constructs. Soluble GalNACT is produced by a proteolytic cleavage occurring near the boundary between the transmembrane domain and the stem region at a site that fits the sequence pattern for sites cleaved by cathepsin D (11).

MATERIALS AND METHODS

Chimeric Constructs—GalNACT/myc, GNT/GalNACT/myc, and ip3/S/ GalNACT/myc constructs have been described elsewhere (20) and are summarized in Fig. 1. In addition a six-histidine sequence was fused to the carboxyl terminus of GalNACT/myc as follows. The GalNACT/myc construct in the pCDM8 vector was used as template in a polymerase chain reaction (PCR). The amplified sequence consisted of the COOH-terminal fragment of GalNACT/myc from the EcoRI restriction site at base pair 1,444 to the terminal fragment of GalNACT/myc including nine base pair 1,444 to the terminal fragment of GalNACT/myc including the COOH-terminus of GalNACT/myc was complementary to amino acid residues 266–270 of protein A and contained an XhoI restriction site to allow subcloning of the PCR product into the vector. The amplified sequence was cloned into pCDM8 containing the GalNACT/myc insert (20). The pROTA plasmid (21) was used as a template in a PCR to amplify the cDNA encoding the transmembrane protein A hybrid sequence. The pROTA plasmid was kindly provided by Dr. B. Macher (San Francisco State University). PCR primers (Bio-synthesis, Lewisville, TX) were the GalNACT sense primer described previously (20) and an antisense primer that contained a DraI restriction site to allow subcloning of the PCR product into the vector. The GalNACT/myc construct in pCDM8 was opened with EcoRI and DraI. The GalNACT/myc construct was as follows: 5'-CTCACCGTGTGTTAGTGGTGGTGGTGGTGGTGCAGGTCTTTCTTCTTATGAGATCATGCAGTTC-3'.

To prepare a soluble chimeric protein consisting of the IgG binding domain of protein A fused to the luminal domain of GalNACT/myc, the pPROTA plasmid (21) was used as a template in a PCR to amplify the cDNA encoding the protein A hybrid sequence. The pPROTA plasmid was kindly provided by Dr. B. Macher (San Francisco State University). PCR primers (Bio-synthesis) were as follows: sense, 5'-GATCAG GTTGCGGGCGACGTAGAAGGTCGTCCGTC-3'; antisense, 5'-AGTCCCCGCTGGATGTGCTTTTAAAGGTC-3'.

The sense primer coded for the first six amino acid residues of transmembrane and contained a HindIII restriction site and a Kozak consensus sequence for proper initiation of translation (22). The antisense primer was complementary to amino acid residues 266–270 of protein A and contained an XmaI site. After partial digestion with HindIII and XmaI, the PCR product was subcloned into pCDM8 containing the GalNACT/myc insert (20).

Cell Culture and Transfection—Wild type CHO cells and mutant Lec3.2 cells were the gifts of J. Baenzerger (Washington University, St. Louis, MO) and P. Stanley (Albert Einstein Medical College, Bronx, NY), respectively, and were grown in α-minimal essential medium containing 10% (v/v) fetal calf serum plus glutamine at 37 °C. Lec3.2 is a sialylation-defective mutant similar to Lec3 (23). Cells were transfected, and clones were selected as described previously (20). In addition CHO cells were transfected with either the GalNACT/myc construct, which contains the IgG binding domain of protein A fused to the luminal domain of GalNACT/myc construct using electroporation (BTX, San Diego, CA) or the protein A/GalNACT/myc construct using DOTAP (Boehringer Mannheim) or electroporation. Transfected cells were maintained in complete medium containing 0.4 mg/ml of active Geneticin (G418, Life Technologies, Inc.). Subclone CE4 of L5178Y murine lymphoma cells was described elsewhere (24) and was grown in RPMI 1640 containing Nutridoma-CS (Boehringer Mannheim).

GalNACT Enzyme Assay—The in vitro assay for GalNACT activity has been described elsewhere (20). Conditioned medium was centrifuged at 250 × g for 10 min at 4 °C, and the supernatant was centrifuged at 100,000 × g for 1 h at 4 °C to remove any cell debris from the culture medium prior to enzyme analysis. In the case of L5178Y clone CE4 cells, the high speed supernatant was concentrated 54-fold using a Centricon 30 device (Amicon, Beverly, MA). The protein concentration of cell extracts was determined by the BCA method (25) (Pierce). Lactate dehydrogenase activity of cells and conditioned media was used to monitor cell lysis and was determined using the CytoTox 96 assay kit (Promega, Madison, WI).

Glycosylation Treatment—N-Glycanase (Genzyme, Cambridge, MA) treatment was performed according to the manufacturer's protocol. Cell extracts (20 μg of total protein) were denatured by boiling for 5 min in 0.2 M Tris-HCl buffer, pH 7.65, containing 0.5% SDS and 50 mM β-mercaptoethanol. Following the addition of 3.75 μl of 10% Nonidet P-40, 0.3 units of N-glycanase, and water to bring the final volume to 30 μl, samples were incubated at 37 °C for 4 h and then analyzed by Western blotting. For neuraminidase treatment, cell extracts (10 μg of total protein) or immunoprecipitated proteins on protein G-agarose beads were incubated in 10 μl of 50 mM sodium acetate buffer, pH 4.5, with 1 milliunit of neuraminidase from Vibrio cholerae (Calbiochem) for 18 h at 37 °C. Samples were analyzed by Western blotting using anti-myc antibody 9E10 (26). Endo-H (Genzyme) treatment was performed according to the manufacturer's protocol. Cell extracts (10 μg of protein) were denatured by boiling in 0.2 M dithiothreitol and a 2-fold excess by weight of SDS. Then samples were diluted with sodium citrate buffer, pH 6.0, to a final concentration of 50 mM and an SDS concentration of 0.2%. After the addition of 1 milliunit of endo-H, samples were incubated for 18 h at 37 °C and then analyzed by Western blotting.

Western Blotting—Details have been described elsewhere (20). Briefly, samples were separated by SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp., Marlborough, MA). Blots were blocked and then incubated in succession with intervening washing with anti-myec 9E10 hybridoma cell culture medium diluted 1:10 and then with horseradish peroxidase-coupled sheep anti-mouse IgG at a dilution of 1:5000. Blots were visualized using the ECL detection system (Amersham Corp.).

Immunoprecipitation of Protein A/GalNACT/myc from Culture Media—Conditioned medium (2.5 ml) was concentrated 10 times using Centricon 30 devices. 50 μl of rabbit IgG-agarose beads (Sigma) was washed three times with washing buffer (20 mM Tris, pH 7.4, 150 mM NaCl) and then incubated overnight at 4 °C in conditioned medium with rotation at 4 °C. After washing the beads five times with washing buffer containing 0.1% SDS, 0.5% Nonidet P-40, an equal volume of 2 × SDS-PAGE sample buffer was added, and the samples were boiled for 5 min and electroblotted.

Subcellular Fractionation—Cells were harvested with a rubber policeman and washed in 10 mM Tris-HCl, pH 7.5, 0.25 M sucrose (Tris-sucrose). The cell pellet was resuspended in an equal volume of Tris-sucrose and homogenized using a ball bearing homogenizer with a 0.1573-inch precision bore and a 0.15625-inch ball bearing (27). After centrifugation at 1,100 × g for 12 min at 4 °C, the postnuclear supernatant was loaded onto a discontinuous sucrose gradient, which consisted of 5 steps of 0.8, 1.2, 1.4, 1.6, and 1.8 M sucrose as described previously (28). The gradients were centrifuged for 5 h at 22,500 rpm (90,000 × g) in an SW 40Ti rotor. One-milliliter fractions were harvested from the top using a density gradient fractionator (Auto Densi-Flow IIc, Buchler, Lenexa, KS). Fractions were assayed for galactosyltransferase activity (Golgi marker) as described previously (29) and for α-glucosidase II activity (ER marker) as we previously described (30), and they were then electroblotted for GalNACT/myc as described previously (20).

Immunoprecipitation of Released GalNACT/myc from Culture Media—Cells were grown in serum-free medium (CHO-S-SFM II; Life Technologies, Inc.) for 4 days. All subsequent steps were at 4 °C. The medium was centrifuged at 500 × g for 10 min to remove cells and then at 100,000 × g for 1 h at 4 °C to remove any cell debris, including membrane vesicles (31). Supernatants were concentrated 10-fold using Centricon 30 devices (Amicon) by centrifugation at 2,000 × g for 1 h. 50 μl of protein G-agarose beads (Sigma) were washed three times with washing buffer and then incubated with rotation for 4 h with 10 times concentrated anti-myec 9E10 hybridoma culture supernatant. After washing the antibody-coated beads three times with washing buffer, they were incubated overnight with rotation with concentrated media from appropriate clones. Beads were then washed five times with washing buffer including 0.1% SDS and 0.5% Nonidet P-40, suspended in an equal volume of 2 × SDS-PAGE reducing sample buffer, boiled for 5 min, and electroblotted on a 6.5% stacking, 10% separating gel.

Metabolic Labeling—Confluent monolayers of clone C3 cells were rinsed twice in methionine- and cysteine-free minimal essential medium (EMEM; ICN, Costa Mesa, CA), incubated for 1 h in EMEM, and then labeled with 100 μCi/ml 35S-label (ICN) in EMEM for 15 min at 37 °C. After chasing in α-minimal essential medium containing 10% fetal calf serum, the cells were extracted as described above, and the chase media were collected. Cell extracts and media were subjected to immunoprecipitation with anti-myec 9E10 as described above followed by electrophoresis. Radiolabeled bands were processed using a phosphor imager (Molecular Devices Corp., Menlo Park, CA).

Amino Acid Sequencing—CHO clone C5 cells transfected with GalNACT/myc were grown to confluence in CHO-S-SFM II medium in 30 150-cm2 flasks. The medium (840 ml) was centrifuged at 500 × g for 20 min to remove cells, and a protease inhibitor mixture was added (32). Following centrifugation at 30,100 × g for 3 h at 4 °C, the supernatant...
was concentrated in Centriprep 30 devices to 7 ml. Protein G-agarose beads were covalently coupled (33) with anti-myc 9E10 as follows. Aliquots of washed beads were incubated with concentrated 9E10 culture supernatant as described above, washed in 0.2 M triethanolamine, pH 8.2, incubated in 30 mM dimethylimidelinate in 0.2 M triethanolamine, pH 8.2, for 45 min at room temperature, centrifuged, and resuspended in 30 mM ethanolamine, pH 8.2, for 5 min at room temperature. After washing in 0.1 M borate buffer, pH 8.2, 50-ml aliquots of beads were each incubated with 1-ml aliquots of concentrated C5 culture medium overnight with rotation at 4 °C. Beads were washed and boiled in reducing sample buffer, and aliquots were subjected to SDS-PAGE followed by electrotransfer onto a Pro-Blott membrane (Perkin-Elmer). After staining with Coomassie Blue, the GalNAcT/myc band was excised, and the amino-terminal sequence was determined on an Applied Biosystems 470A gas-phase sequencer at the Biomolecular Research Facility, University of Virginia.

RESULTS

Release of GalNAcT Activity—We reported elsewhere (20) that CHO cells stably transfected with three constructs (Fig. 1) of GalNAcT/myc contained high levels of GalNAcT activity when assayed in vitro. An enzyme assay of the conditioned media from cells transfected with GalNAcT/myc (clone C5) revealed that a high percentage of the total activity was present in the medium (Table I). As a control for release from broken cells, we measured the activity of the cytoplasmic enzyme lactate dehydrogenase. In contrast to GalNAcT release, less than 2% of the cellular lactate dehydrogenase activity was released from clone C5 cells, indicating that the presence of GalNAcT/myc in the conditioned medium was not the result of cell lysis. For clone B5 cells transfected with the GNT/GalNAcT/myc construct, the majority of the total GalNAcT activity was present in the medium (Table I). In contrast, no GalNAcT activity above background levels could be detected in the conditioned media from clones E6, K16, and K19 transfected with the Iip33/GalNAcT/myc construct (Table I). One explanation for these release data could be that release increased with expression level because the total GalNAcT activity was highest in GalNAcT/myc-transfected clone C5 and lowest in the Iip33/GalNAcT/myc-transfected clones. However, GalNAcT/myc/His6-transfected clones R8 and R12 each also released a high percentage of GalNAcT into the medium although the total activity of these clones was at least 10-fold less than clone C5 and lower than the Iip33/GalNAcT/myc-transfected clones (Table I). Thus, release was not simply a function of expression level.

We also tested for release of GalNAcT from murine lymphoma L5178Y clone CE4 cells, which express this enzyme endogenously (17). As shown in Table I, 30% of the total GalNAcT activity was present in the medium of a 3-day culture of these cells. In contrast, 13.3% of lactate dehydrogenase activity was detected in the medium. The release of lactate dehydrogenase was considerably higher than expected, considering that the cells were >98% viable based on trypan blue exclusion. These cells produce membrane vesicles that bud into the medium (31), and it is possible that the cytoplasmic enzyme lactate dehydrogenase may be released into the medium during this budding process. In summary, although the extent of GalNAcT released from L5178Y clone CE4 cells was not as great as from transfected cells, these results indicate that release of GalNAcT is not restricted to CHO cells and is not an artifact of transfection.

The occurrence of soluble forms of glycosyltransferases has been thought to be the result of proteolytic release of the catalytic domain from the transmembrane domain (1). If GalNAcT was cleaved in this manner, then the two Golgi-targeted constructs of GalNAcT (expressed in clones C5, R8, R12, F9, and B5) might be susceptible to this cleavage, while the ER targeted construct (expressed in clones E6, K16, and K19) would not. To determine the extent of intracellular processing

**Table I**

**Released versus cell-associated GalNAcT transferase activity**

Clones of stable transfactants were assayed for GalNAcT transferase activity using Gα3 as substrate as previously described (17). For all CHO derived cells, the total activity found in cell extracts prepared from an entire flask (75 cm²) of transfected cells is denoted as “cell-associated activity,” whereas the total activity found in the processed media harvested from the same flask after 4 days of culture is termed “released activity.” For L5178Y clone CE4 cells, the data are expressed as the activity present in a culture containing 22 × 10⁶ cells grown in a volume of 20 ml. One unit of transferase represents the amount of enzyme that can transfer 1 pmol of GalNAcT to 15 nmol of Gα3 during a 60-min incubation period, after correction for background transfer.

| Cells        | Construct       | Specific activity | Cell-associated activity | Released activity |
|--------------|----------------|------------------|-------------------------|------------------|
| CHO clone C5 | GalNAcT/myc     | 12.2             | 11,879                  | 192,048 (94.2% of total) |
| CHO clone R8 | GalNAcT/myc/His6| 1.1              | 783.8                   | 6,987 (89.9% of total) |
| CHO clone R12| GalNAcT/myc/His6| 1.2              | 878.4                   | 16,512 (95.0% of total) |
| Lecl.2 clone F9 | GalNAcT/myc     | 5.6              | 5,310                   | 51,824 (90.7% of total) |
| CHO clone B5 | GNT/GalNAcT/myc | 4.6              | 3,050                   | 6,528 (63.8% of total) |
| CHO clone e6 | Iip33/GalNAcT/myc | 4.2            | 5,182                   | ND* |
| CHO clone K16| Iip33/GalNAcT/myc | 5.9          | 6,109                   | ND* |
| CHO clone K19| Iip33/GalNAcT/myc | 3.7          | 3,885                   | ND* |
| CHO clone PM6| Protein A/GalNAcT/myc | 0.51    | 424                     | 12,170 (96.6% of total) |
| L5178Y clone CE4 (Not transfected) | (0.65 | 2,310 | 1,006 (30.2% of total) |

* ND, not detectable. The values were less than those obtained by assay of either fresh culture medium or the conditioned medium from untransfected CHO cells.
of the cellular and released forms of GalNAcT, in the following experiments we conducted Western blot analysis using monoclonal antibody 9E10, which is specific for the myc epitope (26).

Comparison of Cellular and Released GalNAcT—As we reported previously (20), an extract of clone C5 cells transfected with GalNAcT/myc produced a doublet when Western blotted with anti-myc in the presence of reducing agent (Fig. 2A, left lane C) and a broad band of a size consistent with that of a homodimer in the absence of reducing agent (Fig. 2A, right lane C). In contrast, GalNAcT/myc present in the clone C5 conditioned medium produced a single band migrating at or slightly below the upper cellular band in the presence of reducing agent (Fig. 2A, left lane M) and a single band co-migrating with the cellular disulfide bonded species in the absence of reducing agent (Fig. 2A, right lane M). These results suggested that the disulfide bonds responsible for the formation of the cellular disulfide-bonded species of GalNAcT/myc were also present in the released form of GalNAcT/myc.

We also analyzed the secretion of the soluble product that resulted from the fusion of the IgG binding domain of protein A to the luminal domain of GalNAcT/myc (see the construct in Fig. 1). More than 96% of the total GalNAcT activity produced by clone PM6 of CHO cells transfected with this construct was present in the medium (Table I). Protein A/GalNAcT/myc secreted into the culture medium produced, under reducing conditions, a single anti-myc-reactive band (Fig. 2A, left lane PA) of approximately 86,000, a size consistent with that predicted from the cDNA sequence. In the absence of reducing agent, this soluble species formed an intermolecular disulfide-bonded species of a size consistent with that of a homodimer (Fig. 2A, right lane PA). A similar fusion of protein A to α1,3-galactosyltransferase resulted in a soluble product that was a monomer even in the absence of reducing agent (see Fig. 7; Ref. 35), indicating that the cysteine residues of the protein A domain were not capable of forming dimers of that fusion protein. These results suggest that the dimers of protein A/GalNAcT/myc may be the result of disulfide bonds formed between cysteine residues in the lumen of disulfide bonds formed between cysteine residues in the lumen of cellular and released GalNAcT/myc may also be the result of disulfide bonds formed between luminal domain cysteine residues of GalNAcT.

Because the soluble form represented such a high percentage of the total GalNAcT present in C5 cultures after 4 days of cell growth, it was possible that the anti-myc-reactive upper band present in cell extracts might in fact be soluble GalNAcT that had not yet been released from the cells. To test this point, C5 cells were homogenized (see “Materials and Methods”), frozen and thawed twice, and then centrifuged at 100,000 × g for 1 h at 4 °C. The membrane pellet was resuspended to the original volume, and an aliquot was taken for SDS-PAGE under reducing conditions followed by Western blotting with anti-myc 9E10. Lane P, the membrane pellet; lane H, the homogenate.

Glycosidase Digestions—As we reported previously (20), an extract of clone B5 cells transfected with GNT/GalNAcT/myc produced a doublet when Western blotted with anti-myc under reducing conditions (Fig. 3A, lane B5). In contrast, only a single band was produced from extracts of clone E6 cells transfected with Iip33/GalNAcT/myc (Fig. 3A, lane E6). To clarify the relationship between the cellular GalNAcT doublet and the released single band in clones C5 and B5, the following glycosidase digestions were performed. First, the nature of the Gal-
GalNAcT/myc doublet in cell extracts from clones C5 and B5 was explored. Three potential sites for N-glycosylation were predicted from the corrected cDNA sequence (36) of cloned GalNAcT (16). Treatment with N-glycanase, to remove all N-linked oligosaccharide chains, converted the C5 and B5 doublets to essentially a single band that migrated slightly faster than the lower band in untreated samples (Fig. 3A, lanes C5 + and B5 +). N-Glycanase also caused the single band from clone E6 extract to migrate slightly faster than the untreated control (Fig. 3A, compare lanes B6 – and B6 +). Thus, GalNAcT/myc in all three clones contained N-linked chains. Furthermore, the structures responsible for the difference in migration between the two bands comprising the C5 and B5 doublets appeared to reside in the N-linked chains.

To further define the difference between the upper and lower bands, cell extracts were treated with neuraminidase, which converted the doublets in clone C5 and B5 extracts to single bands (Fig. 3B, lanes C5 – and B5 –) that co-migrated with the lower band in untreated C5 and B5 cell extracts (Fig. 3B, lanes C5 – and B5 –). In contrast, the single band in the clone E6 extract was unaffected by neuraminidase treatment (Fig. 3B, lane E6 +). These findings suggested that sialylation differences were responsible for the differences in migration of the two bands comprising the C5 and B5 doublets. We reported elsewhere that GalNAcT/myc in clone C5 was distributed throughout all the cisternae of Golgi stacks, while GNT/GalNAcT/myc in clone B5 was primarily located in the medial Golgi cisternae (20). Furthermore, sialylation of N-linked chains is widely accepted as being a late Golgi function. Therefore, it seems reasonable to propose that those GalNAcT/myc molecules located in the later Golgi cisternae of clone C5 would be available for N-linked sialylation, whereas those molecules remaining in more cis cisternae would not. In the case of GNT/GalNAcT/myc it is possible that although the distribution of this enzyme is centered over the medial Golgi cisternae, in fact a significant portion of this enzyme could overlap the distribution of the sialyltransferase responsible for sialylation of N-linked chains. As a result, a portion of GNT/GalNAcT/myc molecules would become sialylated on N-linked chains.

To test the effect of sialylation on the pattern of anti-myct-reactive species of GalNAcT/myc, a sialylation-defective mutant of CHO cells, Lecl3.2, was transfected stably with GalNAcT/myc, and clone F9 was isolated. GalNAcT/myc in these cells was found by immunoelectronmicroscopy with anti-myct to be distributed throughout the Golgi stack (data not shown). Anti-myct staining of an extract from F9 cells produced only a single band (Fig. 3B, lane F9 –), which co-migrated with the lower band seen in C5 and B5 cells (Fig. 3B, lanes C5 – and B5 –). Neuraminidase treatment had no effect on the migration of the band from F9 cells (Fig. 3B, lane F9 +), supporting the conclusion that sialylation was responsible for the difference in migration between the two anti-myct reactive bands present in C5 and B5 extracts. Interestingly, a high percentage of GalNAcT activity was released from clone F9 cells (Table I), indicating that sialylation was not a prerequisite for release of a soluble form of GalNAcT.

In the final set of glycosidase digestions, endo-H treatment of the clone E6 cell extract produced a band that migrated faster (Fig. 3C, lane E6 +) than the untreated control (Fig. 3C, lane E6 –), indicating that the lip33/GalNAcT/myc fusion protein was completely sensitive to endo-H as expected based on its ER location. Endo-H treatment of C5 and B5 extracts produced more widely spaced anti-myct-reactive doublets (Fig. 3C, lanes C5 + and B5 +) than found in untreated controls (Fig. 3C, lanes C5 – and B5 –). The slower migrating band co-migrated with the upper band of untreated C5 and B5 extracts, while the lower band following endo-H treatment migrated considerably faster than the untreated lower band. These results suggest that the upper band contains endo-H-resistant N-linked chains, while the lower band is endo-H-sensitive. As with the interpretation of neuraminidase results described above, the lower GalNAcT band in C5 and B5 cells may represent molecules located in relatively early Golgi compartments, while the upper band represents molecules located in later cisternae.

**Fig. 4. Subcellular fractionation of GalNAcT/myc-transfected clone C5 cells on a sucrose gradient.** Cells were homogenized, and the postnuclear supernatant was centrifuged on a discontinuous sucrose gradient. Panel A, aliquots (7.5 μl) of fractions 3–13 were analyzed by Western blotting with anti-myct. Panel B, fractions were assayed for α-glucosidase II (ER marker enzyme; open circles) and galactosyltransferase (Golgi marker enzyme; closed circles). Gradient interfaces occurred between fractions 3 and 4 (0.8 and 1.2 M sucrose interface), at fraction 5 (1.2 and 1.4 M sucrose), and between fractions 11 and 12 (1.4 and 1.6 M sucrose).

Subcellular Fractionation—The preceding results suggest that the anti-myct-reactive doublet formed by GalNAcT/myc consists of an upper band of sialylated molecules present in later Golgi cisternae, while the lower band represents nonsialylated enzyme present in earlier compartments of the secretory pathway. We used subcellular fractionation to separate Golgi and ER membranes in order to directly address the subcellular distribution of the two bands. These results revealed that the lower GalNAcT/myc band was present in both Golgi and ER fractions (Fig. 4A), whereas the upper band was strongest in the Golgi peak fractions 3–5 (Fig. 4A) and was not detectable in the ER peak fractions 11–13. Thus, the upper band in fact was composed of sialylated molecules present in the Golgi.

Immunoprecipitation of Released GalNAcT—Having determined that the upper band of the anti-myct-reactive doublets seen in Western blots of clone C5 and B5 cell extracts was neuraminidase-sensitive (Fig. 3B), we next explored the neuraminidase sensitivity of the single band released from these cells. In order to obtain sufficient signal from clone B5 cells, it was necessary to immunoprecipitate the culture media with anti-myct and then to visualize the bands by Western blotting with anti-myct. Immunoprecipitates of conditioned media from clone C5 and B5 cells produced a single anti-myct-reactive band (Fig. 5, lanes C5 M – and B5 M –), which co-migrated with or was slightly faster than the upper cellular band (Fig. 5, lanes C5 C and B5 C). In striking contrast, no band was detectable in the immunoprecipitate from E6 conditioned medium (Fig. 5, lane E6 M), in agreement with the absence of detectable GalNAcT in vitro activity in E6 medium (Table I). Neuraminidase treatment of the C5 and B5 immunoprecipitates produced a single band (Fig. 5, lanes C5 M + and B5 M +), which co-migrated with the lower cellular band (Fig. 5, lanes C5 C and B5 C). These results suggest that only the sialylated form of GalNAcT/myc and GNT/GalNAcT/myc is released from clone C5 and B5 cells, respectively.

Kinetics of Release—GalNAcT/myc-transfected clone C5 cells...
were labeled with \[^{35}S\]Met and \[^{35}S\]Cys, chased in medium containing excess unlabeled methionine and cysteine, and harvested. Aliquots of media and cell extracts were immunoprecipitated with anti-myc. At the end of the 15-min labeling period, nearly all of the labeled species in the cells (Fig. 6A, left panel) consisted of the endo-H-sensitive, neuraminidase-resistant lower band seen in Western blots (Fig. 2), while only 6% of the immunoprecipitated GalNAcT/myc was composed of the slower migrating set of bands corresponding to the endo-H-resistant, neuraminidase-sensitive upper band (Fig. 2). At this zero chase time point no labeled GalNAcT/myc was detectable in the medium (Fig. 6A, right panel). After 1 h of chase, approximately 25% of the lower cellular band had been converted to the endo-H-resistant, neuraminidase-sensitive upper cellular band plus a labeled band in the culture medium that migrated between the cellular upper and lower bands (Fig. 6A).

The amount of labeled GalNAcT/myc in the medium continued to increase with time of chase (Fig. 6B). In addition, roughly 10% of the initial labeled GalNAcT/myc appeared in the cells at 7 h of chase as an unidentified band roughly 20 kDa smaller than intact GalNAcT/myc. Overall, intact GalNAcT/myc in clone C5 cells turned over with a half-time of about 1.7 h. When combined with the results of glycosidase digestions and subcellular fractionation described above, these results suggest that the intracellular processing of GalNAcT/myc proceeded first by the conversion of an endo-H-sensitive, neuraminidase-resistant form to an endo-H-resistant, neuraminidase-sensitive form followed shortly thereafter by the release of this latter form as a soluble species into the culture medium.

**NH2-terminal Amino Acid Sequencing of Released GalNAcT/myc**—Soluble GalNAcT/myc was purified from GalNAcT/myc-transfected clone C5 conditioned medium by immunoprecipitation with anti-myc and subjected to gas-phase sequencing. The relative abundance of amino acids recovered in each sequencing cycle (Table II) suggested the presence of a major species with the NH2-terminal sequence of YASTRDAPGLR plus a mixture of less abundant species including one with the NH2-terminal sequence of STRDAPGLR. The major species corresponded to a region of cloned GalNAcT (16) that began at the COOH-terminal end of the transmembrane domain and extended into the stem region (Fig. 7). The minor species identified in Table II corresponded to the same region but with the two amino-terminal residues removed from the major species, suggesting either aminopeptidase trimming of the major species or endoproteolytic cleavage of intact GalNAcT/myc at more than one site (see “Discussion”).

A common structural motif around the cleavage site produced by the aspartyl proteinase cathepsin D was defined previously (11) (Fig. 7). Beyond the general features shown in Fig. 7, at the P1 position leucine and aromatic residues were strongly favored (11). At position P2, the requirement for a basic residue was not absolute in that other polar or small residues including alanine were possible. However, at least one basic residue at either the P2 or P9 position was required. As shown in Fig. 7, the site in GalNAcT that was cleaved to produce the major soluble species satisfied all these requirements for a cathepsin D cleavage site; i.e., there was a hydrophobic residue at P2, a favored leucine at P1, a permissible tyrosine at P1, and permissible alanine at P9, and a required arginine at P9.

**DISCUSSION**

The results reported here indicate that cloned GalNAcT is released into the culture medium from transfected cells, provided the enzyme is located in the Golgi, but not when it is retained in the ER. The data supporting this conclusion are of two types. First, only GalNAcT proteins encoded by Golgi-targeted constructs were released into the medium, whereas no
TABLE II
Phenylthiohydantoin-derivatives and peptide sequences

| Cycle | Amino acids | pmols |
|-------|-------------|-------|
| 1     | Tyr         | 5.29  |
|       | Leu         | 4.52  |
|       | Ser         | 0.93  |
| 2     | Ala         | 3.72  |
|       | Thr         | 0.65  |
| 3     | Ser         | 1.01  |
|       | Arg         | 0.78  |
| 4     | Asp         | 1.99  |
|       | Thr         | 1.89  |
| 5     | Arg         | 2.36  |
|       | Ala         | 1.08  |
| 6     | Asp         | 2.69  |
|       | Pro         | 1.63  |
|       | Thr         | 0.84  |
| 7     | Ala         | 3.09  |
|       | Gly         | 1.54  |
| 8     | Pro         | 1.77  |
|       | Leu         | 1.69  |
| 9     | Gly         | 2.42  |
|       | Ala         | 1.95  |
|       | Tyr         | 0.8   |
|       | Arg         | 0.75  |
| 10    | Leu         | 3.22  |
| 11    | Arg         | 1.79  |
|       | Ala         | 1.16  |
|       | Glu         | 0.83  |
|       | Gly         | 0.37  |

* Due to a high background, the data are reported as pmol of the amino acid in the respective cycle minus pmol in the preceding cycle.

As described in the Introduction, catalytically active, soluble forms of several endogenously expressed glycosyltransferases have been found in body fluids (reviewed in Ref. 1). In addition, release of a soluble form of endogenous Gal α1,3-GT from mouse teratocarcinoma F9 cells recently was shown to increase in parallel with the increase in cellular levels of this enzyme in response to retinoic acid treatment (38). Also, most cloned glycosyltransferases have been reported to be released into the culture medium from transfected cells, although the sites of cleavage have not been defined as yet. Specifically, those reported to be released were Gal α1,3-GT from transfected COS-1 cells (2), α1,3/1,4-fucosyltransferase from COS-1 cells (4), the CT antigen GalNAc transferase from CHO cells (3), and Galα1,2,8-ST (Gmα3 synthase) from COS-7 cells (5). In striking contrast, only a trace amount of α1,2-fucosyltransferase was released into the medium from transfected COS-1 cells (6).

Interestingly, although a soluble form of O-linked GalNAcT was found in bovine colostrum, the level of this enzyme in the medium of transfected insect Sf9 cells was so low that the authors attributed it to leaking from dead cells (7). These findings suggest the likelihood that there are cell type differences in the susceptibility of any glycosyltransferase to proteolytic cleavage and release as a soluble form. In this regard, we found that endogenous GalNAcT was released from L5178Y murine lymphoma cells (Table I), indicating that cleavage to produce a soluble form of this enzyme is not restricted to the CHO cells used to express cloned GalNAcT.

NH2-terminal sequencing of soluble GalNAcT/myc released from transfected CHO cells indicated that the major cleavage site occurred near the COOH-terminal end of the transmembrane domain (Fig. 7). As described in the Introduction, the cleavage sites of three endogenously expressed glycosyltransferases were at least 12 amino acid residues from the transmembrane domain. However, precedents exist for proteolytic cleavage within the transmembrane domain, such as the production of β-amylod from the amyloid precursor protein (reviewed in Ref. 39). In our NH2-terminal sequence data, the presence of additional residues in each sequencing cycle suggested the possibility of additional cleavage sites in GalNAcT/myc, one of which was identified (Table II). Our pulse-chase data also suggested the generation during chase of a GalNAcT/myc degradation product of about 47 kDa, which remained cell-associated (Fig. 6A). This fragment was also present when samples were electrophoresed under nonreducing conditions. Additional experimentation is required to define this fragment. The protease(s) responsible for cleaving GalNAcT/myc must have been derived from the cells and not from the growth medium because all of the GalNAcT activity data described

Fig. 7. GalNAcT cleavage site sequence compared with the preferred site for cathepsin D proteolysis. The NH2-terminal sequence of the major soluble species of GalNAcT (Table II) is shown aligned with the respective region of intact GalNAcT (16, 36). At the top, the cleavage site at which the major soluble species of GalNAcT/myc was formed is shown in comparison with the preferred cathepsin D cleavage site (11).
above (Table I) as well as the soluble form used for NH₂-
terminal sequencing were obtained with cells grown in the
serum-free supplement CHO-S-SFM II, which lacks known
proteases. Within the cells, there are several proteases associ-
ated with the Golgi secretory pathway. In the ER there is
signal peptidase (40) and a poorly characterized degradative
pathway for incompletely folded or misfolded proteins (41).
In the trans-Golgi network there is a family of ubiquitously ex-
pressed subtilisin-like endoproteases, which include yeast
kexin and mammalian furin (42). Also, in this same compart-
ment is endothelin-converting enzyme-2, a metalloprotease
having an acidic pH optimum of 5.5 and a restricted tissue
distribution (43). Cathepsin D is a aspartyl protease that is
present in the lysosomal and endosomal vesicles of virtually all
mammalian tissues and has an acidic pH optimum (11). Be-
cause of the extensive traffic between the endosomes and the
trans-Golgi network, cathepsin D would have access to glyco-
syltransferases in the trans-Golgi network. We previously re-
ported that GalNAcT/myc was present in all Golgi cisterna-
including the trans-most elements (20), so GalNAcT/myc
and cathepsin D could have interacted in our transfected cells.
In fact, as described under “Results,” the major cleavage site of
GalNAcT/myc (Table II) satisfies the criteria that have been
defined for the common structural motif around the cathepsin
D cleavage site (11). Thus, cathepsin D or another unknown
cathepsin D-like protease appeared to mediate the release of
GalNAcT/myc from transfected CHO cells. Interestingly, the
apparent cathepsin D cleavage site that we have identified in
human GalNAcT, LL ↓ YASTR (Table II), is also present in the
rat homolog (18) and only slightly modified (LL ↓ YSTR) in the
murine homolog.4

The soluble form of GalNAcT released into the culture me-
dium appeared as an intermolecular disulfide-bonded species of
a size consistent with that of a homodimer (Fig. 2). GalNAcT
contains eight cysteine residues, two in the transmembrane
domain and the other six in the lumenal domain (36). There is
a precedent for glycosyltransferase homodimer formation being
dependent on transmembrane domain cysteine residues (33).
However, because we now know that the cleavage of GalNAcT
(Table II) separates nearly all of the transmembrane domain
including the two cysteine residues from the lumenal domain,
we can exclude the possibility that transmembrane domain
cysteine residues participate in disulfide bond formation of the
soluble form of GalNAcT. This conclusion is consistent with other
data. The GNT/GalNAcT/myc construct (Fig. 1), which forms apparent homodimers (20), consists of the lumenal
domain of GalNAcT fused to the cytoplasmic and transmembrane
domains of GlcNAc transferase I, which has no cysteines.
Therefore, intermolecular disulfides must form between lumen-
nal domain cysteines of GalNAcT in that case. Also, protein
A/GalNAcT/myc (Fig. 1), which contains only the lumenal do-
main of GalNAcT, forms an apparent homodimer (Fig. 2A).
Since the protein A domain used here formed only monomers
either by itself (44) or when fused to the lumenaal domain of
another glycosyltransferase (35), it is likely that the protein
A/GalNAcT/myc dimers as well as those of soluble GalNAcT are
the result of disulfide bonds formed between lumenal domain
cysteine residues of GalNAcT.

The release of soluble glycosyltransferases from cells raises
several issues. First, there is little information concerning the
relative stability of the soluble form of a glycosyltransferase,
subsequent to its cleavage and during its passage out of the
cell, to produce its normal product glycoconjugates as compared
with its membrane-bound counterpart. Relevant to the ques-

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