Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Effect of *N*-Glycosylation on Turnover and Subcellular Distribution of *N*-Acetylgalactosaminyltransferase I and Sialyltransferase II in Neuroblastoma Cells

Erhard Bieberich, Tewin Tencomnao, Dmitri Kapitonov, and Robert K. Yu

Department of Biochemistry and Molecular Biophysics, Medical College of Virginia Campus of Virginia Commonwealth University, Richmond, Virginia, U.S.A.

Abstract: Gangliosides are sialylated glycosphingolipids whose biosynthesis is catalyzed by a series of endoplasmic reticulum (ER)- and Golgi-resident glycosyltransferases. Protein expression, processing, and subcellular localization of the key regulatory enzymes for ganglioside biosynthesis, sialyltransferase II (ST-II) and *N*-acetylgalactosaminyltransferase I (GalNAcT), were analyzed upon transient expression of the two enzymes in the neuroblastoma cell lines NG108-15 and F-11. The enzymes were endowed with a C-terminal epitope tag peptide (FLAG) for immunostaining and immunoaffinity purification using a FLAG-specific antibody. Mature ST-II–FLAG and GalNAcT–FLAG were expressed as *N*-glycoproteins with noncomplex oligosaccharides. ST-II–FLAG was distributed to the Golgi apparatus, whereas GalNAcT–FLAG was found in the ER and Golgi. Inhibition of early *N*-glycoprotein processing with castanospermine resulted in a distribution of ST-II–FLAG to the ER, whereas that of GalNAcT–FLAG remained unaltered. In contrast to GalNAcT, the activity of ST-II and the amount of immunostained enzyme were reduced concomitantly by 75% upon incubation with castanospermine. This was due to a fourfold increased turnover of ST-II–FLAG, which was not found with GalNAcT–FLAG. The ER retention and increased turnover of ST-II–FLAG were most likely due to its inability to bind to calnexin upon inhibition of early *N*-glycoprotein processing. Calnexin binding was not observed for GalNAcT–FLAG, indicating a differential effect of *N*-glycosylation on the turnover and subcellular localization of the two glycosyltransferases. **Key Words:** Gangliosides—Glycosyltransferases—Posttranslational modification—Subcellular localization—*N*-Glycosylation—Chaperones.
J. Neurochem. **74,** 2359–2364 (2000).

Gangliosides are sialic acid-containing glycosphingolipids synthesized by a sequence of glycosyltransferases acting in parallel biosynthetic pathways. The composition of gangliosides is correlated with distinct stages of neuronal cell development, indicating that the temporal and spatial expression of biosynthetic enzymes is tightly regulated (Yu, 1994). Recently, it has been shown that *N*-glycosylation of sialyltransferase II (ST-II) or *N*-acetylgalactosaminyltransferase I (GalNAcT) may affect their activity or subcellular localization (Haraguchi et al., 1995; Martina et al., 1998). Elimination of N-linked oligosaccharides reduced the activity of the two enzymes to <10% of the activity as determined with cells expressing the fully glycosylated enzyme (Haraguchi et al., 1995; Martina et al., 1998). It remained to be elucidated, however, whether this phenomenon was due to a reduction of the substrate affinity or catalytic activity of the enzymes or to a decrease of the amount of enzyme by enhanced intracellular turnover. In this investigation, we analyzed the significance of *N*-glycosylation for the specific activity, intracellular stability, and subcellular localization of GalNAcT and ST-II. Intracellular turnover and localization were monitored by pulse chase experiments and immunofluorescence studies with neuronal cells expressing the respective enzymes endowed with a C-terminal tag epitope for immunoaffinity purification and detection. The enzyme activity of the *N*-glycosylated expression products was compared with that of the unglycosylated enzymes translated in vitro in a cell-free system. Optionally, early *N*-glycoprotein processing in transfected cells was blocked by incubation with castanospermine, an inhibitor of trimming glucosidases I and II (Kornfeld and Kornfeld, 1985; Kalz-Füller et al., 1995).

MATERIALS AND METHODS

Materials

NG108-15 and F-11 cells were kindly provided by Drs. Robert Ledeen (New Jersey School of Medicine, Newark, NJ, U.S.A.) and Glyn Dawson (University of Chicago, Chicago, IL, U.S.A.), respectively. A Superscript mouse 15.5-day embryo

Received December 16, 1999; revised manuscript received February 11, 2000; accepted February 14, 2000.

Address correspondence and reprint requests to Dr. E. Bieberich at Department of Biochemistry and Molecular Biophysics, Medical College of Virginia Campus of Virginia Commonwealth University, 1101 E. Marshall St., P. O. Box 980614, Richmond, VA 23298-0614, U.S.A. E-mail: ebieberi@hsc.vcu.edu

Abbreviations used: endo H, endoglycosidase H; ER, endoplasmic reticulum; GalNAcT, *N*-acetylgalactosaminyltransferase I; glyco F, glycopeptidase F; NBD-C6-ceramide, 6-[[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]sphingosine; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ST-II, sialyltransferase II.

cDNA library in pCMV-Sport 2 vector and Lipofectamine were purchased from GibcoBRL (Gaithersburg, MD, U.S.A.). The FLAG epitope mammalian expression system, including pFLAG-CMV-5b expression vector, anti-FLAG BioM2a mouse monoclonal IgG antibody, and anti-FLAG M2-immunoaffinity gel, was purchased from Eastman Kodak Co. (New Haven, CT, U.S.A.). Goat polyclonal anti-calnexin (C-20) IgG and goat anti-mouse IgG–fluorescein isothiocyanate conjugate were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Donkey polyclonal anti-mouse IgG–horseradish peroxidase conjugate was from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Rabbit polyclonal anti-goat IgG-horseradish peroxidase conjugate was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glycopeptidase F (glyco F) and endoglycosidase H (endo H) were from New England Biolabs (Beverly, MA, U.S.A.). The TNT-coupled reticulocyte lysate system for in vitro transcription/translation was from Promega (Madison, WI, U.S.A.). 6-[[*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]sphingosine (NBD-C6-ceramide) and ER-Tracker Blue-White DPX were purchased from Molecular Probes (Eugene, OR, U.S.A.). CMP-*N*-[9-^{3}H]acetylneuraminic acid (33 Ci/mmol) and UDP-*N*-[1-^{3}H]acetylgalactosamine (50 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). [^{35}S]Methionine (1,175 Ci/mmol) was from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.). All other chemicals were of analytical grade or higher, and solvents were freshly redistilled before use.

Cloning of ST-II and GalNAcT cDNAs

A 15.5-day-old mouse embryo cDNA library containing 1×10^7 independent clones in *Escherichia coli* DH-12 S was amplified on 96-well plates. An aliquot (0.5 μl) from each well was screened by PCR for the cDNA of GalNAcT or ST-II following a procedure described elsewhere (Kapitonov et al., 1999). A cDNA encompassing the entire coding region of mouse GalNAcT or ST-II was amplified using two sets of oligonucleotide primers, one with primer position −10 (CCATATCAGGATGCGGCTAG) and 1,730 (AAAGGATCCACACTCGGCGGTCATGCAC) for amplification of GalNAcT-specific cDNA and the other with primer position −14 (ACACCGAGCTGCGATGAG) and 1,096 (AAAGGTACCGGAAGTGGGCTGTGGTGACG) for amplification of ST-II-specific cDNA. The cDNAs were inserted upstream from the FLAG epitope sequence of the pFLAG-5a vector.

In vitro transcription and translation

Cell-free synthesis of ST-II or GalNAcT tagged with the C-terminal FLAG epitope was performed according to the instructions of the manufacturer of the TNT-coupled in vitro transcription/translation kit. For radioactive labeling of the translation product, the incubation reaction mixture was supplemented with 0.5 μl of [^{35}S]methionine (2 μCi). GM3 (final concentration 500 μM) was added when the translation product was used for determination of enzyme activity. Other aliquots of the reaction mixture were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) or immunoaffinity isolation of the translation product.

Transient transfection and immunofluorescence studies

Transient transfection of murine neuroblastoma F-11 or NG108-15 cells was performed with 2 μg of cDNA specific for ST-II– or GalNAcT–FLAG following the Lipofectamine procedure described by the manufacturer. Fifteen to 35 h after transfection, cells were used for metabolic labeling studies or harvested by cell scraping and resuspended in 1 ml of phosphate-buffered saline (pH 7.4). Aliquots of the cells were solubilized with lysis buffer for determination of enzyme activity or immunoaffinity purification of the expression product. For immunofluorescence studies, NG108-15 or F-11 cells were grown on coverslips and transiently transfected with cDNA constructs as described above. Twenty-four hours after transfection, cells were fixed with 4% formaldehyde in phosphate-buffered saline and the expression products detected by confocal laser immunofluorescence microscopy following a procedure described elsewhere (Bieberich and Bause, 1995). Specific staining of the Golgi apparatus or the endoplasmic reticulum (ER) was achieved by incubation of the cells for 10 min at 37°C prior to fixation with 100 μM NBD-C6-ceramide or 500 nM ER-Tracker Blue White DPX according to the instructions of the manufacturer.

Enzyme preparation and activity assay

Transfected or untransfected cells were harvested as described above and the activities of ST-II and GalNAcT determined with detergent-solubilized cells under the condition of substrate saturation as described elsewhere (Bieberich and Yu, 1999). Determination of the activity of the in vitro translated enzymes was performed with one-tenth of the translation reaction mixture using the same composition of assay buffer as with solubilized cells. After the reaction, the radiolabeled glycolipid product was separated from the substrate by gel permeation chromatography on a Sephadex G-50 column or by high-performance thin-layer chromatography as described (Gu et al., 1995). The transferred radioactivity was determined by liquid scintillation counting.

Metabolic labeling and pulse chase experiments

Approximately $2–3 \times 10^6$ NG108-15 or F-11 cells transfected with 5 μg of the plasmid containing epitope-tagged ST-II or GalNAcT were incubated for 1 h at 37°C with 250 μCi of [^{35}S]methionine (1,175 Ci/mmol) in 2 ml of serum-free and methionine-deficient Dulbecco's modified Eagle's medium. For metabolic chase, the medium was replaced by Dulbecco's modified Eagle's medium and 10% fetal calf serum supplemented with 1 mM unlabeled methionine. Immunoaffinity purification of the expression products was performed with an immobilized monoclonal antibody against the FLAG epitope following a procedure given by the manufacturer. The time period for labeling was extended to 15 h for determination of the specific activity of the expression products.

Miscellaneous procedures

The amount of protein in cell preparations was determined according to a modification of the Folin phenol reagent assay as described elsewhere (Wang and Smith, 1975). Protein precipitation was performed according to the Wessel–Flügge method (1984). SDS-PAGE, immunoblotting, and all molecular biology procedures followed standard methods as described elsewhere (Laemmli, 1970; Gershoni and Palade, 1983; Sambrook, 1989).

RESULTS AND DISCUSSION

Enzyme activity of transiently expressed and in vitro translated enzymes

Two neuroblastoma cell lines, NG108-15 and F-11 cells, were used for the transient transfection with plasmid containing ST-II- or GalNAcT-specific cDNA endowed with a C-terminal sequence encoding the FLAG epitope tag. Cells were transfected with the cDNA cod-

TABLE 1. *Expression of ST-II and GalNAcT*

	ST-II			GalNAcT		
	NG108-15	F-11	Cell-free	NG108-15	F-11	Cell-free
Untransfected	450 ± 90	1,500 ± 300	—	5,800 ± 800	2,900 ± 550	—
Transfected	2,500 ± 500	—	—	—	5,900 ± 800	—
Transfected + castanospermine	650 ± 130	—	—	—	4,500 ± 700	—
+ GM3	—	—	1,130 ± 230[a]	—	—	<200
− GM3	—	—	340 ± 70[a]	—	—	<200

Specific enzyme activity is expressed as dpm of transferred [^{3}H]sugar/mg of cell protein/h. NG108-15 or F-11 cells were solubilized in enzyme assay buffer with or without prior transfection with ST-II–FLAG or GalNAcT–FLAG and incubation with or without castanospermine. An aliquot equivalent to 200 μg of cell protein was used for determination of enzyme activity as described in Materials and Methods. ST-II–FLAG or GalNAcT–FLAG was synthesized in a cell-free in vitro transcription/translation system with or without 500 μM GM3, and an aliquot corresponding to the amount of enzyme expressed in transfected cells was used for activity assays. The relative amount of enzyme was determined by the intensity of staining with a FLAG epitope-specific antibody on immunoblots, as shown in Fig. 2. The specific activity of the translation product was standardized based on the protein concentration of the expression product with identical staining intensity.

[a] Specific activity was calculated from the amount of cell protein yielding the intensity of FLAG-positive immunostaining equivalent to that of the in vitro translated enzyme.

ing for the enzyme known to show low endogenous activity in the respective cell line. As shown in Table 1, expression of ST-II in NG108-15 cells resulted in an increase of enzyme activity to 550% of that of untransfected control cells (450 dpm/mg of cell protein/h). The enzyme activity of GalNAcT was enhanced by 100% (to 5,900 dpm/mg of cell protein/h) when F-11 cells were used for transfection. Inhibition of *N*-glycoprotein processing by preincubation of transfected cells with castanospermine (500 μM) resulted in a reduction of the specific activity of ST-II and GalNAcT to 25 ± 5% and 75 ± 15%, respectively. Cell growth was not significantly affected by incubation with castanospermine up to 4 days prior to transfection, indicating that the level of protein translation was not reduced by the inhibitor.

The cDNA of ST-II–FLAG or GalNAcT–FLAG was used to synthesize in vitro the enzymes without N-linked oligosaccharides and analyze their activity. The in vitro synthesis was performed in the presence of the substrate GM3 to stabilize the active protein conformation. The enzyme activity was determined by high-performance thin-layer chromatography analysis and liquid scintillation counting of incorporated radioactivity found in the excised band of the product ganglioside. Based on the staining intensity on immunoblots, the same amount of in vitro translation and expression product was used for determination of enzyme activity (see Fig. 2 for immunostaining). As shown in Table 1, the specific activity of in vitro translated ST-II was calculated to be 55% of the cellular expression product. This observation is in contrast to the conclusion reached by a previous report on a loss of enzyme activity by elimination of N-linked oligosaccharides to <10% that was due to an impairment of the catalytic activity of the enzyme (Martina et al., 1998). The in vitro synthesis of ST-II in the absence of substrate yielded a translation product that was <30% as active as that synthesized in the presence of GM3 (Table 1). This result indicates that the active conformation of the enzyme was significantly stabilized by the substrate. The enzyme activity of the in vitro translated GalNAcT, however, was <20% of the expression product and could not be stabilized by incubation with GM3 (Table 1). It should be noted that in vitro synthesis is more affected by a variety of factors required for enzyme stabilization than is protein translation in vivo. This effect may have accounted for a partial loss of the ST-II activity and the individual differences observed with the activities of in vitro translated ST-II and GalNAcT.

Turnover and *N*-glycoprotein processing of enzymes in neuroblastoma cells

The biosynthesis and turnover of ST-II–FLAG and GalNAcT–FLAG were analyzed by metabolic pulse chase labeling of transiently transfected NG108-15 cells with [^{35}S]methionine in the presence or absence of castanospermine. The expression products were isolated by immunoaffinity chromatography, separated by SDS-PAGE, and visualized by autoradiography. Figure 1 shows the semilogarithmic plot of incorporated [^{35}S]methionine as calculated from a densitometric analysis of various autoradiograms and subsequent scintillation counting of the excised bands corresponding to the labeled expression products. In unaffected NG108-15 cells, ST-II–FLAG was expressed as a protein of 45 kDa that was degraded at a rate of $t_{1/2}$ = 30 h, whereas the half-life of GalNAcT–FLAG (65 kDa) was only 6 h (see Fig. 2 for molecular masses). Incubation of transfected and metabolically labeled cells with castanospermine reduced the half-life of ST-II–FLAG to 8 h, whereas that of GalNAcT remained almost unaltered. From this result, it can be concluded that the reduction of the activity of ST-II upon incubation with castanospermine was due solely to a decrease of the intracellular half-life by enhanced turnover of the enzyme. This conclusion is in line with the observation that neither the activity nor the half-life of GalNAcT was significantly reduced upon incubation with castanospermine.

The type of oligosaccharide N-linked to ST-II–FLAG or GalNAcT–FLAG was characterized by incubation of the expression products with glyco F or

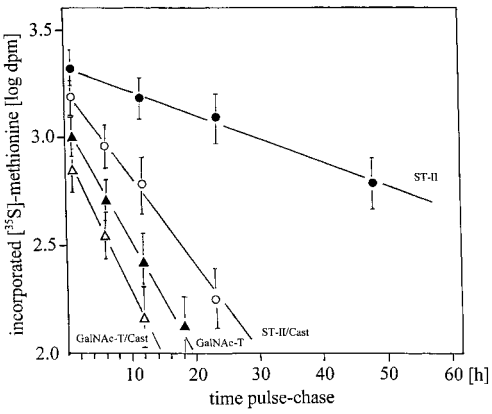

FIG. 1. Pulse chase labeling of ST-II–FLAG and GalNAcT–FLAG in NG108-15 cells. NG108-15 cells were transfected with ST-II–FLAG or GalNAcT–FLAG and after 24-h incubation labeled for 1 h with 250 μCi of [^{35}S]methionine. Optionally, the medium was supplemented with 500 μM castanospermine (Cast) 2 h prior to transfection. After time periods of chase with 1 mM unlabeled methionine as indicated, cells were solubilized and the labeled expression products immunoaffinity purified and analyzed by SDS-PAGE and autoradiography. The radioactivity incorporated into the enzymes was determined by liquid scintillation counting of the excised bands and analyzed as shown in the semilogarithmic plot. Radioactivity as plotted for each labeling reaction corresponds to 100 μg of solubilized protein. Results were obtained from three independent pulse chase experiments for each time course.

endo H prior to SDS-PAGE and immunoblotting. Figure 2A and B shows that the two proteins of 45 kDa (ST-II, Fig. 2A, lanes 1 and 2) and 65 kDa (GalNAcT, Fig. 2B, lane 1) migrated with a lower molecular mass after removal of N-linked oligosaccharides with endo H (Fig. 2A and B, lane 4). This difference of ~3 kDa was also observed after cleavage with glyco F (GalNAcT, Fig. 2B, lane 7) and can be explained by removal of approximately two to three *N*-glycans. The molecular mass after cleavage with glyco F or endo H corresponded to that of the unglycosylated enzymes translated in vitro (ST-II, Fig. 2A, lane 6; GalNAcT, Fig. 2B, lane 6). The sensitivity to endo H indicates that the sugar chains were not converted to complex-type oligosaccharides. Incubation with castanospermine resulted in a reduction of the amount of immunostained ST-II–FLAG by ~75%, whereas that of GalNAcT–FLAG was decreased to a much lesser extent (by 25%). Incubation with castanospermine converted the endo H-sensitive ST-II–FLAG into a form of higher molecular mass, most likely due to preservation of the precursor oligosaccharide N-linked to the protein (Fig. 2A, lane 3). A reduction of the amount of unprocessed ST-II was very likely caused by a decrease of the half-life of the expression product upon inhibition of early *N*-glycoprotein processing by castanospermine. This is corroborated by the observation that metabolic labeling did not reveal a reduction of the rate of protein translation (Fig. 1). The amount of GalNAcT was not significantly reduced upon incubation with castanospermine. Due to the higher molec-

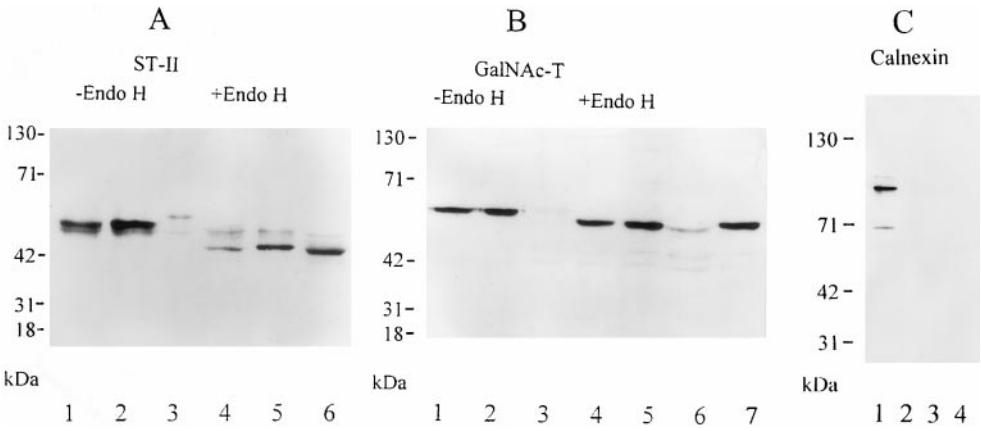

FIG. 2. *N*-Glycoprotein analysis and calnexin binding of ST-II–FLAG and GalNAcT–FLAG. NG105-15 cells transfected with ST-II–FLAG or GalNAcT–FLAG were solubilized and the epitope-tagged enzymes analyzed by SDS-PAGE and immunoblotting, as described. **A:** Immunostaining of ST-II–FLAG with anti-FLAG antibody. Lane 1, control without castanospermine or endo H incubation, 5 μg of cellular protein; lane 2, as in lane 1 but 10 μg of cellular protein; lane 3, incubation with castanospermine but without endo H, 10 μg of cellular protein; lane 4, control without castanospermine but with endo H incubation, 10 μg of cellular protein; lane 5, incubation with castanospermine and endo H, 50 μg of cellular protein; lane 6, in vitro synthesized ST-II, 2 μl of the translation reaction mixture (50 μl). **B:** Immunostaining of GalNAcT–FLAG with polyclonal anti-FLAG antibody. Lane 1, control without castanospermine or endo H, 20 μg of cellular protein; lane 2, incubation with castanospermine but without endo H, 20 μg of cellular protein; lane 3, empty; lane 4, control without castanospermine but with endo H, 20 μg of cellular protein; lane 5, incubation with castanospermine and endo H, 20 μg of cellular protein; lane 6, in vitro synthesized GalNAcT, 1 μl of the translation reaction mixture (50 μl); lane 7, control without castanospermine but with glyco F incubation. **C:** Immunostaining of calnexin co-purified as a complex with ST-II–FLAG or GalNAcT–FLAG from 250 μg of solubilized cellular protein. Lane 1, ST-II–FLAG/calnexin without castanospermine or endo H incubation, corresponding to 20 μg of cellular protein; lane 2, as in lane 1 with castanospermine, corresponding to 100 μg of cellular protein; lanes 3 and 4, as in lanes 1 and 2 with GalNAcT–FLAG, corresponding to 50 μg of cellular protein.

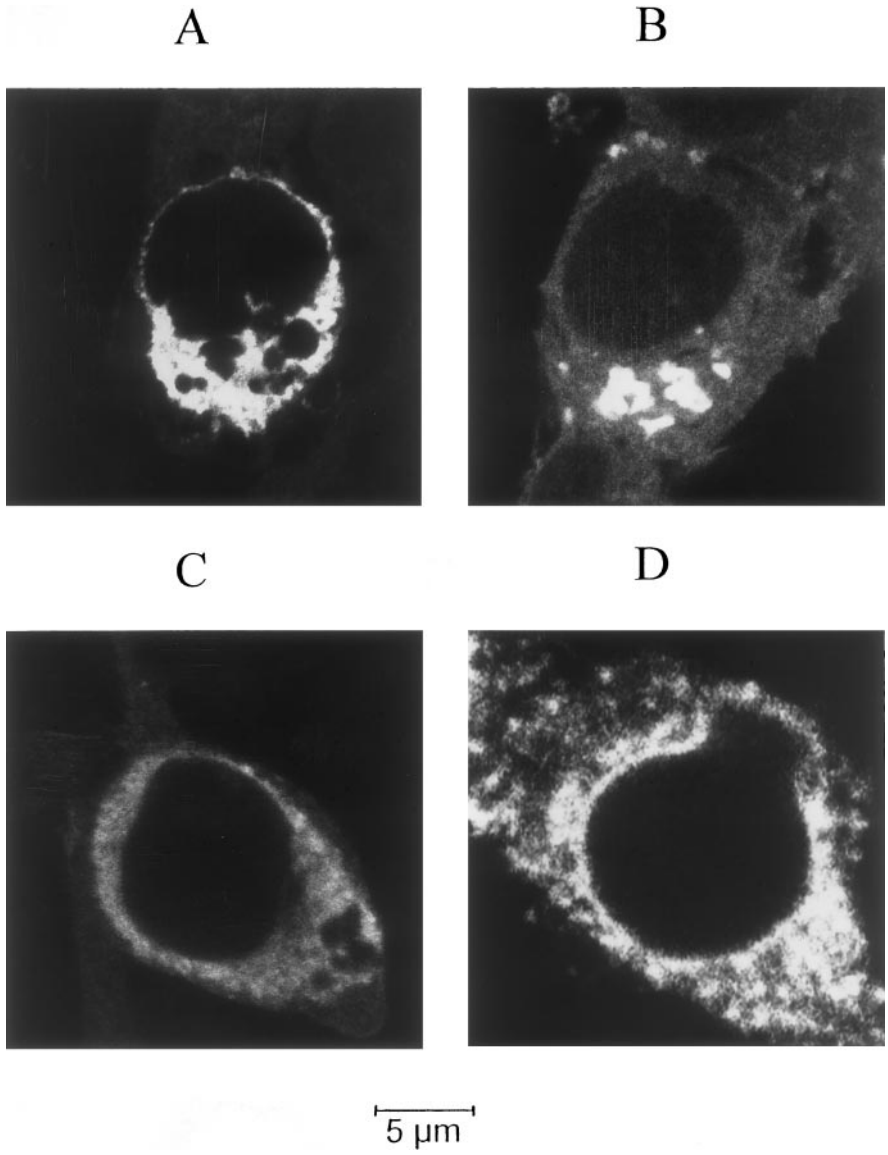

FIG. 3. Subcellular distribution of ST-II–FLAG and GalNAcT–FLAG in NG108-15 cells. NG108-15 cells were transfected with ST-II–FLAG or GalNAcT–FLAG and immunostained with a FLAG-specific antibody and a secondary antibody with fluorescein isothiocyanate or rhodamine label. Cells were analyzed by confocal laser immunofluorescence microscopy. **A:** Staining of GalNAcT–FLAG; **B:** staining of ST-II–FLAG; **C:** staining of ST-II–FLAG after incubation of the transfected cells with castanospermine; **D:** staining with ER-Tracker.

ular mass of GalNAcT, it cannot yet be decided whether this *N*-glycoprotein also undergoes partial processing (Fig. 2B, lanes 1 and 2).

The potential binding to chaperones known to participate in assisting folding of *N*-glycoproteins was analyzed by co-immunoaffinity chromatography of a potential complex with ST-II–FLAG and GalNAcT–FLAG expressed in NG108-15 cells. Up to 500 μg of solubilized protein was applied to the FLAG-specific immunoaffinity gel and the eluate subjected to SDS-PAGE. Immunoblots were probed with an antibody specific for calnexin. Figure 2C shows that calnexin was co-purified as a complex with ST-II–FLAG (lane 1) unless the transfected cells were incubated with castanospermine (lane 2). This result indicates that calnexin binding was dependent upon the removal of the terminal glucose residue of oligosaccharides N-linked to ST-II–FLAG. The lack of calnexin binding to ST-II–FLAG endowed with precursor *N*-glycans is in line with the increased turnover of the enzyme. Recently, it has been reported for a number of *N*-glycoproteins that processing of the precursor oligosaccharide by glucosidase I is a prerequisite for the chaperone activity of calnexin (Helenius, 1994; Hammond et al., 1994; Chen et al., 1995; Hebert and Helenius, 1995; Hebert et al., 1995; Bonen et al., 1998; Cannon and Helenius, 1999). Inhibition of glucosidases I and II by incubation with castanospermine resulted in retention and enhanced degradation of *N*-glycoproteins in the ER. In this study, we demonstrate for the first time that processing of N-linked oligosaccharides is a prerequisite for chaperone binding to a glycosyltransferase participating in glycolipid biosynthesis. Calnexin binding, however, could not be demonstrated for GalNAcT (Fig. 2C, lanes 3 and 4), suggesting that its processing and intracellular translocation may follow a mechanism different from that of ST-II.

Subcellular distribution of ST-II and GalNAcT in neuroblastoma cells

The subcellular distribution of ST-II–FLAG and GalNAcT–FLAG was determined by epitope tag-specific immunostaining combined with confocal laser fluorescence microscopy, as shown in Fig. 3. GalNAcT was distributed over a wide range in the ER or ER and Golgi (Fig. 3A), whereas the localization of ST-II was confined to the Golgi (Fig. 3B). This result is in line with previous reports on the subcellular localization of ST-II; however, the distribution of GalNAcT has been reported to be confined to the Golgi (Haraguchi et al., 1995; Martina et al., 1998). Incubation with castanospermine resulted in an aberrant distribution of ST-II to the ER (Fig. 3C), whereas that of GalNAcT remained unaltered (not shown). For co-localization, Golgi and ER were specifically stained with NBD-C6-ceramide or ER-Tracker, respectively (for ER staining, see Fig. 3D; Golgi staining is not shown). The pattern of subcellular distribution was independent of the time period after transfection, the overall staining intensity, or the cell line used for transfection.

Conclusions

In summary, our results demonstrate that early processing of N-linked oligosaccharides is crucial for the intracellular stability and distribution of ST-II but not for its catalytic activity. Retention and enhanced turnover of ST-II in the ER upon inhibition of early *N*-glycoprotein processing were very likely correlated with a lack of calnexin binding, resulting in incorrect protein folding and aberrant translocation of the expression product. It was shown that this effect cannot be generalized to all of the glycosyltransferases in ganglioside biosynthesis as GalNAcT was not or was only slightly affected by inhibition with castanospermine. Both enzymes are type II transmembrane proteins without any known ER retention signal, which could have accounted for a different subcellular localization (Ellgaard et al., 1999). However, the number of potential *N*-glycosylation sites of GalNAcT is smaller than that of ST-II (three versus four), whereas its molecular mass is greater by ~20 kDa. It is possible that the number or position of N-linked oligosaccharides may have accounted for the individual binding on *N*-glycoproteins to chaperones. This assumption is supported by a recent study on binding of calnexin to pancreatic ribonuclease, which was found to depend on the number of *N*-glycan chains (Rodan et al., 1995). In future studies, we will investigate the molecular mechanism underlying the distinct binding of ST-II and GalNAcT to calnexin. In addition, we will analyze the significance of a differential modulation of turnover and subcellular distribution of these two glycosyltransferases for the expression of specific gangliosides.

Acknowledgment: We are indebted to Dr. Thomas Biber (Department of Physiology, MCV/VCU, Richmond, VA, U.S.A.) who assisted in the confocal microscopy studies. This study was supported by grant USPHS NS11853 (to R.K.Y.) and an A. D. Williams grant (to E.B.).

REFERENCES

Bieberich E. and Bause E. (1995) Man_9-mannosidase from human kidney is expressed in COS-cells as a Golgi resident type II transmembrane *N*-glycoprotein. *Eur. J. Biochem.* **233,** 644–649.

Bieberich E. and Yu R. K. (1999) Multi-enzyme kinetic analysis (MEKA) of ganglioside metabolism. *Biochim. Biophys. Acta* **1432,** 113–124.

Bonen D. K., Nassir F., Hausman A. M., and Davidson N. O. (1998) Inhibition of N-linked glycosylation results in retention of intracellular apo[a] in hepatoma cells, although nonglycosylated and immature forms of apolipoprotein[a] are competent to associate with apolipoprotein B-100 in vitro. *J. Lipid Res.* **39,** 1629–1640.

Cannon K. S. and Helenius A. (1999) Trimming and readdition of glucose to N-linked oligosaccharides determines calnexin association of a substrate glycoprotein in living cells. *J. Biol. Chem.* **274,** 7537–7544.

Chen W., Helenius J., Braakman I., and Helenius A. (1995) Cotranslational folding and calnexin binding during glycoprotein synthesis. *Proc. Natl. Acad. Sci. USA* **92,** 6229–6233.

Ellgaard L., Molinari M., and Helenius A. (1999) Setting the standards: quality control in the secretory pathway. *Science* **286,** 1882–1888.

Gershoni J. M. and Palade G. E. (1983) Protein blotting: principles and applications. *Anal. Biochem.* **13,** 1–15.

Gu X., Preuß U., Gu T., and Yu R. K. (1995) Regulation of sialyltransferase activities by phosphorylation and dephosphorylation. *J. Neurochem.* **64,** 2295–2302.

Hammond C., Braakman I., and Helenius A. (1994) Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. USA* **91,** 913–917.

Haraguchi M., Yamashiro S., Furukawa K., Takamiya K., Shiku H., and Furukawa K. (1995) The effects of site-directed removal of N-glycosylation sites from β-1,4-*N*-acetylgalactosaminyltransferase on its function. *Biochem. J.* **312,** 273–280.

Hebert D. N., Foellmer B., and Helenius A. (1995) Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell* **81,** 425–433.

Helenius A. (1994) How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Mol. Biol. Cell* **5,** 253–265.

Kalz-Füller B., Bieberich E., and Bause E. (1995) Cloning and expression of glucosidase I from human hippocampus. *Eur. J. Biochem.* **231,** 344–351.

Kapitonov D., Bieberich E., and Yu R. K. (1999) Combinatorial PCR approach to homology-based cloning: cloning and expression of mouse and human GM3 synthase. *Glycoconj. J.* **16,** 337–350.

Kornfeld R. and Kornfeld S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54,** 631–664.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Martina J. A., Daniotti J. L., and Maccioni H. J. F. (1998) Influence of *N*-glycosylation and *N*-glycan trimming on the activity and intracellular traffic of GD3-synthase. *J. Biol. Chem.* **273,** 3725–3731.

Rodan A. R., Simons J. F., Trombetta E. S., and Helenius A. (1995) N-linked oligosaccharides are necessary and sufficient for association of glycosylated forms of bovine RNase with calnexin and calreticulin. *EMBO J.* **15,** 6921–6930.

Sambrook J., Fritsch E. F., and Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Press, Cold Spring Harbor, NY.

Wang C.-S. and Smith R. L. (1975) Lowry determination of protein in the presence of Triton X-100. *Anal. Biochem.* **63,** 414–417.

Wessel D. and Flügge U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138,** 141–143.

Yu R. K. (1994) Developmental regulation of ganglioside metabolism. *Prog. Brain Res.* **101,** 31–44.