FULL TEXT:

Influence of N-Glycosylation and N-Glycan Trimming on the Activity and Intracellular Traffic of GD3 Synthase*

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GD3 synthase (ST8Sia I) transfers a sialic acid in α-2–8 linkage to the sialic acid moiety of GM3 to form the ganglioside GD3. The cDNAs of GD3 synthases predict several putative N-glycosylation sites. In this work we have examined the occupancy of these sites in a chicken GD3 synthase and how they affect its activity and intracellular traffic. COS-7 cells were transfected with an influenza virus hemagglutinin (HA) epitope-tagged form of GD3 synthase (GD3 synthase-HA). Cells acquired GD3 synthase activity, cell surface GD3 immunoreactivity, and GD3 synthase-HA immunoreactivity in the Golgi complex. In Western blots, a main GD3 synthase-HA band of 47 kDa was detected, which was radioactive upon metabolic labeling with [2-3H]mannose. Tunicamycin prevented the incorporation of [2-3H]mannose into GD3 synthase-HA, blocked the enzyme activity, and promoted a reduction of the enzyme molecular mass of 6–7 kDa. Timed deglycosylation with N-glycosidase F showed that all three potential N-glycosylation sites of GD3 synthase-HA were glycosylated. The deglycosylated forms were enzymatically more unstable than the native form. Tunicamycin treatment of cells led to retention of GD3 synthase-HA immunoreactivity in the endoplasmic reticulum (ER). Castanospermine and deoxynojirimycin, inhibitors of the ER-processing enzymes a-glucosidases I and II, also prevented the exit from the ER but did not essentially affect the enzyme specific activity. 1-Deoxynojirimycin and swainsonine, inhibitors of mannosidases, did not affect either the enzyme activity or the Golgi localization. Results indicate that (a) N-glycosylation is necessary for GD3 synthase to attain and to maintain a catalytically active folding, and for exiting the ER; and (b) N-glycan trimming in the ER, while not required for enzyme activity, is necessary for proper trafficking of GD3 synthase to the Golgi complex.

GD3 synthase (ST8Sia I, EC 2.4.99.8) catalyzes the addition of sialic acid in α-2–8 linkage to the sialic acid moiety of ganglioside GM3 to form ganglioside GD3, the first intermediate of b-pathway gangliosides. GM3 is also substrate for a N-acetylgalactosaminyl transferase (GM2 synthase, EC 2.4.1.92) which converts it to GM2, the first intermediate of a-pathway gangliosides (1).

The relative efficiency of these conversions, and hence the contribution of a- and b-pathway gangliosides, is regulated in a cell type-specific manner during differentiation, development, or malignant transformation (2–5). The metabolic basis of this regulation is poorly understood. In vitro labeling of endogenous gangliosides of Golgi membranes have shown that GD3 synthase and GM2 synthase compete for a common pool of acceptor GM3 in the synthesis of GD3 and GM2 (6, 7). In this competence, the relative activities of the transferases, as well as their relative saturation with the respective donor sugar nucleotide influence the conversion of GM3 to one or the other product (8, 9). Concerning the activities of the transferases, it is known for some of them that transcriptional (10–13) and post-transcriptional (14, 15) controls affect their relative activities. However, in considering the highly compartmentalized organization of the synthesis along the endoplasmic reticulum (ER) and the Golgi complex, factors that could affect the proper sorting of the transferases to the different subcompartments, and hence their relationships with donors and acceptors and with other transferases, are worth of consideration.

Among posttranscriptional events that could influence the relevance of a given glycosyltransferase for the overall functioning of the glycolipid or glycoprotein glycosylating system, their glycosylation status appears of particular interest due to the presence of potential N-glycosylation sites in their cDNAs (16). In fact, site-directed removal of N-glycosylation sites of GM2 synthase showed that N-glycans were relevant for stabilizing the enzyme structure but not for sorting of the enzyme to the Golgi complex (17). For the case of a glycoprotein α-2,6-sialyltransferase, N-glycan release in vitro resulted in loss of activity, but no information was provided on whether they are relevant for sorting to the proper compartment (18). Very recently, it was reported that transiently expressed GlcNAcT-III, a glycosyltransferase involved in the synthesis of bisecting GlcNAc structures, requires N-glycosylation for its activity and Golgi retention (19).

Human (20), mouse (10), rat (11), and chicken (12) GD3 synthase have been cloned and their expression levels studied in several cancer cell lines (21) and in the CNS of mouse (22),

TBS, Tris-buffered saline; PBS, phosphate-buffered saline; PNGase F, peptide N-a-(N-acetyl-β-glucosaminyl)-asparagine amidase; ER, endoplasmic reticulum; HA, YPYDVPDYA nonapeptide of the influenza virus hemagglutinin; M6PR, mannose 6-phosphate receptor; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

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rat (11), and chicken (12). As for other mammalian glycosyltransferases, the deduced primary sequence of these cloned GD3 synthases predicts a transmembrane disposition of type II membrane proteins and reveals three potential N-glycosylation sites (Asn-X-Ser/Thr) in the chick (Asn-57, -105, and -200) (12) and rat (Asn-70, -213, and -244) (11) enzymes and four in the human (Asn-71, -119, -214, and -245) (20) and mouse (Asn-56, -104, -199 and -230) (10) enzymes. Whether these glycosylation sequences are occupied by oligosaccharides is unknown, so information on the possible influences of carbohydrates on GD3 synthase activity, stability and intracellular trafficking is lacking.

In this work, we examine the occupancy of N-glycosylation sites of an epitope tagged form of chick GD3 synthase expressed in (GD3-) COS-7 cells. In addition, we study the effect of inhibiting en bloc glycosylation and oligosaccharide trimming, on the activity and intracellular localization of the enzyme, and also the effect of the in vitro deglycosylation on the enzyme activity.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—The chick GD3 synthase cDNA was subcloned intact into the mammalian expression vector pCEFL-GD3 synthase as described (12). To generate an epitope-tagged form of GD3 synthase, a viral hemagglutinin (HA) nonapeptide was added at the carboxyl terminus. For this, a 1.1-kilobase pair DNA fragment including the GD3 synthase coding region was amplified by polymerase chain reaction using primer oligonucleotides (sense, 5'-CCGCGATCCGGCAGGATCCAGCGCGTGTCGTAAG-3') and antisense, 5'-CTGTGCAACGATCCGGCAGAAGCGGTGTTAAC-3') and pCEFL-GD3 synthase as template. The sense primer contains a BamHI restriction site and a Kozak initiation sequence (23) upstream of the initiation codon. At the antisense primer, the stop codon TAA was deleted and a CGCGGATCCGCCGCCACCATGGCGGGGCT sequence (23) upstream the initiation codon. The polyadenylation signal was inserted downstream of the last codon. The recombinant plasmid was linearized with SalI site upstream the HA epitope sequence. The linearized plasmid DNA was used to transform E. coli showcasing the pCEFL vector (pCEFL-GD3 synthase-HA) modified vector encoding the HA nonapeptide epitope YPYDVPDYA (24), to generate pCEFL-GD3 synthase-HA. The identity of each construct was confirmed by restriction mapping and that of the final construct by DNA sequencing.

**Cell Culture and Transfection**—African green monkey COS-7 cells were plated on 35-mm tissue culture dishes (2–3×10⁴ cells/dish) and grown for 24 h in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). Cells were transfected with either 0.8 μg of pCEFL or pCEFL-GD3 synthase or pCEFL-GD3 synthase-HA using LipofectAMINE reagent (Life Technologies, Inc.), following the manufacturer's instructions. Fifteen hours after transfection the cells were washed with phosphate-buffered saline (PBS) and either fixed for immunostaining or harvested and homogenized for enzyme activity determinations or lysed for immunoprecipitation experiments. For detection of GD3 synthase activity, the cells were harvested and homogenized. GD3 synthase activity was determined in parental and transfected cell homogenates (30 μg of protein), using GM3 as exogenous acceptor substrate and CMP-[3H]NeuAc as donor nucleotide as described previously (8). Values are the mean ± S.D. of two separated experiments performed in duplicate.

**Characterization of GD3 Synthase-HA**—Homogenates of COS-7 cells transiently transfected with a influenza virus HA epitope-tagged GD3 synthase (GD3 synthase-HA) were assayed for GD3 synthase activity *in vitro*. Table I shows that parent and mock-transfected cells express only background levels of GD3 synthase activity. On the other hand, the activity was clearly expressed in homogenates from GD3 synthase-HA and GD3 synthase-transfected cells. The specific activities were similar in both homogenates, indicating that the added tag did not affect the catalytic activity of the enzyme. High performance thin layer chromatography analysis (not shown) of the radioactive product of the enzyme reaction showed more than 95% of the radioactivity migrating as authentic GD3. Transfected cells also immunoprecipitate cell surface GD3 at variable intensities after immunostaining with the mAb R24 (Fig. 1), indicating that the GD3 synthase-HA was also functional in vivo.

**Subcellular Localization of GD3 Synthase-HA**—Transfected buffer A (50 mM HCl-Tris, pH 7.2, 0.6% Triton X-100, 140 mM NaCl, 3 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 3 mM/ml aprotinin, 1 mM EDTA, 0.05% sodium azide). Lysates were preabsorbed with protein G-agarose beads (75% suspension washed with buffer A prior to use) for 60 min at room temperature and then were incubated overnight on a rotating wheel at 4 °C with mAb anti-HA (1:50) and with 50 μl of protein G-agarose (25).

Samples were pelleted by centrifugation at 2,500 × g for 10 s and then washed five times at 4 °C with buffer A and three times with buffer B (100 mM phosphate buffer, pH 7.2, 10 mM EDTA). Pellets were resuspended in 50 μl of buffer B. For digestion with PNGase F, immunoprecipitates were incubated in the presence or in the absence of 8 units/ml PNGase F in digestion buffer (100 mM phosphate buffer, pH 7.2, 10 mM EDTA, 5% methanol) in a total volume of 100 μl (18). In some experiments the above volumes were scaled up as appropriate. Incubation was for up to 10 h at 37 °C and was stopped by transferring the tubes to ice. The beads were washed four times with 100 mM HCl-cacodylate buffer pH 8.5 prior to sialyltransferase activity determinations and immunoblotting analysis.

**SDS-PAGE, Immunoblotting, and Fluorography**—SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (26). Cell homogenates or immunoprecipitates were boiled in SDS sample buffer with 10% mercaptoethanol and then separated by 10% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes (27) for 1 h at 300 mA. For immunoblotting, nonspecific binding sites on the nitrocellulose were blocked with 2.5% bovine serum albumin, 2.5% polyvinylpyrrolidone 40 in Tris-buffered saline (400 mM NaCl, 100 mM HCl-Tris, pH 7.5, buffer, TBS). Monoclonal antibody anti-HA was used at a 1:800 dilution. Blots were developed with polyclonal anti-mouse IgG horseradish peroxidase diluted 1:40,000, using an enhanced chemiluminescence detection kit (ECL, Amersham Corp.) and Kodak X-Omat-SB5 film. Polypeptide molecular weights were calculated based on calibrated standards (Life Technologies, Inc.) run in every gel. Protein bands in nitrocellulose membranes were visualized by Ponceau S staining.

**RESULTS**

**Characterization of GD3 Synthase-HA**—Homogenates of COS-7 cells transiently transfected with a influenza virus HA epitope-tagged GD3 synthase (GD3 synthase-HA) were assayed for GD3 synthase activity *in vitro*. Table I shows that parent and mock-transfected cells express only background levels of GD3 synthase activity. On the other hand, the activity was clearly expressed in homogenates from GD3 synthase-HA and GD3 synthase-transfected cells. The specific activities were similar in both homogenates, indicating that the added tag did not affect the catalytic activity of the enzyme. High performance thin layer chromatography analysis (not shown) of the radioactive product of the enzyme reaction showed more than 95% of the radioactivity migrating as authentic GD3. Transfected cells also immunoprecipitate cell surface GD3 at variable intensities after immunostaining with the mAb R24 (Fig. 1), indicating that the GD3 synthase-HA was also functional in vivo.

**Subcellular Localization of GD3 Synthase-HA**—Transfected

### Table I

| COS-7 cells | Enzyme activity  |
|------------|-----------------|
| Parental   | 0.09 ± 0.01    |
| Mock transfected | 0.10 ± 0.03   |
| GD3 synthase-HA transfected | 10.60 ± 0.40 |
| GD3 synthase transfected   | 11.00 ± 0.20   |

**Characterization of GD3 Synthase-HA**—Homogenates of COS-7 cells transiently transfected with an influenza virus HA epitope-tagged GD3 synthase (GD3 synthase-HA) were assayed for GD3 synthase activity *in vitro*. Table I shows that parental and mock-transfected cells express only background levels of GD3 synthase activity. On the other hand, the activity was clearly expressed in homogenates from GD3 synthase-HA and GD3 synthase-transfected cells. The specific activities were similar in both homogenates, indicating that the added tag did not affect the catalytic activity of the enzyme. High performance thin layer chromatography analysis (not shown) of the radioactive product of the enzyme reaction showed more than 95% of the radioactivity migrating as authentic GD3. Transfected cells also immunoprecipitate cell surface GD3 at variable intensities after immunostaining with the mAb R24 (Fig. 1), indicating that the GD3 synthase-HA was also functional in vivo.

**Subcellular Localization of GD3 Synthase-HA**—Transfected...
cells were double immunostained with mAb anti-HA and polyclonal anti-M6PR antibody. GD3 synthase-HA was evident from 10 h after transfection, predominantly located in the Golgi complex juxtanuclear zone (Fig. 2B) and colocalizing with M6PR, a trans-Golgi network and late endosome resident protein (Fig. 2C). Treatment of the transfected cells with brefeldin A, which causes Golgi membranes to redistribute into the ER (28), resulted in a redistribution of the expressed GD3 synthase-HA into the ER (Fig. 2D), thus further supporting its Golgi localization.

Metabolic Labeling of GD3 Synthase-HA—To assess if GD3 synthase-HA was N-glycosylated, transfected COS-7 cells were metabolically labeled from [2-3H]mannose added to the culture medium 12 h before harvesting. Homogenates of these cells or immunoprecipitates from Triton X-100 extracts of the homogenate were probed with the mAb anti-HA (Fig. 3). A polypeptide of 47 kDa was detected in homogenates of the transfected cells (lane 1). The polypeptide was immunoprecipitable from Triton X-100 extracts of the homogenate (lane 2) and upon fluorography, it was found radioactively labeled (lane 5), which was consistent with GD3 synthase-HA being a glycoprotein.

Effect of Inhibitors of N-Glycosylation and of N-GlycanTrimming on GD3 Synthase-HA Electrophoretic Mobility—Immunoprecipitates from cells that were labeled from [2-3H]mannose in the presence of tunicamycin lacked the HA-immunoreactive band at 47 kDa and showed a band at ~40 kDa (Fig. 3, lane 3); this band was devoid of radioactivity upon fluorography (lane 6), thus indicating that GD3 synthase-HA was N-glycosylated. The immunoprecipitated polypeptide of 47 kDa was not detected in homogenates from mock transfected cells (lanes 4 and 7). The molecular mass of the polypeptide synthesized in the presence of tunicamycin (~40-kDa) was the expected calculated from the cDNA of GD3 synthase-HA and strongly suggest that all three potential N-glycosylation sites of GD3 synthase-HA were occupied by oligosaccharides (2–3-kDa N-glycan) (see also Fig. 6).

The effect of inhibitors of the oligosaccharide trimming on the electrophoretic mobility of GD3 synthase-HA was also examined (Fig. 4). Castanospermine, which inhibits ER glucosidase I and II, showed GD3 synthase-HA as a single polypeptide of molecular mass slightly higher (~48 kDa) than in untreated cells (compare lanes 1 and 3). In cells treated with deoxynojirymycin, which also prevents trimming of glucose, two forms of

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**Fig. 1.** Immunooexpression of GD3 by transfected COS-7 cells. COS-7 cells were transiently transfected with pCEFL-GD3 synthase-HA. Fifteen hours after transfection, cells were fixed with paraformaldehyde and stained for cell surface GD3 by indirect immunofluorescence using mAb anti-GD3 (R24) as described under “Experimental Procedures.” A, phase contrast; B, fluorescence. Arrowheads point to untransfected (GD3−) cells.

**Fig. 2.** Immunooexpression of GD3 synthase-HA by transfected COS-7 cells. COS-7 cells transfected as in Fig. 1 were fixed and permeabilized with cold methanol and double immunostained for GD3 synthase-HA (B) and M6PR (C) as indicated under “Experimental Procedures.” A, phase contrast; arrowheads in A–C point to cells that immunoexpress both GD3 synthase-HA and M6PR. D, GD3 synthase-HA immunostaining in cells after treatment with 1 μg/ml brefeldin A for 2 h before fixation.

**Fig. 3.** Metabolic labeling of GD3 synthase-HA from [2-3H]mannose, effect of tunicamycin. Transfected and mock transfected COS-7 cells were metabolically labeled by adding 20 μCi/ml [2-3H]mannose (Life Science Products, specific activity, 21 Ci/mmol) to the culture medium, in the presence or in the absence of 10 μg/ml tunicamycin as indicated at the top. After 12 h of labeling, cell lysates were prepared and GD3 synthase-HA was immunoprecipitated with mAb anti-HA. Homogenate (lane 1) and immunoprecipitates (lanes 2–7) were run on two paired 10% SDS-PAGE gels under reduction conditions, one for immunoblotting with mAb anti-HA (lanes 1–4) and the other for fluorography (lanes 5–7). The asterisk marks the mouse IgG heavy chain of the bead immunocomplexes. For other details, see “Experimental Procedures.”
Inhibitor | - | TUN | CST | DNJ
--- | --- | --- | --- | ---
48 kDa | 1 | 2 | 3 | 4
47 kDa | | | | |
40 kDa | | | | |

Fig. 4. Effect of N-glycosylation and oligosaccharide trimming inhibitors on GD3 synthase-HA electrophoretic mobility. Transfected cells were incubated in the absence or in the presence of either 10 μg/ml tunicamycin (TUN) or 75 μg/ml castanospermine (CST) or 5 mM deoxyojirimycin (DNJ) during the last 8 h of the 15-h transfection period, as indicated above each lane. Fifty micrograms of proteins of cell homogenates were run in SDS-PAGE, immunoblotted with mAb anti-HA, and developed as described under “Experimental Procedures.” Migration positions and sizes of the different GD3 synthase-HA forms are indicated on the left.

GD3 synthase-HA were detected (lane 4); a minor one of molecular mass ~45 kDa and a major one of molecular mass ~47.5 kDa, slightly higher than the fully glycosylated GD3 synthase-HA. 1-Deoxymannojirimycin, inhibitor of mannosidase I, and swainsonine, inhibitor of mannosidase II, did not detectably affect the electrophoretic mobility of GD3 synthase-HA; however, careful examination of Western blots from 1-deoxymannojirimycin-treated cells showed two GD3 synthase-HA forms in almost equal amounts, one having the electrophoretic mobility of the fully glycosylated enzyme, and the other one with a molecular mass about 0.5 kDa smaller (not shown).

It should be noticed that densitometric quantification of Western blots from control and inhibitor-treated cells as the one shown in Fig. 4, evidenced that in cells treated with castanospermine and deoxyojirimycin, the mass of GD3 synthase-HA immunoreactive band was about 40% lower than in control cells (taking the value of intensity of the band in control cells relative to the sum of background bands intensities as 1.0, the values in tunicamycin, castanospermine and deoxyojirimycin treated cells were 1.1, 0.62, and 0.57, respectively).

Effect of Glycosylation Inhibitors on GD3 Synthase-HA Activity—Table II shows that GD3 synthase-HA expressed in the presence of tunicamycin (40-kDa) was devoid of activity toward exogenous GM3 acceptor. On the other hand, the enzyme activity of the forms expressed in the presence of inhibitors of N-glycan trimming was less affected or not affected at all. Homogenates of cells treated with castanospermine and deoxyojirimycin showed enzyme activity about 30 and 40% lower than untreated cells, respectively (Table II). However, as mentioned above, a reduction of about 40% of the mass of GD3 synthase-HA was observed in Western blots of these homogenates, which indicates that the enzyme-specific activity was not modified by treatment with these inhibitors. 1-Deoxymannojirimycin and swainsonine, which act late in the Golgi complex by inhibiting the addition of galactose and sialic acid, did not affect the activity. It should be mentioned that when determined in the presence of inhibitors added to the sialyltransferase assay, the GD3 synthase-HA activity was as in control homogenates (Table II). This indicates that the inhibitors added to the cells in culture affected the activity by affecting N-glycan incorporation or trimming, and not by a direct inhibitory effect on GD3 synthase-HA activity.

In Vitro Deglycosylation of GD3 Synthase-HA—The decrease of GD3 synthase-HA from 47 to 40 kDa (Fig. 3) strongly suggest that all three putative N-glycosylation sites of GD3 synthase-HA were occupied by carbohydrate. This was confirmed by in vitro treatment of GD3 synthase-HA immunoprecipitates

**TABLE II**

| Additions to | Cells in culture | Cell homogenate | GD3 synthase-HA |
| --- | --- | --- | --- |
| | | | nmol/mg protein/h |
| Control | - | - | 10.90 ± 0.20* |
| Tunicamycin | + | - | 0.10 ± 0.05 |
| Castanospermine | - | + | 7.74 ± 0.06 |
| Deoxyojirimycin | + | - | 6.50 ± 0.30 |
| 1-Deoxymannojirimycin | - | + | 9.30 ± 0.10 |
| Swainsonine | - | + | 9.70 ± 0.20 |

* Values are the mean ± S.D. of two separated experiments performed in duplicate.

**Effect of inhibitors of N-glycosylation and N-glycan trimming on GD3 synthase-HA activity**

COS-7 cells were transiently transfected with 0.8 μg of pCEFL-GD3 synthase-HA. Eight hours after transfection the medium was replaced with fresh culture medium supplemented or not with either 10 μg/ml tunicamycin, 75 μg/ml castanospermine, 5 mM deoxyojirimycin, 5 mM 1-deoxymannojirimycin, or 20 μg/ml swainsonine. Eight hours later the cells were harvested and homogenized. GD3 synthase-HA activity was determined as indicated in the legend of Table I in homogenates of inhibitor-treated and untreated cells (Control), and in homogenates of inhibitor untreated cells supplemented with inhibitors at the same concentration as they were in the culture medium.

with PNGase F. PNGase F is a peptide N-glycosidase that cleaves N-linked oligosaccharides, regardless of its complexity, by hydrolyzing the asparagine oligosaccharide bond. As shown in Fig. 5A, incubation of the immunoprecipitates with PNGase F for different periods of time generated three polypeptide bands of 45, 43, and 40 kDa. The simplest interpretation for these changes is that the new polypeptides generated by PNGase F correspond to forms with two, one, and none of the N-linked oligosaccharides. The mobility of the band of 40 kDa is identical to that of a sample of the enzyme from an homogenate of tunicamycin treated cells (not shown). Ten-hour incubation in the absence of PNGase F but in otherwise identical conditions did not modify the electrophoretic pattern of GD3 synthase-HA.

Fig. 5B is a densitometric quantification of the percentage contribution of the GD3 synthase-HA forms generated by PNGase F. A fast decrease of about 60% of the fully glycosylated form (47 kDa) was observed during the first 0.5 h of treatment, which was followed by a slower decrease in the next periods. Conversely, the fully deglycosylated form (40 kDa) increased rapidly in the first 0.5 h and more slowly thereafter. The percentage of the partially deglycosylated forms (45 and 43 kDa) remained essentially constant in the 0.5–2-h period of the treatment, and dropped below 10% between 2 and 10 h of incubation.

Fig. 5C shows the GD3 synthase-HA activity of the preparation after deglycosylation with PNGase F. In the presence of PNGase F, the activity remained essentially intact at 0.5 and 1 h of incubation, decreased slightly up to 2 h and dropped to near zero in the period between 2 and 10 h. These results indicate that the fully and partially deglycosylated forms generated during the 2-h incubation period were partially as active as the fully glycosylated form. However, they inactivate as the incubation period at 37 °C was prolonged beyond 2 h, suggesting a loss of stability following deglycosylation.

**Inhibition of Glycosylation Affects the Exit of GD3 Synthase-HA from the ER**—As shown in Fig. 2, GD3 synthase-HA localized to the Golgi complex of the transfected cells. Fig. 6A, shows that cells expressing the transfected GD3 syn-
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Fig. 5. Effect of PNGase F treatment on the electrophoretic mobility and activity of GD3 synthase-HA. Immunoprecipitates of GD3 synthase-HA were incubated at 37 °C for the different times with or without PNGase F, as indicated. At the end of the incubation periods, aliquots of the incubates were analyzed by Western blots with mAb anti HA (A) as described under “Experimental Procedures” and for sialyltransferase activity (C) as in Table I. B is a densitometric quantification of the Western blot in A. In A, migration positions and sizes of the different GD3 synthase-HA forms are indicated on the left. Asterisk marks the mouse IgG heavy chain of the bead immunocomplexes. In C, the activity after 10 h of incubation in deglycosylation conditions without PNGase F was taken as 100%.

PNGase F - + + + +

Time (h) 10 0.5 1.0 2.0 10

A

B

C

d organizati from the corresponding cDNA (39.9 kDa), and the shape of the immunoreactive band, with a main core and diffuse borders, were compatible with GD3 synthase-HA being a glycoprotein. Incubation of transfected cells with [2-3H]mannose resulted in radioactive labeling of the 47-kDa polypeptide and suggested the N-glycan nature of these oligosaccharides.

Treatment of the immunoprecipitated GD3 synthase-HA with PNGase F lends further support to the notion that GD3 synthase-HA bears N-linked oligosaccharides. The appearance in the immunoblots of transient intermediates of 45 and 43 kDa, that were finally converted to a 40-kDa form, which likely represent the GD3 synthase core protein plus the 9 amino acids of the HA epitope, confirmed that the three potential N-glycosylation sites were in fact glycosylated.

GD3 synthase-HA activity and intracellular sorting were differently affected when cells were exposed to optimized concentrations of inhibitors of N-glycosylation and of N-glycan processing. Tunicamycin, which inhibits en bloc glycosylation by inhibiting the formation of the chitobiose moiety on dolichol-phosphate, led to the synthesis of the 40-kDa form of GD3 synthase-HA. Homogenates of cells expressing the unglycosylated form showed background levels of GD3 synthase-HA activity in vitro, evidencing a role of these N-linked oligosaccharides in conferring a catalytically active folded state. An interesting observation emerged from the timed deglycosylation of the active GD3 synthase-HA with PNGase F, although a substantial fraction of totally and partially deglycosylated forms appeared in the first 2 h of incubation, the sialyltransferase activity was essentially unchanged in the same period. After 2 h, however, the activity dropped to near zero, indicating that the stability at 37 °C was seriously affected by the loss of N-glycans and suggesting that these are important to keep the GD3 synthase-HA in a more stable form.

Immunoblots of homogenates from cells treated with castanospermine, which prevents the removal of the three glucose residues from N-linked oligosaccharides and hence the oligosaccharide processing at the ER level, lead to a form of GD3 synthase-HA having a molecular mass slightly higher than in untreated cells. This form most probably still contains the glucose residues in the three N-linked oligosaccharide chains. Deoxynojirimycin, which also inhibits glucosidases I and II, showed in addition to the major band of ~45 kDa, a minor band of ~45 kDa. The major band probably represent the form with glucose residues, while the minor band probably represent a form lacking one N-linked oligosaccharide, due to inefficient en bloc glycosylation in the presence of deoxynojirimycin, as it has been already suggested (29).

The GD3 synthase-HA activity was similar in homogenates occupied by carbohydrate and studied if these oligosaccharides are relevant for the catalytic activity and proper subcellular sorting of the enzyme. We have previously observed that heterologous expression of GM2 synthase and of GD3 synthase in Escherichia coli resulted in insoluble forms of these enzymes having no activity toward GM3 as exogenous acceptor suggesting that glycosylation could be of importance for proper folding. Here we addressed this question for the case of chicken GD3 synthase, whose primary sequence predicts three potential N-glycosylation sites. For this, COS-7 cells lacking GD3 synthase activity were transfected with an HA-epitope tagged form of the enzyme, and the effect of drugs affecting N-glycosylation on both the enzyme activity and subcellular localization was examined.

Both the molecular mass of GD3 synthase-HA (47 kDa) in Western blots, which was about 7 kDa larger than expected from the corresponding cDNA (39.9 kDa), and the shape of the immunoreactive band, with a main core and diffuse borders, were compatible with GD3 synthase-HA being a glycoprotein. Incubation of transfected cells with [2-3H]mannose resulted in radioactive labeling of the 47-kDa polypeptide and suggested the N-glycan nature of these oligosaccharides.
from castanospermine and deoxynojirimycin treated cells, and 30–40% lower than in untreated cells. However, the GD3 synthase-HA mass determined in the Western blots was also decreased by about 40% with respect to control cells, suggesting that these inhibitors either inhibited the synthesis or increased the degradation of the enzyme. It has already observed that deoxynojirimycin produced partial inhibition of cruzipain synthesis (29) and that castanospermine induced rapid degradation of major histocompatibilty class I molecules (30).

Immunocytochemical examination of cells treated with the different inhibitors showed that, in those treated with tunicamycin, castanospermine, and deoxynojirimycin, HA-immunoreactivity was mostly associated with the ER. However, whereas the unglycosylated form was enzymatically inactive in vitro, the glycosylated but untrimmed forms in castanospermine- and deoxynojirimycin-treated cells were in a folding state having full enzyme activity. The simplest interpretation of these results is that N-glycosylation is critical for both enzyme activity and exiting the ER, while removal of glucose residues from the N-glycan is necessary for efficient trafficking of the enzyme from the ER to the Golgi complex. Inhibitors of mannosidase I (1-deoxymannojirimycin) or mannosidase II (swainsonine) acting late in the Golgi complex were without effect on GD3 synthase-HA subcellular localization.

Concerning GD3 expression at the surface of inhibitor-treated transfectants, those treated with tunicamycin were essentially negative, although a weak immunostaining could be detected in few of these cells; on the other hand, GD3 was expressed in most castanospermine- or deoxynojirimycin-treated transfectants at levels comparable to those in untreated cells, suggesting that in these transfectants the enzyme was active also in vivo (results not shown). We cannot ascertain in the later cases if GD3 synthesis was carried out by the enzyme retained in the ER or by the fraction that reached the Golgi complex or by both.

Although the effect of inhibiting N-glycosylation on activity and/or intracellular transport has been examined in diverse proteins, the high variability found in the requirements makes difficult to define a role for N-glycan chains on their biological activity. N-Glycans were found essential for proper folding and cellular transport of the α subunit of the lysosomal β-hexosaminidase A (31), while lysosomal delivery of GM2 activator protein, a singly glycosylated protein involved in glycolipid catabolism, was not impaired by tunicamycin (32). On the other hand, inhibition of glycosylation did not affect activity, but affected intracellular transport of secretory proteins as the platelet-derived growth factor B-chain (33), or plasma membrane proteins as the norepinephrine (34) and GLYT1 glycine (35) transporters. For the case of the Golgi glycosyltransferases examined up to now, the common observation is that N-glycans would be necessary for folding into a catalytically active state (17, 18) (this work). However, while N-glycosylation and proper trimming are critical for trafficking of GD3 synthase and GlcNAcT-III (19) to the Golgi complex, this is not so for the case of GM2 synthase (17).

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