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Glycoconjugate glycosyltransferases

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Abstract Assay methods for representative glycosyltransferases were described, covering those involved in the synthesis of glycosphingolipids, *N*-glycans, *O*-glycans, and proteoglycan glycosaminoglycans. In addition, intracellular localization of glycosyltransferase was comprehensively summarized. Lastly, complex formation of glycosyltransferase proteins with related molecules including subunits, chaperones, and enzyme regulators, that have been recently reported was also summarized.

Keywords Glycosyltransferase · Golgi · Substrate · Enzyme · Glycan

Introduction

There have been a number of enzymes (about 170 cDNAs are well characterized) reported that are involved in the synthesis and modification of carbohydrates. Above all, enzymes that catalyze the transfer of monosaccharide are essentially involved in the fundamental structures (core) of glycans on glycoproteins and glycolipids. Before molecular cloning of glycosyltransferase cDNAs, presence of those enzymes has been recognized by the biochemical assay to measure the enzyme activity *in vitro*. Just a few enzymes could be handled as purified homogenous proteins. However, success of molecular cloning of those enzymes has enabled us to directly observe the reaction, substrate specificity, enzyme kinetics and interaction with other molecules [1]. In fact, individual enzymes have their own characteristics, and it seemed difficult to summarize natures of all enzymes reported to date with fixed criteria. In this review, various aspects of glycosyltransferases such as measurement of their activity, localization of the enzymes, formation of molecular complexes with subunit, chaperone or other regulatory molecules were described by selecting representative enzymes among several series of glycosyltransferases responsible for the synthesis of glycolipids, *N*-glycans, *O*-glycans and proteoglycan glycosaminoglycans.

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Enzyme activity (assay)

Measurement of enzyme activity of glycosyltransferases responsible for the synthesis of glycolipids

Example 1: GM2/GD2 synthase (β1,4GalNAc transferase)
Ref: Yamashiro 1993 [2]

Reagents: sodium cacodylate (Wako pure chemical)

CDP-choline (Kohjin)
UDP-GalNAc (Sigma)
GM3 (or GD3) as an acceptor (Sigma)
[^{14}C]-UDP-GalNAc (NEN)
Extracts as an enzyme source
Triton CF54 (Sigma)

Apparatus:

N_2 cavitation apparatus
water bath
N_2 evaporator

TLC plate
imaging analyzer
ultracentrifuge and swing type bukette
SepPak C18 cartridge (Water)

Procedure:

1. Preparation of membrane fraction
   1) N_2 cavitation of cell pellets at 400 psi on ice for 30 min.*
   2) Centrifuge at 1,000 rpm for 10 min at 4°C.
   3) Ultracentrifuge the supernatant at 34 K (Beckman, SW 55Ti) for 1 h at 4°C.
   4) Resuspend the pellets in 0.1 M cacodylate buffer, pH 7.2.
2. Enzyme assay
   1) Evaporate the following items in a glass tube

      UDP-GalNAc (400 μM)
      GM3 (or GD3) (325 μM)
      [^{14}C]-UDP-GalNAc (3.5×10^5 dpm)
      CDP-choline (10 mM)

   2) Dissolve in 0.1 M cacodylate buffer (adjust to make final volume 50 μl)
   3) Add 10 mM $MnCl_2$, 0.3% Triton CF54 in cacodylate buffer.
   4) Sonicate for 10 sec.
   5) Add membrane proteins (200 μg).
   6) Incubate at 37°C for 2–3 h with shaking.
   7) Add DW 1 ml to stop the reaction.
   8) Separate the products by SepPak C18 column.
   9) Dry with N2 stream.
   10) Count radioactivity of 1/5 of the products by scintillation counter.
   11) Analyze 4/5 of the products in TLC and autoradiography**

*Can be replaced by homogenizer.
**Activity is usually presented by calculating from count as unit (pmol/mg protein/h)

Example 2: GD3 synthase (α2,8-sialyltransferase) (Fig. 1A)
Ref: Ruan, S. 1992 [3]

Reagents: sodium cacodylate (Wako pure chemical)

CMP-NeuAc (Sigma)
GM3 as an acceptor (Sigma)
[^{14}C]-CMP-NeuAc (NEN)
Extracts as an enzyme source
Triton CF54 (Sigma)

Apparatus:

essentially as shown in GM2/GD2 synthase

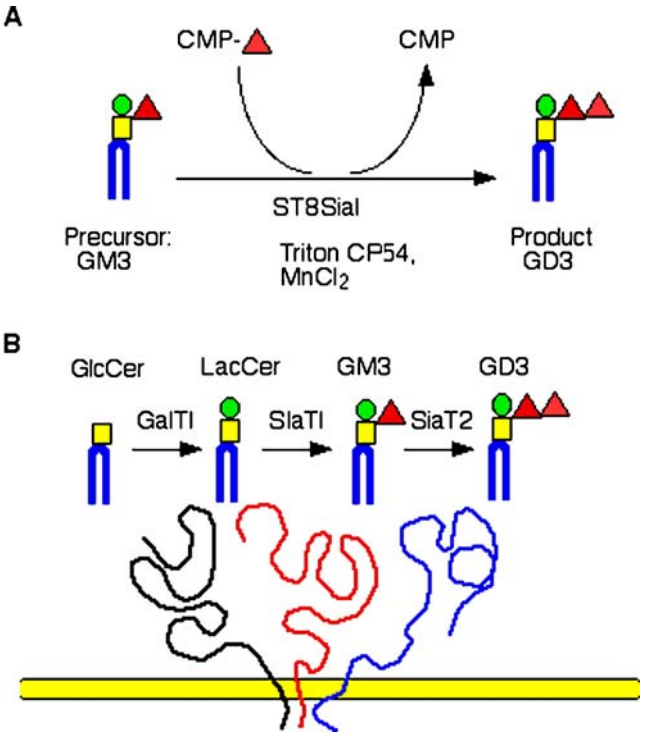

Fig. 1 Enzyme reaction of a glycosyltransferase, GD3 synthase (ST8SiaI) and its linkage with other related glycosyltransferases in Golgi. (A) GD3 synthase catalyze transfer of NeuAc from CMP–NeuAc onto the precursor GM3. Appropriate detergent and cation enhance the activity. (B) Co–precipitation experiments suggested that three enzymes essential for the synthesis of gangliosides physically associate in Golgi apparatus to promote efficient synthesis of GD3 [26]. N-terminal domains participate in these interactions

Procedure:

1. Preparation of membrane fraction
   1) N2 cavitation of cell pellets at 400 psi on ice for 30 min.*
   2) Centrifuge at 1,000 rpm for 10 min at 4°C.
   3) Ultracentrifuge the supernatant at 34 K (Beckman, SW 55Ti) for 1 h at 4°C.
   4) Resuspend the pellets in 0.1 M cacodylate buffer, pH 6.0.
2. Enzyme assay:
   1) Evaporate the following items in a glass tube

      CMP-NeuNc (500 μM)
      GM3 (325 μM)
      [^{14}C]-CMP-NeuNc (9.2×10^3 dpm)

   2) Dissolve in 0.05 M cacodylate buffer (adjust to make final volume 50 μl)
   3) Add 5 mM $MnCl_2$, 0.3% Triton CF54 in cacodylate buffer.

4) Sonicate for 10 sec.
5) Add membrane proteins (200 μg).
6) Incubate at 37°C for 1–2 h with shaking.
7) Add DW 1 ml to stop the reaction.
8) Separate the products by SepPak C18 column.
9) Dry with N_2 stream.
10) Count radioactivity of 1/5 of the products by scintillation counter.
11) Analyze 4/5 of the products in TLC and autoradiography**

*Can be replaced by homogenizer.

**Activity is usually presented by calculating from count as unit (pmol/mg protein/h)

Example 3: Gb3/CD77 synthase: Ref: Kojima 2000 [4]

Reagents: sodium cacodylate (Wako pure chemical)

UDP-Gal (Sigma)
Lactosylceramide as an acceptor (Sigma)
[^{14}C]-UDP-Gal (NEN)
Extracts as an enzyme source
Triton X-100 (Sigma)
Galactonolactone
Phosphatidylglycerol (Sigma)

Apparatus:

N_2 cavitation apparatus
water bath
TLC plate
imaging analyzer
ultracentrifuge and swing type bukette
SepPak C18 cartridge (Water)

Procedure:

1. Preparation of membrane fraction

1) N_2 cavitation of cell pellets at 400 psi on ice for 30 min.*
2) Centrifuge at 1,000 rpm for 10 min at 4°C.
3) Ultracentrifuge the supernatant at 34 K (Beckman, SW 55Ti) for 1 h at 4°C.
4) Resuspend the pellets in 0.05 M cacodylate HCl, pH 6.0.

2. Enzyme assay

1) Evaporate the following items in a glass tube

UDP-Gal (200 μM)
LacCer (0.4 mM)
[^{14}C]-UDP-Gal (2.5×10^5 dpm)
galactonolactone (5 mM)
phosphatidylglycerol (2.9 mM)

2) Dissolve in 0.05 M cacodylate buffer (adjust to make final volume 50 μl)
3) Add 10 mM $MnCl_2$, 0.3% Triton X-100 in cacodylate buffer.
4) Sonicate for 10 sec.
5) Add membrane proteins (50 μg).
6) Incubate at 37°C for 2–3 h with shaking.
7) Add DW 1 ml to stop the reaction.
8) Separate the products by SepPak C18 column.
9) Dry with N_2 stream.
10) Count radioactivity of 1/5 of the products by scintillation counter.
11) Analyze 4/5 of the products in TLC and autoradiography**

*Can be replaced by homogenizer.

**Activity is usually presented by calculating from count as unit (pmol/mg protein/h)

Measurement of enzyme activities of glycosyltransferases responsible for the synthesis of *N*-glycans

Example 1: GnT-V Ref: Nishikawa BBA 1990 [5]

Reagents: 2-aminopyridylated sugar
(Fluorescence labeling and purification based on amino-pyridyl derivatization are in ref. Hase *et al.* [6–8])

MES (2-morphorinoethansufonate)(Katayama)
UDP-GlcNAc (Uridine 5'-diphspho-*N*-acetyl-glucosamine)(Sigma)
GlcNAc (*N*-acetylglucosamine)(Sigma)
biotinated leukoagglutinating phytohemagglutinin (bitotin-labeled L-PHA) (Seikagaku Co.)
horseradish peroxidase avidin D (Vector)
ECL-kit (Amersham Life Science)
Reaction mixture (25 μl)

125 mM MES buffer (pH 6.25)
40 mM UDP-GlcNAc
200 mM GlcNAc
0.5% Triton X-100
10 mM EDTA

Apparatus

HPLC (Shimazu)
HPLC reverse phase column (TSK gel, ODS-80TM)(Shimazu)
fluorescence spectrophotometer (F-1000) (Hitachi)
ultrasound sonicator (Bioruptor)
protein gel electrophoresis apparatus
immunoblotting apparatus

Procedure

1. Preparation of crude samples.

   Tissues

   1) homogenate tissues with 4 x vol of 10 mM Tris–HCl (pH 7.4), 0.25 M sucrose and protease inhibitors (1 mM benzamidine, 10 μM APMSF etc).
   2) centrifuge at 900 g for 10 min at 4°C.
   3) Remove supernatants to be used for enzyme assay.

   Cell lines

   1) wash cultured cells with PBS twice.
   2) centrifuge at 2,000 rpm for 5 min at 4°C.
   3) resuspend in 200 μl PBS
   4) sonicate with Bioruptor

2. Enzyme reaction of GnT-V

   1) mix in 1.5 ml Eppendorf tube

      substrate (PA-oligosaccharide, 0.77 mM) 10 μl
      crude sample as an enzyme source 10 μl
      reaction mixture 25 μl

   2) incubate for 4 h at 37°C.
   3) heat at 100°C for 1 min.
   4) centrifuge at 15,000 rpm for 10 min.
   5) subject supernatants containing enzymatic products to HPLC.

3. Analysis of the enzyme products with HPLC

   1) switch on HPLC and fluorescence spectrophotometer
   2) set an excitation and an emission wavelength of the spectrophotometer at 320 and 400 nm, respectively.
   3) keep the HPLC column at 55 and equilibrate it with 0.1 M acetate buffer as follows
   4) apply 10 μl of enzymatic products to HPLC
   5) elute with 0.1 M acetate buffer (pH 4.0) containing 0.15% *n*-butanol at a flow rate 1.0 ml/min.
   6) calculate the specific activity of GnT-V and express the activity as pmol/h/mg protein.

Example 2: HNK antigen synthase Ref: Oka, S., J. Biol. Chem. 1992 [9]

Reagents: UDP-GlcA (Sigma)

UDP-[^{14}C]GlcA (10.2 GBq/mmol) (ICN)
Asialoorsomucoid (ASOR)
Agalactoorsomucoid (AGOR)
Asialoorsomucoid glycopeptide (ASOR-GP)
Agalactoorsomucoid glycipeptides (AGOR-GP)
Synthetic glycolipid, neolactotetraose-phenyl-C14H29 (nLcPA14)(Bio Carb)
Neolactotetraosylceramide (Dia-Iatron)
UDP-GlcA-Sepharose CL-6B
ASOR-Sepharose 4B
AGOR-Sepharose 4B

Preparation of enzyme

1. Ten grams of rat brain was homogenized with five volumes (50 ml) of 10 mM HEPES buffer, pH 6.5, containing 0.32 M sucrose, I mM EDTA, 0.1% (*v*/*v*) 2-ME, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 100 μg/ml benzamidine, and I mM PMSF.
2. Centrifuge the homogenate at 105,000×*g* for 1 h.
3. The resulting pellet was suspended in five volumes of 10 mM HEPES buffer, pH 6.5, containing 2% NP-40, I mM EDTA, 0.1% (*v*/*v*), 2-ME, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 100 μg/ml benzamidine, and 1 mM PMSF.
4. Stir with a magnetic stirrer for 1 h to extract the membrane-bound enzyme.
5. The suspension was recentrifuged at 105,000×*g* for 0.5 h.
6. The supernatant was stored after addition of 20% glycerol at −20°C.

Glucuronyltransferase assay

GlcAT-P

Method I: ASOR-Sepharose 4B (glycoprotein acceptor)
Method II: Oligosaccharides and glycopeptides (acceptors) for kinetic study and inhibition experiments

GlcAT-L

Method III: synthetic glycolipid as an acceptor

Method I: Assay mixture containing following components (total 100 μl)

ASOR-Sepharose 4B: 40 μg/20 μl
50 μM UDP-[^{14}C]GlcA (2×10^6 cpm)
100 mM HEPES buffer pH 6.5
10 mM $MnCl_2$
5 mM ATP
0.4% (*v*/*v*) Nonidet P-40
enzyme solution
Incubation for 3 h at 37°C.
Terminate the reaction by adding EDTA (50 mM).

Centrifuge and wash with 0.5 ml of 0.5% (w/v) SDS six times.
Radioactivity on the resin was counted.
Blank reaction is with heat-inactivated enzyme or resin without ASOR.

Method II: Assay mixture containing following components (total 50 μl)

ASOR-Sepharose 4B: 5 μg
100 μM UDP-[^{14}C]GlcA (2×10^6 cpm)
100 mM HEPES buffer pH 6.5
10 mM $MnCl_2$
2.5 mM ATP
0.4% (v/v) Nonidet P-40
enzyme solution

1. Incubation for 3 h at 37°C.
2. The reaction mixture was spotted onto a 2.5-cm Whatman No.1 paper disc.
3. The disc was dipped in a 10% (w/v) trichloroacetic acid solution.
4. Then rinsed twice in a fresh TCA solution, then in ethanol/ether (2/1,v/v) and finally in ether.
5. The radioactivity was counted after air-dry.

Method III: Assay mixture containing following components (total 50 μl)

150 μM nLc-PA_{14} or nLc-Cer
100 μM UDP-[^{14}C]GlcA (2×10^6 cpm)
200 mM HEPES buffer pH 4.5
10 mM $MnCl_2$
10 mM ATP
0.4% (v/v) Nonidet P-40
enzyme solution

1. Incubation for 3 h at 37°C.
2. Terminate the reaction by adding 20 volumes of chloroform/methanol (2/1, v/v).
3. The radioactive reaction products were separated by passing through a Sephadex G-25 column (0.5×5.0 cm), which had been equilibrated with chloroform/methanol/water (60/30/4.5, v/v/v).
4. Radioactivity was counted with a Beckman LS-6000 scintillation counter.

Measurement of enzyme activities of glycosyltransferases responsible for the synthesis of *O*-glycans

Example 1: ppGalNAc-T Ref: Yoshida A Glycoconj J. 1995 [10]

Reagents: imidazole-HCl (Sigma)

Bovine submandibular gland apomucin
UDP-GalNAc (Sigma)
[^{3}H]-UDP-GalNAc (NEN)
Enzyme sample
Triton X-100 (Sigma)

Apparatus:

water bath
SepPak C18 cartridge (Water)
vacuum manihold (Supelco)

Procedure:

1. Transfer of GalNAc to apomucin

1) Prepare Mix I and Mix II

Mix I (5 mM UDP-GalNAc, 2.4 μl; [^{3}H]-UDP-alNAc (6×10^4 dpm/tube), 3.0 μl; H_2O, 94.6 μl) total 100 μl (for 10 samples)
Mix II (1 M imidazole-HCl pH 7.2, 40 μl; 0.2 M $MnCl_2$, 40 μl; 5% Triton X-100,60 μl) total 160 μl (for 10 samples)

2) Prepare incubation mixture (Mix I, 10 μl; Mix II, 16 μl; 10 mg/ml apomucin 15 μl; enzyme* 2–15 μl)
3) Add H_2O to a final volume of 80 μl.
4) Incubate at 37°C for 5–30 min.
5) Stop the reaction by adding 10 μl of 0.25 M EDTA.
6) Move samples to ice and add 50 μl of 10 mg/ml BSA and 0.5 ml of 10% TCA including 1% PTA (phosphotungustic acid) to the incubation mixture on ice.
7) Let samples precipitate on ice for 5 min and centrifuge at 3,000 g for 1 min.
8) discard the supernatant and wash precipitates with 10% TCA including 1% PTA 3 times**
9) wash with 0.5 ml of ether/ethanol (3:1, v/v) and dry samples in Speed-Vac for a few min.
10) Suspend the dried pellets in 1 ml of 10% SDS by sonication.
11) Heat at 100°C for 5 min to dissolve the precipitates.
12) Count in 10 ml scintillator.

2. Transfer of GalNAc to peptides

1) Prepare Mix I and Mix II

Mix I (5 mM UDP-GalNAc, 14.4 μl; [^{3}H]-UDP-GalNAc (2×10^5 dpm/tube), 14.4 μl; H_2O, 91.2 μl) total 120 μl (for 10 samples)
Mix II (1 M imidazole-HCl pH 7.2, 30 μl; 0.2 M $MnCl_2$, 30 μl; 5% Triton X-

100, 60 μl) total 120 μl (for 10 samples)
2) Prepare incubation mixture (Mix I, 10 μl; Mix II, 8 μl; peptide solution, 2–17 μl; enzyme* 5 μl)
3) Add H_2O to a final volume of 40 μl.
4) Incubate at 37°C for 5–30 min.
5) Stop the reaction by adding 1 ml of 0.1% TFA in H_2O.
6) Wash the C18 cartridge with 2 ml MeOH followed by 2 ml of 0.1% TFA in H_2O.
7) Apply assay samples to the C18 cartridge.
8) Wash with 5 ml of 0.1% TFA in H_2O.
9) Elute with 1.5 ml of 0.1% TFA in 35% acetonitrile/65% H_2O and collect the eluate directly into a scintillation vial.
10) Count in 10 ml of scintillator.

*Enzyme should be added last.
**Disperse the pellets in the tubes by sonication.

Example 2: POMT-1/2 Ref: Manya, H. PNAS, 2004 [11]

Reagents: 20 mM Tris–HCl (pH 8.0)

Dol-P-[^{3}H]Man
n-octyl-β-D-thioglucoside (Dojindo)
GST-α-dystroglycan

POMT enzyme assay

The reaction mixture (20 μl) contained following components.

20 mM Tris–HCl (pH 8.0)
100 nM Dol-P-[^{3}H]Man (125,000 dpm/mol) (American Radiolabeled Chemicals)
2 mM 2-ME
10 mM EDTA
0.5% *n*-octyl-β-D-thioglucoside (Dojindo)
10 μg of GST-β-dystroglycan
80 μg of microsomal membrane fraction

1. The reaction was initiated by adding the protein extract.
2. Incubate for 1 h at 20°C.
3. The reaction was stopped by adding 200 μl of PBS containing 1% Triton X-100.
4. The reaction mixture was centrifuged at 10,000 x g for 10 min.
5. The supernatant was removed, mixed with 400 μl of PBS containing 1% Triton X-100 and 10 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia Biosciences)
6. The mixture was rotated at 4°C for 1 h, and washed 3 times with 20 mM Tris–HCl (pH 7.4) containing 0.5% Triton X-100.
7. The radioactivity absorbed to the beads was measured by using a liquid scintillation counter.
8. The incorporation of radioactive mannose into GST-α-DG was detected by SDS-PAGE and subsequent autoradiography.

Measurement of enzyme activities of glycosyltransferases responsible for the synthesis of glycosaminoglycans

Example 1: Galactosyltransferase I (XGalT-1) Ref: Okajima, T. [12], Nakamura [13]

Reagents: MES (2-morphorinoethansufonate) (Katayama)

UDP-Gal (Sigma)
DMSO (Sigma)
[^{14}C]-UDP-Gal (NEN)
Extracts as an enzyme source
Triton X-100 (Sigma)

Apparatus:

N_2 cavitation apparatus
water bath
N_2 evaporator
imaging analyzer
ultracentrifuge and swing type bukette
SepPak C18 cartridge (Water)

Procedure:

1. Preparation of membrane fraction is essentially same as described for GM2/GD2 synthase except that the pellets of ultracentifugation were resuspend in 0.1 M MES buffer, pH 6.0.
2. Enzyme assay
   1) Evaporate the following items in a glass tube

      UDP-Gal (600 μM)
      Substrate (acceptor) p-Nph-β-D-Xyl (2 mM)
      [^{14}C]-UDP-Gal (5×10^3 dpm/μl)

   2) Dissolve in 0.1 M MES buffer (adjust to make final volume 25 μl)
   3) Add 15 mM $MnCl_2$, 50 mM KCl, 1 μl of DMSO, 1% Triton X-100 in MES buffer.
   4) Sonicate for 10 sec.
   5) Add enzyme (3–20 μg).
   6) Incubate at 37°C for 30–120 min with shaking.
   7) Add DW 1 ml to stop the reaction.

8) Separate the products by SepPak C18 column by eluting with methanol.
9) Dry with N_2 stream.
10) Count radioactivity of 1/5 of the products by scintillation counter.
11) Analyze 4/5 of the products in TLC and autoradiography*

*Activity is usually presented by calculating from count as unit (nmol/mg/min)

Enzyme localization of glycosyltransferases

Examples of various patterns for glycosyltransferase localization

Majority of glycosyltransferases are localized in Golgi apparatus. However, some of them are localized in ER, plasma membrane and/or outside of cells such as blood. Generally, glycosyltransferases involved in the synthesis of precursor structures or in early glycosylation steps of various lineages tend to be in ER, and some are exceptionally localized in the plasma membrane.

GlcCer synthase is present on the ER membrane facing outside of ER. After the synthesis of GlcCer, it is incorporated inside of ER by flippase, then undergo following modification and/or transport to Golgi. On the other hand, GalCer synthase is localized in the luminal side of ER.

In contrast, hyaluronan synthases, Has1, Has2 and Has3 are localized in the plasma membrane [14]. The three isoforms differ in kinetic characteristics and product size. The existence of three different HAS isoforms with different characteristics implies that the broad range of biological and physiological roles performed by HA are regulated by controlling the activities and expression of the HAS isoforms.

O-fucosyltransferase 1 (O-Fut1) catalyzes the first step of the synthesis of *O*-fucosyl type glycans, and became attractive since its role in the Notch signaling was demonstrated. O-Fut1 transfers fucose onto Thr/Ser residues in EGF domains (C2XXX(A/G/S)(*S/T*)C3). Sequential addition of α3GlcNAc by Fringe, β4galactose, and α2-3/6-sialic acid occurs, first two of which seem specific for *O*-fucose type glycans. It was recently demonstrated that O-Fut1 resides in ER and plays as a chaperone molecule in the secretion of EGF repeats or enhancement of binding with ligands [15].

Sometimes, glycosyltransferases are cleaved at the stem regions by proteases, and are secreted out side of cells. They are present in blood at a fairly high level. Whether these soluble form enzymes play significant roles in the body is unknown, and they are rather being utilized as a marker for cancers [16] and tissue degeneration or inflammation [17]. ST6Gal-I is a most investigated enzyme among soluble glycosyltransferases [18]. It is digested by BACE1 [17] that is a critical protease in Alzheimer disease [19]. The fact that secretion of ST6Gal-I is regulated by BACE1 was confirmed using BACE1 knockout mice [20].

All enzymes except ALG4 involved in the synthesis of *N*-glycan precursor are present in ER, although the orientation is switched by Rft1 (flippase) at the point, where Man_5-$GlcNAc_2$-PP-Dol is formed from outside to in side of ER. Enzymes involved in the synthesis of GPI also exist in ER, but the orientation is similarly switched from outside to inside between the synthetic reactions of PIG-L and PIG-W [21].

Analyses of localization of glycosyltransferase proteins

Example 1: Detection of Golgi retention of ST6Gal I [18]

1. Construction of ST6Gal I-FLAG:
   1) FLAG peptide was fused to the carboxy-terminus of ST6Gal I by fusing the ST sequences (generated from the ST-bs + using primers GCCTCGAGCT GGACCATTCATTATGATT and CGCAAGCTTACAACGAA TGTTCC GGAAG) to the FLAG-encoding oligonucleotide which was made by annealing primers at an oligonucleotide-encoded *Hin*dIII site.
   2) The ST-FLAG construct was cloned into bs + in the *Xho*I and *Bam*HI sites and then ligated into the pSVL expression vector at the *Xho*I and *Bam*HI sites.
2. Transfection of COS cells
   1) Cos-1 or 7 cells were plated on 10-cm dishes and incubated until 50–70% confluent.
   2) Cells were transfected using Lipofectin method and Opti-MEM I with 55 μM β-mercaptoethanol according to the manufacturer's instructions.
   3) Expression of transfected proteins was typically allowed to continue for 16–36 h, then used for immunofluorescence assay.
3. Immunofluorescence localization and SNA lectin staining
   1) Following the fixation and blocking steps, cells were incubated for 45 min with ×100 dilution of either rabbit anti-soluble rat liver ST6Gal I (affinity purified) or the M2 mouse monoclonal anti-FLAG antibody in blocking buffer.
   2) Following PBS washes, FITC-conjugated secondary antibodies diluted (x100) in blocking buffer were incubated with the cells.
   3) For staining transfected CHO cells with FITC-conjugated SNA lectin (Sambucus nigra aggluti-

nin), the blocking step was eliminated and unpermeabilized cells were incubated with FITC-conjugated SNA in PBS (x 200 dilution) for 45 min.
4) Immunofluorescence staining was visualized and photographed using a Nikon Axiophot or Optiphot 2 microscope.

Example 2: Detection of ST6Gal I cleaved and secreted into plasma [20]

1. Detection of ST6Gal I in plasma of mice.
   1) Plasma from mice (50 μl) were diluted with 10 volumes of buffer A (20 mM Tris–HCl, pH 8.0)
   2) The diluted plasma was loaded onto a HiTrap Q-Sepharose column (1 ml of gel volume, Amersham Biosciences) using an Akta Prime system.
   3) The column was washed with 5 ml of buffer A, and protein was then eluted with a gradient of 0–0.5 M NaCl for 30 min at a flow rate of 0.5 ml/min.
   4) Fractions were collected every minute, and the ST6Gal I elution pattern was monitored by immunostaining with anti-ST6Gal I antibody.
   5) ST6Gal I-containing fractions were pooled and precipitated with ice-cold acetone.
2. Detection of ST6Gal I in rat plasma
   1) Plasma from rats (600 μg of protein) were treated by the Montage Albumin Depletion kit from Millipore (Bedford, MA) and then precipitated with ice-cold acetone.
   2) Samples were suspended in PBST buffer (20 mM sodium phosphate, pH7.2, 0.15 M NaCl, 0.1% Tween 20).
   3) Suspended samples were subjected to SDS-polyacrylamide gel electrophoresis (5–20% gradient), and then transferred to a nitrocellulose membrane.
   4) The membrane was incubated with anti-ST6Gal I (1:100).
   5) Horseradish peroxidase-goat anti-rabbit IgG (Cappel, 1:10,000) was used as the secondary antibody, and chemiluminescent substrate (Pierce) was used for detection.
   6) ST6Gal I levels were quantified with a Luminoimage Analyzer LAS-1000 PLUS (Fuji).

Complex formation of glycosyltransferases

Novel findings on the complex formation of various glycosyltransferases

Generally, one enzyme reaction to transfer a monosaccharide to an acceptor structure with some linkage has been believed to be catalyzed by a single enzyme. However, there have been many reports recently to indicate that some enzyme reactions are achieved on the basis of collaboration with two or multiple enzyme subunits. The subunit protein is usually difficult to fully express the enzyme activity as a single component, and complex formation (Table 1) can tremendously enhance the enzyme activity. Actually, it is frequently difficult to distinguish, whether some subunit is really a part of the enzyme complex, or a chaperone molecule. Here, various associating molecules to glycosyltransferases, such as subunit, chaperone, and regulator are discussed.

The processing of *N*-glycans was strictly regulated. A long time ago, it was shown that only GnTI and Man-ase II were linked among enzymes involved in the processing [22]. Rat liver Golgi stacks were extracted with Triton X-100 and the low speed centrifugation pellet contained two medial-Golgi enzymes, *N*-acetylglucosaminyltransferase I and mannosidase II. Both were present in the same structure termed matrix.

It was reported that a basic amino acid motif (RKXRK) and its direct binding to Sar1, a component of COPII is important in the selective transport of glycosyltransferases from ER to Golgi [23]. It was also demonstrated that cytoplasmic tails of SiaT2 (ST8SiaI) and β1,4GalNAc-T (GM2/GD2 synthase) impose their respective proximal and distal Golgi localization [24]. On the other hand, it was demonstrated that enzymes responsible for the synthesis of gangliosides formed two distinct clusters in the different compartments in Golgi. One was a cluster of GM2/GD2 synthase and GM1 synthase, and the other was that of LacCer synthase, GM3 synthase and GD3 synthase (Fig. 1B) [25, 26]. These results suggested that there should be a novel system to efficiently synthesize individual series of glycosphingolipids.

For the synthesis of heparan sulfate repetitive sugar chains, EXT1 and EXT2 were reported to have activities of both GlcNAcT-II and GlcAT-II [27]. EXT1 and EXT2 formed hetero-complex in cells, leading to the expression of much higher activity than a single form [28]. This complex had GlcNAcT-I activity to initiate the synthesis of heparan sulfate sugar chain [29]. These results were essentially common to the case of *Drosophila melanogaster* [30] and *C. elegans* [31]. As for the synthesis of chondroitin sulfate sugar chains, similar complex of ChSy and ChPF (chondroitin polymerizing factor) was identified, and its activity in the extension of sugar chains and initiation of chondroitin sulfate was demonstrated [32]. Analogous results were demonstrated in *C. elegans* [33].

In the synthesis of the core 1 *O*-glycan, core 1 β3-galactosyltransferase (C1 β 3Gal-T) generate Galβ1–3GalNAc 1-Ser/Thr (T antigen). Cosmc (*co*re 1 β3-Gal-T-*s*pecific molecular *c*haperone) demonstrating physical association

Table 1 Complex formation of glycosyltransferases and their counterparts (association, subunit, chaperone, regulator)

Main synthetic pathway	Enzyme and its counterpart	Remarks	Reference No.
1. Processing of *N*-glycans	GnTI and Man-ase II		22
2. Ganglioside synthesis	GM2/GD2 synthase and GM1 synthase		25
3. Ganglioside synthesis	LacCer synthase, GM3 synthase and GD3 synthase		26
4. Heparan sulfate (GAG) synthesis	EXT1 and EXT2	GlcNAcT-II and GlcAT-II, GlcNAcT-I activity	28
5. Chondroitin sulfate (GAG) synthesis	ChSy and ChPF	chondroitin polymerizing factor, extension and initiation of chondroitin sulfate of sugar chains	32
6. Core 1 *O*-glycan	C1 β 3Gal-T and Cosmc	*co*re 1 β3-Gal-T-*s*pecific molecular *c*haperone	34
7. Synthesis of *N*-glycan precursor	Alg13 and Alg14	transfer GlcNAc onto GlcNAc-PP-Dol	35–37
8. *O*-glycosylation (O-Man)	POMT1 and POMT2	*O*-glycosylation of alpha-dystroglycan	39
9. HNK synthesis	GlcAT-P(S) and sulfotransferase		44
10. Elongation of N-glycans	β3Gn-T2 and β3Gn-T8	elongation of multiantennary *N*-glycans	46
11. N-glycosylation	oligosaccharyltransferase (OT) 9 OT subunits	the first step of N-glycosylation	47
12. GPI-anchor synthesis	GPI-GlcNAc-T at least seven components	the first reaction (transfer of GlcNAc)	21

with the core 1 enzyme was identified [34], and C1 β3Gal-T activity requires expression of Cosmc. Cosmc acts as a specific molecular chaperone in assisting the folding stability of C1 3Gal-T.

In the synthesis of the oligosaccharide precursor for *N*-glycan, yeast Alg13 and Alg14 were identified recently [35–37]. They catalyze the transfer of GlcNAc onto GlcNAc-PP-Dol using UDP-GlcNAc, forming a complex, which was critical for both ER localization and enzyme activity. Human Alg13 and Alg14 homologues were also identified [37].

In the *O*-glycosylation of α-dystroglycan, POMT1 and POMT2 co-expression was essential for the expression of the enzyme activity [38]. This was confirmed by the co-precipitation of POMT1 and POMT2 showing definite enzyme activity, although transfection of either cDNA or even mixture of the extracts from cells transfected by individual cDNAs could not show the enzyme activity [39]. These results suggested two enzyme proteins needed to form a complex under the translation. There are also two POMT gene homologues in *Drosophila* that require co-expression to show enzyme activity [40]. In the yeast system, multiple PMTs showed different modes of complex formation [41, 42].

HNK antigen is synthesized by sequential actions of GlcAT-P(S) and sulfotransferase onto non-reducing end of *N*-acetyllactosamine structures in NCAM or PO etc. Main glucuronyltransferase (GlcAT) in nerve tissues is GlcAT-P, and it was confirmed in the KO mice of GlcAT-P [43]. Except for kidney tissue, HNK-1 is sulfated at C3 of glucuronic acid, and HNK-1 structure significantly plays its role with sulfation. It was demonstrated that both GlcAT-P and GlcAT-S form a complex with Sulfo-T to raise the efficiency of Sulfo-T action [44]. Surprisingly, Sulfo-T could precipitate with GlcAT-I that is essential for the synthesis of the common linkage sugar chain among many glycosaminoglycans with unknown reason [45].

β3Gn-T2 and β3Gn-T8 also form a complex [46]. They had similar substrate specificities and recognized tetraantennary *N*-glycans and 2-6-branched triantennary glycans in preference to 2-4-branched triantennary glycans, diantennary glycans, and lacto-*N*-neotetraose (LNnT). When soluble recombinant β3Gn-T2 and β3Gn-T8 were mixed, the Vmax/Km value of the mixture was 9.3- and 160-fold higher than those of individual β3Gn-T2 and β3Gn-T8, respectively. This was the first report showing that *in vitro* mixed glycosyltransferases show enhanced enzymatic activity via the formation of a heterocomplex. These results suggested that β3Gn-T8 and β3Gn-T2 are cooperatively involved in the elongation of specific branch structures of multiantennary *N*-glycans.

Asparagine-linked glycosylation is the most ubiquitous protein co-translational modification in the ER [47]. The first step of *N*-glycosylation is catalyzed by oligosaccharyltransferase (OT). OT transfers an oligosaccharyl moiety (Glc_3Man_9 $GlcNAc_2$) from the dolichol-linked pyrophosphate donor to the side chain of Asn within a consensus sequence of Asn-X-Thr/Ser. This enzyme exists as a heteromeric, multisubunit complex in the ER membrane. Genes encoding 9 OT subunits isolated in *Saccharomyces cerevisiae* have homologues in human genome with high

homology and similarity, suggesting highly conserved nature of this system.

Glycosylphosphatudylinositol (GPI) anchor is synthesized by the sequential additions of sugars and other components to phosphatidylinositol (PI) in the ER [48, 49]. Eight reaction steps are required for generation of GPI that is competent for attachment to proteins. The first reaction in the biosynthetic pathway is the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to PI on the cytoplasmic side of ER, generating the first intermediate, GlcNAc-PI. The enzyme that catalyzes this reaction, *i.e.* GPI-*N*-acetyl-glucosaminyltransferase is a multisubunit enzyme consisting of at least seven components, PIG-A, PIG-C, PIG-H, PIG-P, PIG-Q, PIG-Y, and DPM2 [21].

Analyses of complex formation of representative glycosyltransferases

Example 1. Complex formation between GM2/GD2/GA2 synthase (GalNAc-T) and GM1/GD1b /GA1 synthase (Gal-T2) [25]

1. Expression vector:
   1) Human GalNAc-T cDNA was subcloned into eukaryotic expression vector pCI-neo (Promega), and the sequence coding for the *c-myc* epitope was introduced at the C-terminus before the stop codon.
   2) Mouse Gal-T2 bearing a 9-AA hemagglutin (HA) epitope at C-terminus was cut out from pCEFL-Gal-T2-HA with *Eco*RI and *Not*I and subcloned into pCI-neo vector (pCI-Gal- T2-HA).
   3) cDNA for amino acids 1–52 of Gal-T2 tagged with the HA was also constructed (pCI-Gal-$T2_{1-52}$-HA).
2. Immunoprecipitation
   1) Cells (200 μg of protein) were lysed for 60 min on ice with 500 μl of lysis buffer (50 mM Tris–HCl, pH7.2, 1.0% Triton X-100, 300 mM NaCl, 3 mg/ml leupeptin, 1 mM PMSF, 3 mg/ml aprotinin, 2 mM pestatin, 1 mM EDTA, 0.05%, sodium azide).
   2) Lysates were preabsorbed with protein G Sepharose beads (Amersham Pharmacia, 75% suspension washed with lysis buffer before use) for 60 min on ice.
   3) Aliquots of supernatants were incubated overnight on a rotating wheel at 4°C with individual specific monoclonal antibodies. Dilutions were as follows:

      anti-*HA* (12CA5, Babco, Richmond CA), 1:150;
      anti-*c-myc* (CRL1729 immunopurified supernatant), 1:50;
      anti-GFP (mixture of clones 7.1 and 13.1, Boehringer Mannheim), 1:200;
      polyclonal antibody anti-ManII (from K. Moremen), 1:50;

   4) Then incubated with 100 μl of protein G-Sepharose beads (50% slurry).
   5) Immunocomplexes were pelleted by centrifugation at 2,500 g and then washed with lysis buffer and with 150 mM phosphate buffer, pH 7.2, 10 mM EDTA for immunoblotting.
   6) For glycosyltransferase activity assays, final wash was done with 100 mM HCl cacodylate, pH 7.2.
3. Western Blotting
   1) The membrane fraction (100 μg of protein) or immunocomplexes obtained were taken in Laemmli sample buffer and subjected to SDS-PAGE.
   2) For immunoblotting, the following antibodies were used:

      polyclonal anti-*HA* (1:800),
      anti-*c-myc* (1:400, Babco),
      anti-ManII (1:400),
      monoclonal anti-GFP (1:1,000)
      followed by horseradish peroxidase-protein A (1:85,000, Sigma).

   3) For monoclonal antibodies, rabbit anti-mouse IgG was used before addition of protein A.
   4) All incubations were carried out in 0.25% polyvinylpirrolidone 0.25% BSA, 0.05% Tween 20 in TBS (TTBS) for 1 h at room temperature, followed by three washes with TTBS.
   5) Blots were processed by using the Renaissance Chemiluminescence Reagent Plus (NEN) and exposed to Kodak BioMax MS x-ray film.
   6) Resulting bands were quantified by densitometry (SCION IMAGE 1 software, National Institutes of Health).

Example 2. Microscopic measurement of FRET in living cells [25].

1) For FRET study, GalNAc-T_{1-27}-ECFP and GalNAc-T_{1-27}-EYFP were generated using pECFP-N1 and pEYFP-N1 (CLONTECH) with PCR.
2) For FRET study, Gal-$T2_{1-52}$-HA-ECFP and Gal-$T2_{1-52}$-HA-EYFP were also generated by PCR amplification. In addition, Man-II_{1-76}-ECFP and $ManII_{1-76}$-EYFP were generated.
3) Cells stably expressing N-terminal domains of Golgi proteins fused to ECFP or EYFP were grown

in DMEM on coverslips adhered with Sylgard 182 silicone elastomer (Dow-Corning) to the bottom of perforated culture dishes.

4) For FRET determinations, cells washed with PBS were observed in a Zeiss Axiovert 135 M inverted microscope equipped with a 633, 1.4 NA oil immersion objective while kept at 37°C in an atmosphere of 5% CO_2.
5) Details for actual conditions for analyses were in the original reference [25].

Ending remark

As described above, fundamental enzyme reactions for the individual steps of glycosylation in complex carbohydrates have been well characterized. However, knowledge about molecular complex formation of glycosyltransferaes with associating molecules such as subunits, chaperone, and regulators will be more expanded. Furthermore, direct and indirect linkages between enzymes for different reactions based on the compartmentalization will be more clarified. There might be common regulatory factors for various steps of glycosylation, *e.g.* transcription factors, molecular chaperones, proteases, and unknown regulatory proteins such as Large [50].

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