Quantitative Transcriptomic Profiling of Branching in a Glycosphingolipid Biosynthetic Pathway

Received for publication, February 24, 2011, and in revised form, June 8, 2011. Published, JBC Papers in Press, June 10, 2011, DOI 10.1074/jbc.M111.234526

Hiromu Takematsu‡1, Harumi Yamamoto‡§, Yuko Naito-Matsui‡, Reiko Fujinawa‡, Kouji Tanaka¶, Yasushi Okuno**, Yoshimasa Tanaka‡¶, Mamoru Kyogashima‡‡, Reiji Kannagi‡, and Yasunori Kozutsumi‡§

From the 1Laboratory of Membrane Biochemistry and Biophysics, Graduate School of Biostudies, **Department of System-bioscience for Drug Discovery, Graduate School of Pharmaceutical Sciences, and 1Laboratory of Membrane Biochemistry and Biophysics, Graduate School of Biostudies, Graduate School of Medicine, Kyoto University, Sakyo, Kyoto 606-8501, the 2Supra-macromolecular System Research Group, RIKEN Frontier Research System, RIKEN, Wako, Saitama 351-0198, the 3Division of Molecular Pathology, Aichi Cancer Center, Nagoya, Aichi 464-8681, and the 4Department of Oncology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Aichi 464-8681, Japan

Cellular biosynthesis of macromolecules often involves highly branched enzyme pathways, thus cellular regulation of such pathways could be rather difficult. To understand the regulatory mechanism, a systematic approach could be useful. We genetically analyzed a branched biosynthetic pathway for glycosphingolipid (GSL) GM1 using correlation index-based responsible enzyme gene screening (CIRES), a novel quantitative phenotype-genotype correlation analysis. CIRES utilizes transcriptomic profiles obtained from multiple cells. Among a panel of B cell lines, expression of GM1 was negatively correlated with and suppressed by gene expression of CD77 synthase (CD77Syn), whereas no significant positive correlation was found for expression of GM3 synthase (GM3Syn; encoded by B3GNT5) (10, 14). The commitment for various series of GSL biosynthesis takes place at the usage of LacCer, a common precursor for complex GSLs. Globo series GSLs are biosynthesized by CD77/Gb3 synthase (CD77Syn; encoded by A4GALT) (15, 16), and lacto series GSLs are biosynthesized by LC3 synthase (LC3Syn; encoded by B3GNT5) (17), whereas ganglio series are biosynthesized by GM3 synthase (GM3Syn; encoded by ST3GAL5) (18). Due to the complexity of branching at LacCer, the identification of the regulatory mechanisms of GSL expression has been a major challenge, even after identification of the enzymes involved (Fig. 1A) (19). Ganglio series biosynthesis may be an efficient enzyme step because LacCerSyn and GM3Syn participate in multienzyme complexes, by which channeling of LacCer to the GM3Syn reaction may occur (20, 21). This complex, however, may change its sub-Golgi compartment localization in response to other expressed proteins (22, 23) and the regulatory mechanism has not yet been fully clarified. Nevertheless, GSL expression is regulated by various cellular events, such as the activation of human B cells in the germinal center (24).

2 The abbreviations used are: GSL, glycosphingolipid; CD77, Gb3Cer/Galα1→3Galβ1→4Glcβ1→ceramide; Cer, ceramide; FCM, flow cytometry; GM1, Galβ1→4GalNAcβ1→4(Siaα2→3)Galβ1→4Glcβ1→ceramide; GM3, Siaα2→3Galβ1→4Glcβ1→ceramide; LacCer, lactosylceramide (Galβ1→4Gloβ1→3Galβ1→4Glcβ1→ceramide); MFI, mean fluorescent intensity; MSCV, murine stem cell virus; PDI, peptidyl disulfide isomerase; EGFP, epidermal growth factor protein; CTxB, cholera toxin B; GM2, GalNAcβ1→4(Siaα2→3)Galβ1→4Gloβ1→3Cer; GD1a, Siaα2→3Galβ1→4GalNAcβ1→4(Siaα2→3)Galβ1→4Gloβ1→3Cer.
Different GSL species appear to function in cellular signaling events, although individual functional differences have not been fully explored. Moreover, the expressions of particular GSL species are targeted by various bacterial toxins, and thus have pathological significance. For example, the shiga toxin, cholera toxin, and heat-labile enterotoxin target CD77, GM1, and GD1a, respectively (Fig. 1A) (25–27).

The expressions of many cellular phenotypes are mainly controlled by the regulation of gene expression (28). Correlation index-based responsible enzyme gene screening (Cires) is a novel systematic strategy to quantitatively assess the phenotype-genotype correlation. This method was developed to identify genetically dominant enzyme gene(s) that may quantitatively regulate the expression of cell surface glycans (29). Cires involves the statistical comparison of multisample profiles between genotypes (glycan-related gene expression profiles obtained by DNA microarray) and cell surface phenotypes (glycan expression). Correlations found between these profiles are used to build hypotheses, and subsequent genetic manipulation of cells provides experimental verification (Fig. 1B). Cires has been successfully used to screen glycosyltransferase genes responsible for the level of glycan expression on the cell surface (29, 30). Thus, we used cires to analyze other complicated GSL biosynthetic pathways, with a focus on the regulation of branching.

We analyzed the branching point of LacCer in GSL biosynthesis using Cires to understand the genetic characteristics of the branching regulatory mechanism, which is a frequent subject of biochemical studies. Our genetic analyses of B cell lines revealed that the globo series dominated the ganglio series in this biosynthetic pathway at the branching point of the common precursor, LacCer. This genetic dominance is based on LacCerSyn regulation by CD77Syn (residues 192 and 194) were point mutated to threonine residues by PCR-based mutagenesis to give rise to the CD77Syn-TXT mutant. CHO-K1 cells were successively transfected with three vectors harboring resistance genes to antibiotics obtained from Nacalai Tesque (Kyoto, Japan) or Invivogen (San Diego, CA): G418 (1000 μg/ml), blasticidin (10 μg/ml), and zeocin (125 μg/ml).

**Antibodies and Other Probes**—Biotin-xx-conjugated cholera toxin B subunit was obtained from Invitrogen. Anti-CD77 monoclonal antibody (mAb) (clone 38-13) was obtained from IMMUNOTECH (Marseille, France). Anti-giantin rabbit polyclonal antibodies (pAb) (RPB-114C) were kindly provided by Dr. H-M. Shin (Kyoto University). Anti-GM130 (clone 35) and calnexin (clone 37) were from BD Transduction Laboratories (Franklin Lakes, NJ). Anti-HA mAb (HA.11) was from Covance (Berkeley, CA) and anti-PA pAb (y-11) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG mAb (M2) and anti-PDI pAb (P7496) were from Sigma. The following labeled probes were used to detect signals: anti-rat IgM (phycoerythrin, Rockland), streptavidin (phycoerythrin, Caltag), anti-mouse IgG1 (Alexa 488, Invitrogen), anti-rabbit IgG (Alexa 568, Invitrogen, or HRP, DAKO), and anti-mouse IgG (HRP, Zymed Laboratories Inc.).

**Flow Cytometry (FCM)**—Approximately 5 × 10^6 cells in 100 μl of FACS buffer (1% BSA and 0.1% NaN₃ in PBS(−)) were incubated with anti-glycan probes at room temperature for 1 h. Secondary staining was carried out for 30 min. Data were obtained using FACSscan (BD Bioscience) and analyzed using Flowjo software (Tristar, San Carlos, CA). For cross-comparison of the staining signal among cell lines with different autofluorescence, the mean florescence intensity (MFI) of background staining was roughly adjusted to MFI = 10 and the relative staining signal was expressed as a ratio of staining MFI divided by control MFI as previously reported (29).

**Statistical Analyses of Glycan Expression Profiles**—Other than the use of different glycan-binding probes, the correlation index analyses involved in the Cires procedure, which included a systematic comparison of the relative profiles of gene expression obtained by cDNA microarray and glycan expression obtained by FCM in six cell lines, were essentially the same as those previously reported (29, 30). A full list of the gene expression profiles used to estimate Pearson correlation coefficients is available as a supplemental table in previous reports (29, 30).

**Retrovirus-mediated Gene Transfer**—MSCVs carrying the intended glycosyltransferases were prepared by transient transfection of the modified MSCV vector, pMSCV-IRES-EGFP, which bicistronically encodes an enhanced green fluorescent protein (EGFP) open reading frame under control of an internal ribosomal entry site. Vectors were transiently transfected into a Plat-A packaging cell line (31) and culture supernatant was filtered and used for spin infection. Infected cells were cultured for 2 weeks to stabilize expression and stained with anti-glycan probe, whereby a GFP-positive population was regarded as containing gene-transferred cells and GFP-negative cells were considered controls. Thus, a mixture of both populations was stained in a single tube for two-color FCM. The resultant MFI for phycoerythrin was used as an indication of the cell-surface expression of the glycan epitope. Staining of the GFP-negative population was monitored to confirm that it was similar to that of non-infected/vector-infected cells. This method ensured reliable staining with anti-glycan probes to evaluate quantitative differences in glycan expression.
Genetic Dominance to Regulate Biosynthetic Branching

Thin Layer Chromatographic Analysis of GSLs—The GFP-positive population of retrovirus-infected cells was sorted by FACS AriII (BD Biosciences) to enrich glycosyltransferase-expressing cells. Cellular GSLs were isolated as previously reported (32). In short, neutral GSLs contained in the lower phase of the Folch partition were per-acetylated and purified with a Florisil column, whereas the acidic GSL fraction was prepared by repeated partitioning with the salt-containing upper phase of the Folch partition. GSL fractions equivalent to 1 mg of cellular protein were applied to a silica-based TLC plate and separated with a solvent (chloroform, methanol, 0.2% CaCl₂, 60:35:8). Orcinol was used for the visualization of sugar moieties in GSLs and charring was carried out to gain sensitivity for the detection after orcinol staining (33). For detection of GM1, 670 ng/ml of biotinylated CTXb was overlaid and the signal was detected with Konica Immunostain HRP-1000 (Seikagaku). This system could detect roughly 30 ng of GM1 on TLC plate.

Immunofluorescent Detection of Glycosyltransferase Localization—COS-7 cells were grown on glass coverslips and transiently transfected with C-terminal HA-tagged glycosyltransferases harboring the CMV promoter for expression with Lipofectamine reagent (Invitrogen). Cells were harvested 30 h after transfection to avoid overexpression of glycosyltransferases. Cells were fixed with methanol at -20 °C for 10 min. Slips were blocked with 5% BSA, 0.05% Tween 20 in TBS and stained with anti-HA tag antibody (HA.11) and organelle markers. Fluorescent images were obtained using an inverted microscope (IX70; Olympus, ×100 objective lens) equipped with a CCD camera (Cool SNAP HQ/OL: Photometrics) and Methamolph software (Olympus, Tokyo, Japan). The obtained images were subsequently processed with Photoshop software (Adobe). To avoid overexpression-induced mislocalization of glycosyltransferase, cells with moderate HA.11 signals were chosen to obtain images, although strongly stained cells also showed essentially the same staining pattern 30 h post-transfection. The use of COS-7 cells resulted in a more defined intracellular localization of the Golgi-localized enzyme when compared with that of CHO-K1, HeLa, or HT1080 cells. Thus, the images of transfected COS-7 cells were analyzed in detail.

Transfection and Co-immunoprecipitation of Glycosyltransferases—LacCerSyn–FLAG was transiently co-transfected with CD77Syn–HA or GM3Syn into COS-7 cells using Lipofectamine (Invitrogen). Cells were harvested 48 h after transfection by trypsinization. Cell pellets were washed with PBS and lysed with sonication in TDE lysis buffer (50 mM Tris-HCl, pH 7.6, 1 mM DTT, 1 mM EDTA) containing protease inhibitor mixture (Nacalai Tesque). Post-nuclear supernatants were ultracentrifuged (55,000 rpm for 30 min) in MLA-130 rotor (Beckman) and the resultant pellets were sonicated in TL buffer (1% Triton X-100, 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA) containing a protease inhibitor mixture to extract the membrane fraction of the cells. The ultracentrifuge supernatants of these membrane extracts were used as membrane fractions. These were immunoprecipitated with either HA.11 (anti HA antibody) or M2 (anti-FLAG antibody) and Protein G-Sepharose (Amersham Biosciences, Uppsala, Sweden), and co-immunoprecipitates were detected by immunoblotting with M2 or γ-11, respectively. To assess competition between GM3Syn–HA and non-tagged CD77Syn/CD77Syn–TXT in complex formation with LacCerSyn–FLAG, the respective genes were successively introduced into CHO-K1 cells. CHO-K1 cells were employed because this cell line tends to stably hold introduced transgenes. To ensure polyclonality of the cells, a double transfectant clone (expressing LacCerSyn–FLAG and GM3Syn–HA) of CHO-K1 cells (selected with G418 and blastcandin) was transfected with the third constructs and polyclonally selected with zeocin.

Metabolic Labeling of LacCerSyn–FLAG—The polyclonal triple stable cells described above were metabolically pulse labeled with [35S]cysteine and methionine (EXPRESS35S) Protein labeling mix, PerkinElmer Life Sciences) for 30 min and chased in the medium containing enriched cold cysteine and methionine for the indicated times. Cells were recovered with trypsinization and frozen in a deep freezer as cell packs. Cells were lysed with Nonidet P-40/Triton X-100 (1% each) containing lysis buffer and immunoprecipitated with M2 anti-FLAG antibody. The amounts of cell lysate used for immunoprecipitation were adjusted according to the size of the cell pack. Immunoprecipitated samples were subjected to 10% SDS-PAGE and the signals of the visualized bands were quantified with BAS2500 (Fujifilm, Tokyo) after the gels were dried.

RESULTS
Dominant Effect of CD77Syn on GSL Expression in B Cells—Within the biosynthetic pathway of GSLs, LacCer can be used by a variety of enzymes to give rise to various series of GSLs. Therefore, this branching point in the pathway could be interpreted as the point of lineage commitment to specific biosynthetic pathways (Fig. 1A). CIRES may be useful for understanding the regulation of glycan biosynthesis at pathway branching positions given its ability to identify regulatory enzyme genes even when the enzyme reaction is not directly involved in the formation of the glycan in question (29). The B subunits of two bacterial toxins, the shiga toxin and cholera toxin, were employed to probe CD77 and GM1, respectively, in FCM. When the relative expression of a panel of six B cell lines was compared, the staining profiles of CD77 and GM1 were distinct. CD77 was strongly expressed in the germinal center-like B lymphomas Daudi, Ramos, and Raji (Fig. 2A), whereas prominent expression of GM1 was found only in Namalwa cells (Fig. 2A) that lacked expression of CD77. Given that the shiga toxin B subunit and the anti-CD77 antibody showed identical staining patterns for these cell lines, we used anti-CD77 for the remainder of the study. The GSL expression profiles were compared with the gene expression profiles for glycan-related genes in identical sets of cell lines. The expression profile of the CD77Syn (A4GALT) gene positively correlated (r = 0.83) with that of CD77 and negatively correlated with that of GM1 (r = -0.75; Fig. 2B and supplemental Table S1). In contrast, none of the gene expression profiles for GM3Syn (ST3GAL5), GM2 synthase (B4GALNT4), or GM1 synthase (B3GALT4) exhibited significant positive correlations, although these are genes whose products are involved in actual GM1 biosynthesis. A full list of the correlated glycan-related genes is provided under supplemental Table S1. Here, we focus on the presence of the...
Genetic Dominance to Regulate Biosynthetic Branching

CD77Syn Expression Determines the Expression of GSL Species—First, we hypothesized that the CD77Syn reaction would dominate at the branching point using LacCer. Consistent with this hypothesis, retrovirus-mediated ectopic expression of CD77Syn changed the expression profile of GSL in Namalwa cells, where induction of CD77 (Fig. 2C) and concomitant reduction of GM1 was found in FCM (Fig. 2D). In this assay, we utilized a vector in which EGFP is conjugated with an internal ribosomal entry site to monitor glycosyltransferase gene expression, which provided an internal staining control in the same tube. Moreover, when GFP-positive populations were pooled by a cell sorter and their GSL fractions were analyzed by thin layer chromatography (TLC), prominent induction of CD77 and reduction of GM3 were observed upon CD77Syn expression (Fig. 2E). When CTxB was overlaid, a reduction in the GM1 band was also observed (Fig. 2F). These results indicate that CD77Syn controls a global change in the GSL lineage. In contrast to the Namalwa cells, the introduction of CD77Syn to Ramos cells had a minimal effect on GSL biosynthesis (Fig. 2F). We reasoned that strong endogenous expression of CD77Syn (Fig. 2B) could be responsible for the lack of effect in Ramos cells because the total flow of GSL biosynthesis is less likely to be changed by further introduction of CD77Syn.

Minor Contribution of Non-correlated Enzyme Genes in GSL Expression—To determine the regulatory competence of other glycosyltransferases on the GSL biosynthetic pathway (Fig. 1A), we also introduced genes encoding synthases for GlcCer, LacCer, GM3, GM2, GM1, and Lc3 to Namalwa and Ramos cells (Fig. 3, A–D). GM3Syn may occur in alternatively spliced forms (34). Because RT-PCR analysis showed that both Namalwa and Ramos cells expressed the longer isoform of GM3Syn, we used the cDNA of this isoform for further examinations. Consistent with the correlation results, introduction of the CD77Syn gene had the most prominent effect on the expression of both GM1 and CD77 in Namalwa cells (Fig. 3, A and B). In Ramos cells, in which CD77Syn is endogenously expressed, overexpression of GlcCer synthase had the most prominent effect; i.e. a greater than 6-fold increase in CD77 expression was detected without altering the GM1 expression, indicating that GlcCer synthase determines the total flow of GSL biosynthesis in Ramos cells (Fig. 3C). The GSL fraction of the GlcCer synthase-infected GFP-positive pool also exhibited clear induction of CD77 by TLC analysis, whereby a subtle increase in GlcCer but not LacCer or ganglio series GSL was noted (Fig. 3E). This was consistent with the hypothesis that LacCer utilization can be determined by the expression of CD77Syn. In Ramos cells, GM3Syn increased the expression of GM1 up to 4-fold (Fig. 3D). Thus, the abundant expression of GM3Syn was somewhat able to overcome dominance to use LacCer for ganglio series GSLs. These results suggest the presence of simple competition among enzymes to utilize LacCer at the biosynthetic branch. However, previous reports have indicated that branching enzymes CD77Syn, Lc3Syn, and GM3Syn exhibit the same order of Km values toward LacCer, although the reports varied in their values, i.e. 54.5 μM for CD77Syn (15), 80 μM for GM3Syn (35), 3.2 μM for GM3Syn, and 8.0 μM for Lc3Syn (36). Moreover, these data may not take into account molecular crowding, another factor to be considered within the Golgi apparatus (37). Therefore, we asked whether enzymatic conversion of LacCer to CD77 is required for CD77Syn to exhibit dominant “activity” to suppress ganglio series expression.

Enzyme Activity-independent Dominance of CD77Syn over Other Enzyme Genes in the Pathway—CD77Syn is a member of the conventional glycosyltransferase family, whose luminal catalytic domain contains a DXD motif for enzyme activity (38–40). We site directly point-mutagenized the DXD motif into TXT to create a catalytically inactive mutant. When the TXT mutant was introduced to Namalwa cells, no induction of CD77 was observed (Fig. 3F), as expected. However, a reduction in
GM1 was still observed (Fig. 3F). To measure the reduction rate between CD77Syn and its TXT mutant, sorted GFP-positive cells were compared via side by side staining. The rate of GM1 reduction was greater in CD77Syn-introduced cells than in TXT mutants (supplemental Fig. S1A). This difference could be due to some degree of catalytic consumption of the LacCer. Alternatively, because the GFP signal, which infers the degree of transgene expression, was greater in CD77Syn cells, the level of protein expression between the two cell types could account for the difference. The expression level of the mutant appeared to be important because the CTxB signal tended to be weaker in the cells exhibiting stronger expression. Cells with the strongest expression were set to 100% (Fig. 3A).

In any case, the observed reduction in GM1 with the TXT mutant provides evidence against the possibility that enzymatic conversion of LacCer to CD77 by CD77Syn overexpression is the primary cause of the dominant effect of CD77Syn in suppressing GM1 expression.

When the TXT mutant was introduced into Ramos cells, which express endogenous CD77Syn, CD77 expression was reduced, indicating that CD77Syn-TXT competes with the endogenous CD77Syn for the intracellular niche (Fig. 3F). Consistent with wild-type CD77Syn expression, the TXT mutant exhibited only subtle inhibition of GM1 in Ramos cells, indicating that the inhibitory effect of wild-type and TXT mutants on GM3 biosynthesis is similar, resulting in no additive effect in CD77Syn-sufficient Ramos cells. Taken together, these results indicate that genetic dominance of CD77Syn was caused by the occupation of a putative intracellular niche; therefore, catalytically inactive TXT mutants are able to exhibit a dominant-negative phenotype. Moreover, these results clearly rule out the notion that the substrate in the Golgi apparatus is distributed evenly, and is thus accessible to downstream enzymes in the pathway. Rather, depending on the CD77Syn, the accessibility of LacCer to GM3Syn appeared to be regulated in the B cells that endogenously regulate GSL expression. To determine whether or not CD77Syn exhibit a dominant function at branching of GSL in different cell types that is not known for GSL alteration, we expressed CD77Syn and CD77Syn-TXT in COS-7 cells expressing GD1b and GM1 as major and minor gangliosides, respectively (41). Non-conjugated GFP was co-transfected to monitor transfected cells in transient expression experiments. GFP positive cells in CD77Syn-expressed cells did not increase CD77 staining, indicating that dominance in the CD77Syn expression could be cell-type specific. CD77Syn-TXT cells showed suppression of GM1 (supplemental Fig. S2),
thus CD77Syn-TXT could alter glycosylation even in the cell type that does not regulate GSL species.

**Subcellular Localization of Branching Enzymes**—To visualize the putative intracellular niche that CD77Syn may be occupying, we compared the intracellular localization of CD77Syn with other GSL synthases related to LacCer in B cells. The localization of these branching glycosyltransferases was not directly compared in the same cells. Because B cell lines have limited cytoplasmic space, we used COS cells, which have been extensively used for examining Golgi retention signaling of glycosyltransferases (42), thus could be useful in this study. The short N-terminal tail may be involved in the determination of intracellular localization of glycosyltransferase, thus C-terminal HA-tagged glycosyltransferase constructs were transiently expressed in COS-7 cells. Attempts to conjugate a larger tag, such as GFP, resulted in the failed expression of glycosyltransferases, probably due to folding problems. To visualize intracellular localization, we compared the localities of tagged enzymes for LacCerSyn, CD77Syn, Lc3Syn, and GM3Syn to conventional markers for the Golgi apparatus, giantin, or GM130, and the endoplasmic reticulum, calnexin, or peptidyl disulfide isomerase (PDI) in co-staining experiments (Fig. 4, A–F). In B lymphoma cells, among 1–4-galactosyltransferases reported to biosynthesize LacCer, B4GALT6 rather than B4GALT5 (43) was prominently expressed by the cDNA microarray (29), and shRNA-mediated knockdown of B4GALT5 did not alter GSL expression. These results are similar to those previously reported in knock-out mouse liver cells (44). Thus, B4GALT6 was used for subsequent experiments. This assay showed that LacCerSyn-HA and CD77Syn-HA were primarily localized to giantin-positive compartments, likely the Golgi apparatus (Fig. 4, C–F). In contrast, Lc3Syn-HA were primarily localized to the endoplasmic reticulum, as judged from co-localization with PDI (Fig. 4, G and H). GM3Syn-HA showed widespread localization, with partial co-localization with both PDI and giantin (Fig. 4, I and J). Golgi localization could be key for biosynthetic branching, as the degree of Golgi localization was in good correlation with the effect of GSL biosynthetic dominance in the retrovirus-mediated gene transfer experiment; i.e., the efficiency was similar to that of CD77Syn (exhibited 88% reduction in GM1 in Namalwa cells), GM3Syn (exhibited 42% reduction in CD77 in Ramos cells), and Lc3Syn (exhibited no reduction; Fig. 3, B and C). In any case, in the putative intracellular niche, CD77Syn seemed to localize closer to LacCerSyn. However, this was not sufficiently conclusive evidence for the dominance of

![Figure 3. Effect of overexpression of relevant enzymes in the pathway on the cell surface expression of CD77 or GM1.](image-url)

**FIGURE 3. Effect of overexpression of relevant enzymes in the pathway on the cell surface expression of CD77 or GM1.** A–D, expression of CD77 (A and C) or CTxB epitope (B and D) on Namalwa (A and B) or Ramos (C and D) cells infected with various glycosyltransferase genes. The log-scaled signal strength (MFI) in staining was plotted for the indicated glycosyltransferases. Probe(-) indicates the background level of fluorescence detected for the fluorochrome-conjugated secondary probe alone. E, GSL profile analysis of GlcCerSyn-infected Ramos cells on TLC. A GFP-positive population was sorted for vector control (Vector) and GlcCerSyn (GCS)-infected Ramos cells. TLC was carried out as described in the legend to Fig. 2E and GSLs were visualized with orcinol staining. U and L indicate the upper and lower fractions from Folch partitioning, respectively. The standard GSL (Std) used are indicated on the left. Each lane contains a GSL fraction from cells corresponding to 0.65 mg of protein. F, effect of the CD77Syn mutant on GSL expression. A retrovirus vector for CD77Syn-TXT was prepared and used to infect Namalwa or Ramos cells as described in the legend to Fig. 2C. Expression of GSL was monitored with FCM using anti-CD77 or CTxB. Dashed lines indicate the negative control of the staining and bold gray lines indicate probe staining with control virus infection. Thin lines indicate probe staining of infected cells with retrovirus encoding CD77Syn-TXT.
Genetic Dominance to Regulate Biosynthetic Branching

CD77Syn given that GM3Syn could also co-localize with LacCerSyn in the Golgi apparatus (Fig. 4I), indicating that compartmentalization is not completely rigid.

Complex Formation of CD77Syn with LacCerSyn—It was previously reported that LacCerSyn and GM3Syn are co-complexed, and this complex formation is offered as evidence for the efficient conversion of LacCer to GM3, as this physical interaction would ease the process of channeling the substrate from one to the other (20). However, it was not clear how CD77Syn fit into this process, because, at least genetically, the association could ease the process of channeling the substrate and GM3Syn function primarily in the same compartment of the Golgi apparatus can be differentially sorted into their own destinations in relationship to glycosylation (45). We hypothesized that ganglio series and globo series GSL biosynthesis could be distinctly functionally compartmentalized to explain the above findings. Alternatively, if CD77Syn and GM3Syn function primarily in the same compartment of the Golgi apparatus, LacCerSyn should be accessible to both of the distal enzymes. Therefore, one could expect competition between complexes for LacCerSyn. To examine these possibilities, we tested whether CD77Syn could dominantly form a complex with LacCerSyn. To examine these possibilities, we tested whether CD77Syn could dominantly form a complex with LacCerSyn.

Possible Compartmentalization of CD77Syn and GM3Syn to Utilize the LacCerSyn Complex—Because both CD77Syn and GM3Syn can localize to the Golgi apparatus and form complexes with LacCerSyn, an important question is whether LacCerSyn is freely accessible to these distal enzymes. Cargo proteins that traverse the Golgi apparatus can be differentially sorted into their own destinations in relationship to glycosylation (45). We hypothesized that ganglio series and globo series GSL biosynthesis could be distinctly functionally compartmentalized to explain the above findings. Alternatively, if CD77Syn and GM3Syn function primarily in the same compartment of the Golgi apparatus, LacCerSyn should be accessible to both of the distal enzymes. Therefore, one could expect competition between complexes for LacCerSyn. To examine these possibilities, we tested whether CD77Syn could dominantly form a complex with LacCerSyn to out-compete GM3Syn, resulting in dominance in product formation. We first developed a double transfectant cell line expressing both LacCerSyn-FLAG and GM3Syn-HA, which form an intra-Golgi complex (Fig. 6A). We then polyclonally introduced CD77Syn or CD77Syn-TXT and compared them to a control vector to assess changes in complex formation between LacCerSyn and GM3Syn. Expression of CD77Syn and CD77Syn-TXT both resulted in reduced Lac-
CerSyn in the membrane fraction used for immunoprecipitation (Fig. 6A), which is somewhat consistent with the reduction in LacCer found in Ramos cells and CD77Syn-expressed Namalwa cells in TLC (Figs. 2E and 3E). Accordingly, GM3Syn-LacCerSyn co-immunoprecipitation was reduced. In contrast, expression of GM3Syn in the membrane fraction did not change, suggesting that the reduction was specific to LacCerSyn. These data indicate that LacCerSyn was not freely available.
to GM3Syn when CD77Syn was expressed; thus, CD77Syn and GM3Syn are less likely to compete for LacCerSyn in the same compartment of the Golgi apparatus. Rather, it is likely that CD77Syn acts more proximally to LacCerSyn in the pathway than GM3Syn, possibly in different subcompartments, thus favoring globo series GSL biosynthesis.

Change in the Turnover of LacCerSyn in the Presence of CD77Syn—The apparent loss of LacCerSyn upon CD77Syn expression (Fig. 6A) indicated possible functional compartmentalization between biosynthetic enzymes for the globo and ganglio series. Yet, when immunofluorescent signals were compared, neither GM3Syn nor CD77Syn seemed to localize to the specified sub-Golgi structure, given that they were both co-localized with the authentic Golgi marker giantin, indicating that such putative compartmentalization may not be seen at the microscopic level even if it exists. As an alternative approach, we focused on LacCerSyn turnover given the apparent loss of LacCerSyn, because a considerable number of reports have demonstrated that changes in the sorting of a protein may alter its turnover (46–49). Moreover, the sorting of the Golgi glycosyltransferase could be a regulated event (50). We carried out pulse-chase experiments on the above polyclonal triple stable cells to determine whether the turnover rate of LacCerSyn expression would increase. We pulsed these triple stable cells with [35S]cysteine and methionine, then chased the cells for the indicated amounts of time. The radioactivity of immunoprecipitated LacCerSyn did not differ between controls and CD77Syn—expressing cells immediately after the labeling. However, the reduction rate was greater in CD77Syn—expressing cells as a function of time (Fig. 6B). It was unlikely that CD77Syn expression caused overall acceleration of protein turnover, because the expression of GM3Syn appeared unaffected (Fig. 6A). Taken together, these data indicate that complex formation with CD77Syn may change the turnover rate of LacCerSyn within the cells. These data do not conflict with the notion that expression of CD77Syn may trigger alternate sorting of LacCerSyn to a functional compartment that is distinct from the putative GM3—biosynthesizing compartment. Collectively, we propose that a change in CD77Syn expression in activated B cells may cause the conversion of LacCer by separate mechanisms, namely, the catalytic consumption of LacCer (although it may not be a major cause, as anticipated), complex formation with LacCerSyn to ease substrate transfer from upstream enzymes, and alteration of LacCerSyn turnover to limit LacCerSyn accession to other branching enzymes. Supplemental Fig. S4 illustrates this using a flow model.

DISCUSSION

We used genetic techniques to analyze biosynthetic pathways by applying novel quantitative phenotype-genotype correlation analysis; specifically, we used cell surface GSL expression and glycan-related gene expression to examine phenotype and genotype characteristics, respectively. We focused on genes that showed negative correlations between phenotype and genotype, because such a relationship could represent a novel regulatory mechanism. Subsequent analysis revealed a mechanism explaining the genetic dominance of CD77Syn in GSL regulation at a biosynthetic branching point. The genetic analyses presented here provide novel insights into the architecture of biosynthetic pathway branching regulation. Our data support the possible glycosyltransferase interaction-mediated alteration of functional compartmentalization of enzymes involved in pathway branching.

The Impact of Pathway Branching Control at LacCer in the Regulation of GSL Biosynthesis—It is noteworthy that ectopic expression of CD77Syn and CD77Syn—TXT both resulted in a reduction in LacCerSyn by Western blotting (Fig. 6A). However, being similar to Ramos cells, CD77Syn—introduced Namalwa cells exhibited very strong CD77 expression, even when using charring detection, which mirrors the level of GSLs better than orcinol detection. Therefore, the question remains how CD77Syn could suppress LacCerSyn yet efficiently produce CD77.

We found that only CD77Syn—sufficient Ramos cells induced glove series—skewed expression by overexpression of GlcCerSyn (Fig. 3), thus the quantity of GlcCerSyn, not LacCerSyn is likely to control the total flow rate of the GSL biosynthesis in CD77Syn—sufficient cells. Nevertheless, regulation of the GSL species seemed to take place at the LacCer branching point. Therefore, we concentrated on branching regulation to address the above question. One clue was the lack of LacCer accumulation upon expression of CD77Syn—TXT (Fig. 5D). When CD77Syn forms a physical complex with LacCerSyn, CD77Syn complexing may limit the free GlcCer accessibility of LacCerSyn. Taken together, we propose a feasible possibility: induced CD77Syn may compartmentalize LacCerSyn so that ongoing biosynthesis of LacCer is efficiently coupled with subsequent branching to CD77 biosynthesis. In such a scenario, the CD77Syn compartment has a shorter turnover of contents, and thus accelerated LacCerSyn turnover is observed (Fig. 6B).

Regardless of the mechanism, CD77Syn appears to play a key role in regulating LacCerSyn during GSL expression in B cells, where activation-dependent CD77 biosynthesis is potentiated (supplemental Fig. S4). The above mentioned effect of CD77Syn—TXT supports the idea that functional compartmentalization takes place in GSL biosynthesis branching. This may be achieved by the glycosyltransferase—resident protein interaction (23). More recently, Yamaji et al. (51) reported that induced expression of transmembrane BAX inhibitor motif—containing family proteins could suppress CD77Syn. Thus, CD77Syn activity at the branching point can be dependent on the microenvironment of the Golgi, which would include the expression of BAX inhibitor motif—containing family proteins or other unidentified protein(s). Expression profiles of these proteins may determine cell—type specificity in GSL expression. What is needed for a more precise understanding of this putative compartmentalization model is mutant B cells that lack the ganglio series suppressive activity of CD77Syn. Such synthetic biological analysis of a rescuing CD77Syn—TXT mutant would provide information with regards to the putative molecule responsible for compartmentalization. We are currently attempting to isolate such mutant cells for this purpose.

Global Change in GSL to Globo Series—The characterizations in this study indicated that CD77Syn is a good candidate enzyme to globally change the expression of the GSL species at LacCer branching (10, 52), limiting the expression of LacCerSyn available.
to other pathways. Thus, induction of globo series GSL and a concomitant reduction in ganglio series GSL could be regulated by transcriptional activation of CD77Syn alone. Expression of CD77 is specific to the germinal center B cells in secondary lymphoid organs, thus CD77 has been utilized to mark germinal center cells such as centroblasts. Indeed, induction of CD77Syn was reported in in vitro-activated human B cells in a microarray study (53). Thus, the above mechanism appears to be responsible for GSL expression in activated B cells during human germinal center reactions. Similarly, CD77-negative (and ganglio series GSL-expressing) M1 cells can be converted into a strongly CD77-positive state in 72 h in response to differentiation to macrophagic cells (54). Such a drastic change in the GSL profile could be explained by the function of CD77Syn to control LacCerSyn. The physiological/functional meaning of such global GSL conversion in each specific cell type is an important issue to be examined in the future, and which we are currently pursuing using manipulated cells only with the regulatory enzyme(s) identified.

Advantages of the CIRES Technique for Genetic Screening at Branching Points—CIRES is a novel method for phenotype-genotype analysis that utilizes the quantitative cellular phenotype to determine the gene(s) regulating the strength of the phenotype as modulator(s) of the system (29, 55). In the present study, we applied CIRES to quantitatively evaluate differences in GSL expression. From correlation analysis and overexpression experiments, we first hypothesized that the biosynthetic pathway branches for the utilization of LacCer two steps upstream of the GM1 synthase reaction, which could be a key step for GM1 biosynthesis, and that this branching is regulated by CD77Syn, although this enzyme is not in the biosynthetic pathway of GM1. This hypothesis was based on the observed negative correlation, and was a key step in identifying the presence of transferase activity-independent complex formation of LacCerSyn with CD77Syn and changes in LacCerSyn turnover by CD77Syn. To the best of our knowledge, CIRES is the only systematic approach for determining such negative relationships between enzymes, and thus could be an especially powerful tool for the identification of regulatory enzymes in highly branched glycan biosynthesis pathways, where a bonafide regulatory enzyme may not be directly mapped within the biosynthetic pathway of the glycan (e.g. the CD77Syn reaction for GM1 expression). Such regulatory enzyme step mapping should be combined with the introduction of “dominant-negative” glycosyltransferase, rather than knocking down the gene, to informatively analyze the regulation. We strongly recommend this strategy, especially when a negative correlation is found via profile analyses.

Acknowledgments—We thank Dr. Hisashi Narimatsu and Dr. Takashi Sato (National Institute of Advanced Industrial Science and Technology) for providing full-length cDNA clones of glycosyltransferases, Dr. Yasuyuki Imai (University of Shizuoka) for shiga-like toxin B subunit, and Dr. Toshio Kitamura (University of Tokyo) for Plat-A retrovirus packaging cells.

REFERENCES
1. Roseman, S. (1970) Chem. Phys. Lipids 5, 270–297
2. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
3. van Echten, G., and Sandhoff, K. (1993) J. Biol. Chem. 268, 5341–5344
4. Young, W. W., Jr. (2004) J. Membr. Biol. 198, 1–13
5. Schachter, H. (2000) Glycobiol. 17, 465–483
6. Roseman, S. (2001) J. Biol. Chem. 276, 41527–41542
7. Kolter, T., Proia, R. L., and Sandhoff, K. (2002) J. Biol. Chem. 277, 25859–25862
8. Varki, A., Esko, J. D., and Colley, K. J. (2009) in Essentials of Glycobiology (Varki, A., Cummings, R. D., Esko, J. D., Reeve, H. Z., Stanley, H. P., Bertozzi, C. R., Hart, G. W., and Etzler, E. E., eds) 2nd Ed., Chapter 3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
9. Hakomori, S. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 10231–10233
10. Lannert, H., Gorgas, K., Meissner, I., Wieland, F. T., and Jeckel, D. (1998) J. Biol. Chem. 273, 2939–2946
11. Ichikawa, S., and Hirabayashi, Y. (1998) Trends Cell Biol. 8, 198–202
12. D’Angelo, G., Polishchuk, E., Di Tullio, G., Santoro, M., Di Campli, A., Godi, A., West, G., Bielawski, J., Chuang, C. C., van der Spoel, A. C., Platt, F. M., Hannun, Y. A., Polishchuk, R., Mattijss, P., and De Matteis, M. A. (2007) Nature 449, 62–67
13. Lala, P., Itou, S., and Lingwood, C. (2000) J. Biol. Chem. 275, 6246–6251
14. Nomura, T., Takizawa, M., Aoki, J., Arai, H., Inoue, K., Watanabe, M., Yoshizuka, N., Imokawa, G., Dohmae, N., Takio, K., Hattori, M., and Matsumo, N. (1998) J. Biol. Chem. 273, 13570–13577
15. Kojima, Y., Fukushima, K., Okajima, T., Takeda, M., Yocupi, K., Suzuki, Y., Urano, T., Ohta, M., and Furukawa, K. (2000) J. Biol. Chem. 275, 15152–15156
16. Keusch, J. J., Manzella, S. M., Nyame, K. A., Cummings, R. D., and Baeniger, J. U. (2000) J. Biol. Chem. 275, 25315–25321
17. Togayachi, A., Akashima, T., Okubo, R., Kudo, T., Nishihara, S., Iwaki, H., Natsume, A., Mio, H., Inokuchi, J., Irimura, T., Sasaki, K., and Narimatsu, H. (2001) J. Biol. Chem. 276, 22032–22040
18. Ishii, A., Ohta, M., Watanabe, Y., Matsuda, K., Ishiyama, K., Sakoe, K., Nakamura, M., Inokuchi, J., Sanai, Y., and Saito, M. (1998) J. Biol. Chem. 273, 31652–31655
19. Levine, T. P. (2007) J. Cell Biol. 179, 11–13
20. Giraud, C. G., Daniotti, J. L., and Maccioni, H. J. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 1625–1630
21. Giraud, C. G., and Maccioni, H. J. (2003) J. Biol. Chem. 278, 40262–40271
22. Uliana, A. S., Crespo, P. M., Martina, J. A., Daniotti, J. L., and Maccioni, H. J. (2006) J. Biol. Chem. 281, 32852–32860
23. Quintero, C. A., Valdez-Taubs, J., Ferrari, M. L., Haedo, S. D., and Maccioni, H. J. (2008) Biochem. J. 412, 19–26
24. Schwartz-Albiez, R., Dörken, B., Müller, P., Brodin, N. T., Monner, D. A., and Kniep, B. (1990) Int. Immunol. 2, 929–936
25. Jacewicz, M., Clausen, H., Nudelman, E., Donohue-Rolfé, A., and Keusch, G. T. (1986) J. Exp. Med. 163, 1391–1404
26. Fishman, P. H., Moss, J., and Osborne, J. C., Jr. (1978) Biochemistry 17, 711–716
27. Connell, T. D., and Holmes, R. K. (1995) Mol. Microbiol. 16, 21–31
28. Barton, N. H., and Keightley, P. D. (2002) Nat. Rev. Genet. 3, 11–21
29. Yamamoto, H., Takemitsu, H., Fujinawa, R., Naito, Y., Okuno, Y., Tsujiimoto, G., Suzuki, A., and Kozutsumi, Y. (2007) PLoS One 2, e1232
30. Naito, Y., Takemitsu, H., Koyama, S., Miyake, S., Yamamoto, H., Fujinawa, R., Sugai, M., Okuno, Y., Tsujiimoto, G., Yamaji, T., Hashimoto, Y., Itohara, S., Kawasaki, T., Suzuki, A., and Kozutsumi, Y. (2007) Mol. Cell. Biol. 27, 3008–3022
31. Morita, S., Kojima, T., and Kozutsumi, T. (2000) Gene Ther. 7, 1063–1066
32. Saito, T., and Hakomori, S. I. (1971) J. Lipid Res. 12, 257–259
33. Kyogashima, M., Tamiya-Koizumi, K., Ehara, T., Li, G., Hu, R., Hara, A., Aoyama, T., and Kannagi, R. (2006) Glycobiology 16, 719–728
34. Uemura, S., Yoshida, S., Shishido, F., and Inokuchi, J. (2009) Mol. Biol. Cell 20, 3088–3100
35. Preuss, U., Gu, X., Gu, T., and Yu, R. K. (1993) J. Biol. Chem. 268, 26273–26278
Genetic Dominance to Regulate Biosynthetic Branching

36. Nakamura, M., Tsunoda, A., Sakoe, K., Gu, J., Nishikawa, A., Taniguchi, N., and Saito, M. (1992) J. Biol. Chem. 267, 23507–23514
37. Weiss, M., and Nilsson, T. (2003) Traffic 4, 65–73
38. Breton, C., Bettler, E., Joziassé, D. H., Geremia, R. A., and Imberty, A. (1998) J. Biochem. 123, 1000–1009
39. Munro, S., and Freeman, M. (2000) Curr. Biol. 10, 813–820
40. Li, J., Rancour, D. M., Allende, M. L., Worth, C. A., Darling, D. S., Gilbert, J. B., Menon, A. K., and Young, W. W., Jr. (2001) Glycobiology 11, 217–229
41. Anastasia, L., Holguera, J., Bianchi, A., D’Avila, F., Papini, N., Tringali, C., Monti, E., Villar, E., Venerando, B., Muñoz-Barroso, I., and Tettamanti, G. (2008) Biochim. Biophys. Acta 1780, 504–512
42. Colley, K. J. (1997) Glycobiology 7, 1–13
43. Kumagai, T., Sato, T., Natsuka, S., Kobayashi, Y., Zhou, D., Shinkai, T., Hayakawa, S., and Furukawa, K. (2009) Biochem. Biophys. Res. Commun. 379, 456–459
44. Scheiffele, P., Peränen, J., and Simons, K. (1995) Nature 378, 96–98
45. Stack, J. H., Horazdovsky, B., and Emr, S. D. (1995) Annu. Rev. Cell Dev. Biol. 11, 1–33
46. Schekman, R., and Orci, L. (1996) Science 271, 1526–1533
47. Wendland, B., Emr, S. D., and Riezman, H. (1998) Curr. Opin. Cell Biol. 10, 513–522
48. Klionsky, D. J., and Emr, S. D. (2000) Science 290, 1717–1721
49. Weiss, M., and Nilsson, T. (2000) FEBS Lett. 486, 2–9
50. Yamaji, T., Nishikawa, K., and Hanada, K. (2010) J. Biol. Chem. 285, 35505–35518
51. Allende, M. L., Li, J., Darling, D. S., Worth, C. A., and Young, W. W., Jr. (2000) Glycobiology 10, 1025–1032
52. Shaffer, A. L., Emre, N. C., Lamy, L., Ngo, V. N., Wright, G., Xiao, W., Powell, J., Dave, S., Yu, X., Zhao, H., Zeng, Y., Chen, B., Epstein, J., and Staudt, L. M. (2008) Nature 454, 226–231
53. Kannagi, R., Levery, S. B., and Hakomori, S. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 2844–2848
54. Aderem, A. (2005) Cell 121, 511–513