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Synthesis of fluorescent glycosphingolipids and neoglycoconjugates which contain 6-gala oligosaccharides using the transglycosylation reaction of a novel endoglycoceramidase (EGALC)

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Endoglycoceramidase is a glycohydrolase capable of hydrolysing the *O*-glycosidic linkage between oligosaccharides and ceramides of various glycosphingolipids. However, no endoglycoceramidase reported so far can hydrolyse 6-gala series glycosphingolipids which possess the common structure R-Galβ1-6Galβ1-1'Cer. Recently, we found a novel endoglycoceramidase (endogalactosylceramidase, EGALC) which specifically hydrolyses 6-gala series glycosphingolipids. Here, we report that EGALC catalyses the hydrolysis as well as transglycosylation. An intact sugar chain of neogalatriaosylceramide (Galβ1-6Galβ1-6Galβ1-1'Cer) was found to be transferred by EGALC to a primary hydroxyl group of various alkanols and non-ionic detergents such as Triton X-100 generating corresponding alkyl- and Triton-trigalactooligosaccharides. Furthermore, fluorescent 6-gala series glycosphingolipids were synthesized by transglycosylation in a reaction with EGALC using fluorescent ceramides as acceptors. Because of high efficiency and broad acceptor specificity, EGALC would facilitate the synthesis of fluorescent glycosphingolipids and neoglycoconjugates which contain 6-gala oligosaccharides.

Key words: endoglycoceramidase, endogalactosylceramidase (EGALC), fluorescent glycosphingolipid, 6-gala series glycosphingolipid, transglycosylation.

Abbreviations: BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; CDS, ceramide disaccharide, Galβ1-6Galβ1-1'Cer; CTS, ceramide trisaccharide, Galα1-6Galβ1-6Galβ1-1'Cer; CTeS, ceramide tetrasaccharide, Galα1-6Galα1-6Galβ1-6Galβ1-1'Cer; DHB, 2,5-dihydrobenzoic acid; EGALC, endogalactosylceramidase; EGCase, endoglycoceramidase; GalCer, galactosylceramide; GSLs, glycosphingolipids; 6-gala series GSLs, GSLs containing a R-Galβ1-6Galβ1-1'Cer structure; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; NBD, 7-nitro-2,1,3-benzoxadiazole; TLC, thin-layer chromatography; TGC, trigalactosylceramide (neogalatriaosylceramide), Galβ1-6Galβ1-6Galβ1-1'Cer.

Glycosphingolipids (GSLs), amphipathic compounds consisting of sugar and ceramide moieties, are ubiquitous components of the plasma membrane (*1*). More than 500 species of GSLs differing in structure in the sugar moiety have been found in vertebrates and invertebrates (http://lipidbank.jp). Among them, 6-gala series GSLs possessing the common structure R-Galβ1-6Galβ1-1'Cer have been found in the mollusk (*2*), the leech (*3*), the earthworm (*4*) and some pathogenic cestode parasites (*5–7*). Recently, some anti-fungal reagent-resistant fungi were found to synthesize 6-gala series GSLs instead of inositol phosphoceramide (IPC), the synthesis of which is specifically inhibited by anti-fungal reagents such as aureobasidin A (*8*). Thus the synthetic pathway of 6-gala series GSLs in pathogenic fungi and cestode parasites is a possible target for the development of new anti-fungal/parasite reagents, although the metabolic pathway of 6-gala series GSLs has yet to be elucidated.

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Endoglycoceramidase (EGCase, EC3.2.1.123, also known as ceramide glycanase) is a GSL-specific enzyme that hydrolyses acidic and neutral GSLs to produce intact oligosaccharides and ceramides of various GSLs (*9*, *10*). However, none of the EGCases/ceramide glycanases reported so far were able to degrade 6-gala series GSLs (*11–14*), except Rhodococcal EGCase III (*15*). Recently, we cloned the EGCase III which specifically hydrolyses the internal β1-1'galactosyl linkage between the oligosaccharide and ceramide of 6-gal series GSLs (*16*). Based on its specificity, the enzyme was tentatively designated 'endogalactosylceramidase (EGALC)' (*16*). EGALC belongs to glycohydrolase (GH) family 5, members of which hydrolyse glycosides or glycoconjugates retaining the stereochemistry at the anomeric centre via a double displacement mechanism (http://afmb.cnrs-mrs.fr/CAZY/fam/GH5.html).

Here, we report that EGALC transfers the sugar moiety of neogalatriaosylceramide to the primary hydroxyl group of fluorescent ceramides, various alkanols and non-ionic detergents with high efficiency, generating fluorescent GSLs and neoglycoconjugates which contain 6-gala oligosaccharides. Thus, EGALC will facilitate the study of 6-gala series GSLs.

MATERIALS AND METHODS

Materials—Neogalatriaosylceramide (trigalactosylceramide, TGC, Galβ1-6Galβ1-6Galβ1-1'Cer) was prepared from a turban shell, *Turbo cornutus*, by a method described previously (*2*). The recombinant EGALC and its mutants were expressed in *Escherichia coli* BL21(DE3) and purified as reported previously (*16*). Triton X-100 was purchased from Sigma-Aldrich Co. (USA). A pre-coated Silica gel 60 TLC plate was obtained from Merck (Germany). C6-NBD-Cer was obtained from Matreya Inc. (USA). C5-BODIPY-Cer was obtained from Invitrogen Co. (USA). C12-NBD-Cer was prepared as described in (*17*). CDS, CTS and CTeS from a fungus, *Mucor hiemalis* (*8*), were kindly donated by Dr K. Yamamoto, Kyoto University, Japan. All other reagents were of the highest purity available.

Protein Assay—Protein content was determined by the bicinchoninic acid protein assay (Pierce, USA) with bovine serum albumin as a standard.

Assay of EGALC Activity—The activity of EGALC was measured using TGC as a substrate as described in (*16*). One unit of EGALC was defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol of TGC/min.

Transglycosylation Reaction with EGALC—The transglycosylation reaction with the enzyme was carried out using TGC as a donor substrate and various acceptor substrates as described later. (i) For the reaction using alkanols as an acceptor substrate, the reaction mixture contained 2.5 nmol of TGC and 0.2 mU of the enzyme in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 and 10% 1-alkanols. (ii) For the reaction using Triton X-100 as an acceptor substrate, the reaction mixture contained 2.5 nmol of TGC and 0.1 mU of the enzyme in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% of the detergent. (iii) For the reaction using fluorescent ceramides as acceptors, the reaction mixture contained 2.5 nmol of TGC, an appropriate amount of C6-/C12-NBD-Cer or C5-BODIPY-Cer, and an appropriate amount of the enzyme in 10 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.2% Triton X-100. After incubation at 37°C for the times indicated, the reaction was stopped by heating in a boiling water bath for 5 min. The reaction mixture was evaporated dry, re-dissolved in 10 μl of methanol, and applied to a TLC plate, which was then developed with chloroform/methanol/0.02% $CaCl_2$ (5/4/1, v/v/v). GSLs and transglycosylation products generated were visualized by spraying the TLC plate with orcinol-H_2SO_4 reagent and quantified with a Shimadzu CS-9300 chromatoscanner with the reflection mode set at 540 nm. Ceramide and GSLs labeled with fluorescent were quantified with the fluorescent detector (excitation 475, emission 525 nm).

Purification of Fluorescent TGC—Hundred nanomoles of TGC was incubated with 160 μU of the enzyme in 160 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 in the presence of 8 nmol of C5-BODIPY-Cer, C6- or C12-NBD-Cer. After incubation at 37°C for 1 h, the reaction mixture was dried with a speed vac concentrator, dissolved in 5 ml of methanol/water (1/1, v/v), and then applied to a Sep-Pak plus C18 cartridge (Waters) previously equilibrated with methanol/water (1/1, v/v). Fluorescent Cer and fluorescent TGC were eluted with methanol/water (9/1, v/v), while unlabelled TGC was eluted with methanol. The methanol/water fraction was dried, dissolved in chloroform/methanol (9/1, v/v), and then applied to a Sep-Pak silica cartridge previously equilibrated with chloroform/methanol (9/1, v/v). Fluorescent TGC was eluted from the cartridge with methanol.

Preparation of Samples for MALDI-TOF MS—Transglycosylation products were separated from acceptor and donor substrates on a TLC plate as described earlier. The plate was immersed in a solvent, isopropanol/methanol/0.2% $CaCl_2$ (40/7/20, v/v/v), for 20 s (*18*). The product on the TLC plate was then transferred to a PVDF membrane at 180°C for 30 s using a TLC blotter (Atto Inc., Tokyo, Japan). The part of the membrane corresponding to the area containing the product was cut off and the product was then extracted with 20 μl of 50% (v/v) methanol with sonication for 1 min.

MALDI-TOF MS Analysis—The MALDI-TOF MS analysis was performed by a Voyager-DE mass spectrometer (PE-Biosystems, USA) using 10 mg/ml of 2,5-dihydroxybenzoic acid (DHB, Sigma-Aldrich, USA) dissolved in H_2O/ethanol (9/1, v/v) as a matrix. After the sample was mixed with the matrix on the sample plate, it was dried completely. The operation was conducted in the positive polarity mode and the accelerating voltage was 20 kV.

RESULTS

Transglycosylation Reaction of EGALC for 1-Alkanols as Acceptors—It was reported that jellyfish EGCase catalysed the transglycosylation reaction in which the intact sugar chain was transferred from GM1a to the primary hydroxyl group of various 1-alkanols (*19*). In this study, it was thus examined whether EGALC can catalyse the transglycosylation reaction using TGC as a donor substrate and methanol as an acceptor substrate. Interestingly, an orcinol-H_2SO_4-positive band having a different R_f to that of TGC or TGC-oligosaccharide was observed on the TLC plate when the enzyme was incubated with TGC in the presence of methanol (Fig. 1A, lane 3). This product was not generated when mutant EGALCs whose acid/base catalyst (Glu^{234}) or nucleophile (Glu^{341}) was mutated with Gln were used for the reaction (Fig. 1A, lanes 4 and 5), suggesting that generation of the product was catalysed by the same amino acid residues for the hydrolysis reaction with EGALC. Next, the product of the reaction of TGC with EGALC in the presence or absence of methanol was analysed by MALDI-TOF MS. A pseudo-molecular ion detected at *m/z* 527.8 in the absence of methanol coincided with the sodium adduct, $[M+Na]^+$, of the TGC-oligosaccharide (Gal-Gal-Gal-), while that at *m/z* 541.8 in the presence of methanol may be attributed to methyl-TGC-oligosaccharide (Fig. 1B). The proposed structure of the transglycosylation product from the reaction of TGC with EGALC in the presence of methanol is shown in Fig. 1C.

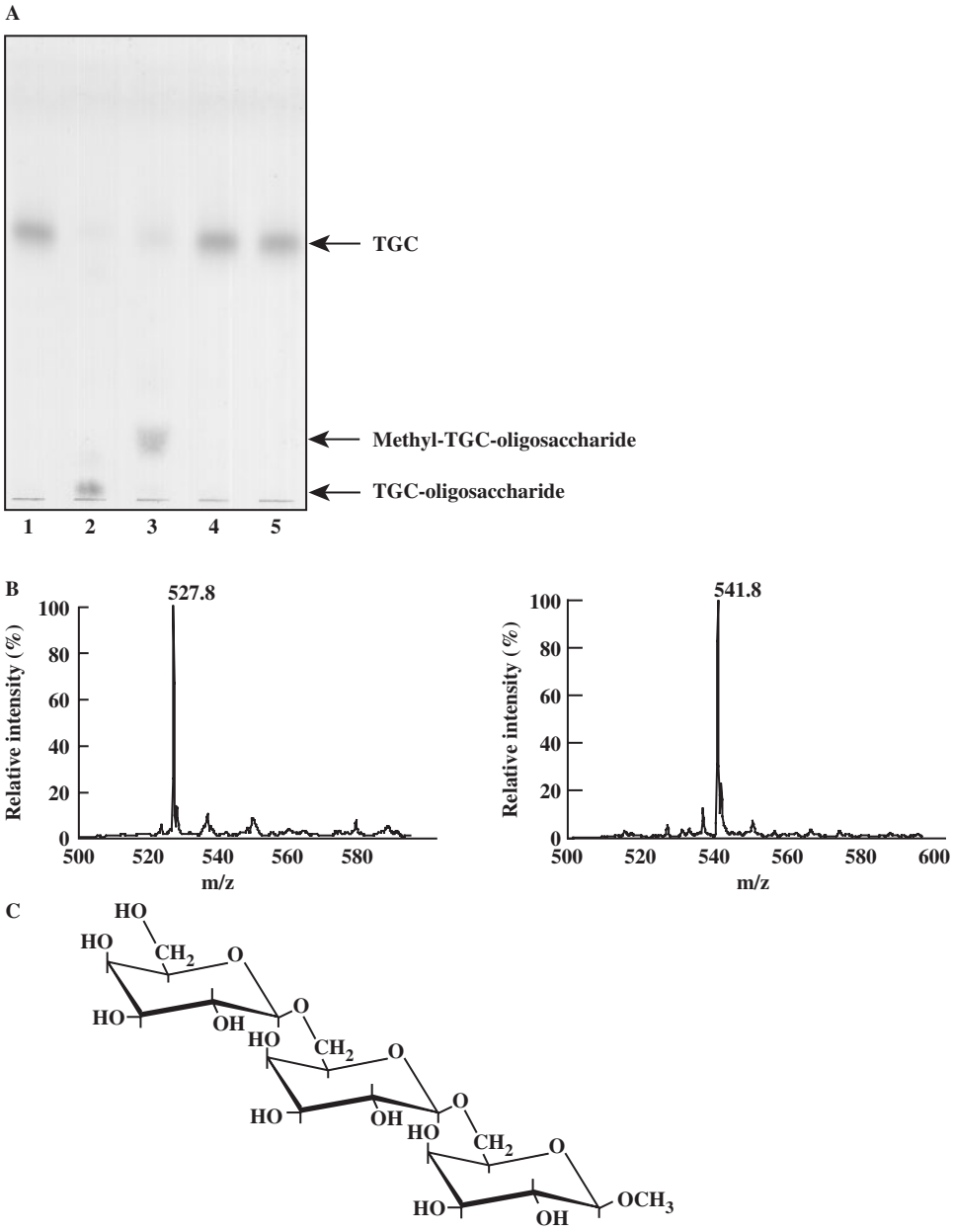

Fig. 1. **Transfer of galactooligosaccharide from TGC to several alkanols by EGALC.** (A) TLC analysis of the transglycosylation product using methanol as an acceptor. The reaction mixture containing 0.1 mU of EGALC or 100 ng of EGALC mutants, 2 nmol of TGC and 10% methanol in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 was incubated at 37°C for 1 h. Lane 1, TGC standard; lane 2, TGC + EGALC; lane 3, TGC + EGALC + methanol; lane 4, TGC + E234Q EGALC mutant + methanol; lane 5, TGC + E341Q EGALC mutant + methanol. (B) MALDI-TOF-MS spectrometric analysis of the galactooligosaccharide obtained from TGC (left) and transglycosylation product from the reaction of TGC with EGALC in the presence of methanol (right). (C) Proposed structure of Methyl-TGC-oligosaccharide.

To examine the acceptor specificity of EGALC, various 1-alkanols and TGC were used for the transglycosylation reaction. As shown in Fig. 2A, new bands having different R_f values were generated which may be attributed to the 1-alkyl-TGC-oligosaccharides corresponding to 1-alkanols used. The acceptor specificity and transfer efficiency of EGALC were quite broad and high, i.e. TGC-oligosaccharide was efficiently transferred from TGC to not only methanol, but also ethanol, 1-propanol, 1-butanol, 1-pentanol and 1-hexanol (Fig. 2A and B). The optimum concentration of all 1-alkanols used was 10% when TGC was used as a donor substrate. However, both transglycosylation and hydrolysis were strongly inhibited by 20% of 1-propanol in the reaction mixture (data not shown).

Transglycosylation Reaction with EGALC Using Detergents as Acceptors—Similar to the reaction in the presence of 1-alkanols, orcinol-H_2SO_4-positive bands

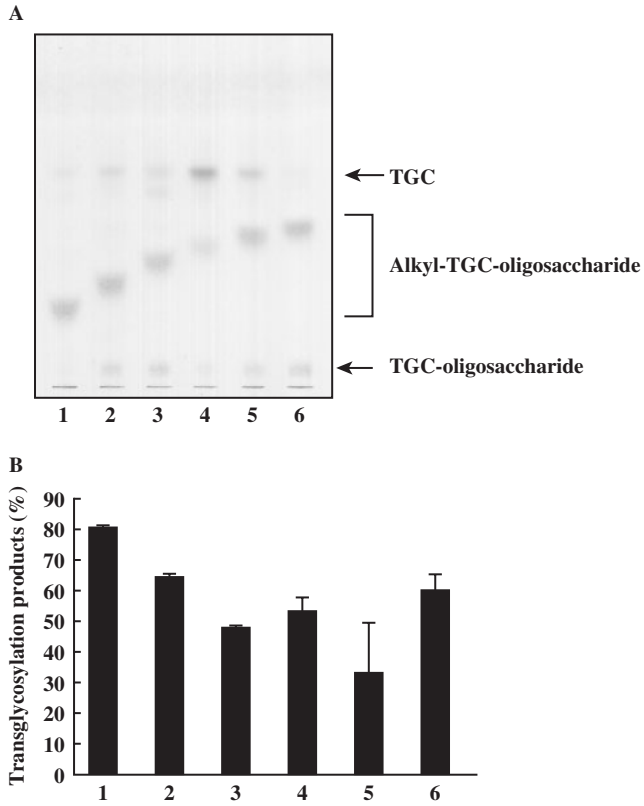

Fig. 2. **The efficiency of the transglycosylation reaction toward 1-alkanols by EGALC.** (A) TLC showing the transglycosylation products from the reaction of TGC with EGALC using various 1-alknols as acceptors. The reaction mixture containing 0.1 mU of EGALC, 2 nmol of TGC and 10% alkanols in 20 µl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 was incubated at 37°C for 1 h. Lane 1, methanol; lane 2, ethanol; lane 3, 1-propanol; lane 4, 1-butanol; lane 5, 1-pentanol; lane 6, 1-hexanol. (B) Quantification of A by TLC chromatoscanner. The extent of transglycosylation was calculated as follows: DR for alkyl-TGC-oligosaccharide × 100/DR for TGC + DR for TGC-oligosaccharide + DR for alkyl-TGC-oligosaccharide, where DR = densitometric response at 540 nm. The results are means of three independent experiments. Lane 1, methanol; lane 2, ethanol; lane 3, 1-propanol; lane 4, 1-butanol; lane 5, 1-pentanol; lane 6, 1-hexanol.

were observed on the TLC plate when TGC was incubated with EGALC (25 µU) in the presence of non-ionic detergents such as Triton X-100 (Fig. 3A, lane 2). On the other hand, ionic detergents such as sodium cholate and taurodexycholic acid strongly inhibited both the transglycosylation and hydrolysis (data not shown). The product from the incubation of TGC with EGALC in the presence of Triton X-100 was purified and then analysed by MALDI-TOF MS. Pseudo-molecular ions at *m/z* 891.7, 935.6, 979.6, 1023.5 and 1067.5 were detected (Fig. 3B), which coincided with the sodium adduct, $[M+Na]^+$, of Gal-Gal-Gal-$C_{14}H_{21}O(C_2H_4O)_4$, Gal-Gal-Gal-$C_{14}H_{21}O(C_2H_4O)_5$, Gal-Gal-Gal-$C_{14}H_{21}O(C_2H_4O)_6$, Gal-Gal-Gal-$C_{14}H_{21}O(C_2H_4O)_7$ and Gal-Gal-Gal-$C_{14}H_{21}O(C_2H_4O)_8$, respectively. These results suggest that EGALC transferred the intact sugar chain from

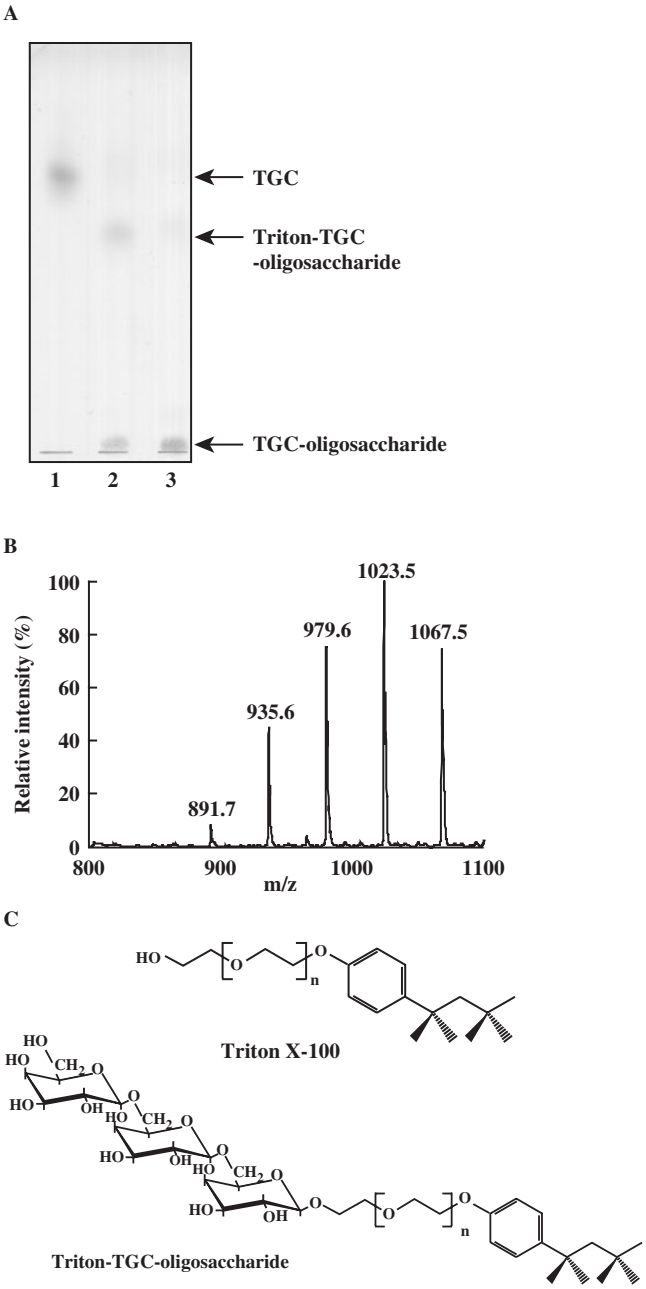

Fig. 3. **Transfer of trigalactooligosaccharide from TGC to Triton X-100 by EGALC.** (A) TLC analysis of transglycosylation products using Triton X-100 as an acceptor. The reaction mixture containing an appropriate amount of EGALC, 2 nmol of TGC and 0.1% (w/v) of Triton X-100 in 20 µl of 50 mM sodium acetate buffer, pH 5.5, was incubated at 37°C for 1 h. Lane 1, TGC standard; lane 2, TGC + 0.1% Triton X-100 + EGALC (25 µU); lane 3, TGC + 0.1% Triton X-100 + EGALC (0.1 mU). The transglycosylation product was indicated as Triton-TGC-oligosaccharide. (B) MALDI-TOF-MS spectrometric analysis of the transglycosylation products. The pseudo-molecular ions, $[M+Na]^+$, were consistent with the molecular mass of the trigalactooligosaccharides attached with Triton X-100 ($n = 4$–8), respectively. (C) Proposed structures of Triton X-100 and Triton-TGC-oligosaccharides.

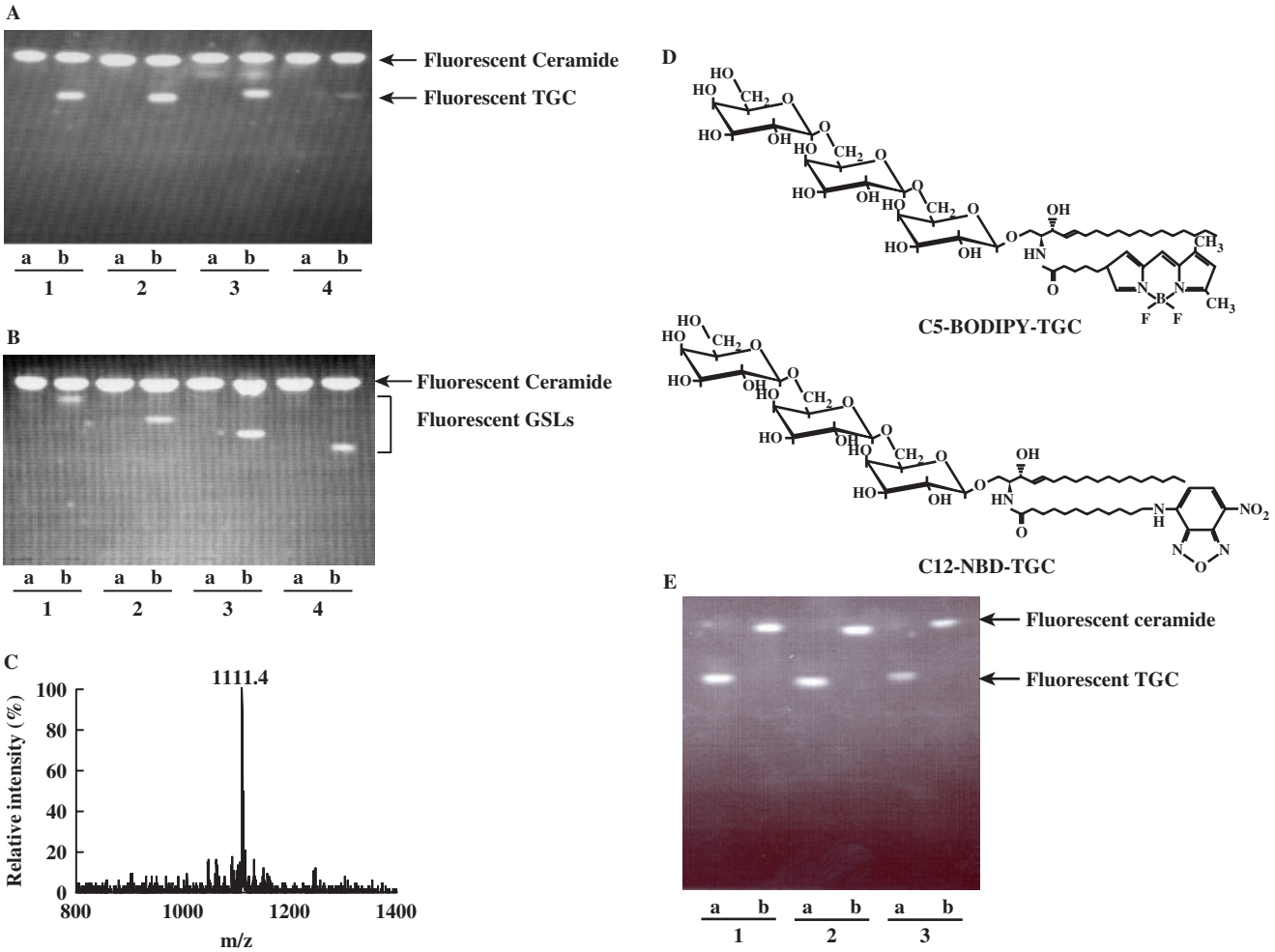

Fig. 4. **Transfer of oligosaccharide from gala-series GSLs to various fluorescent ceramides by EGALC.** (A) TLC analysis of transglycosylation products using various fluorescent ceramides as acceptors. Lane 1, C5-BODIPY-Cer; lane 2, C6-NBD-Cer; lane 3, C12-NBD-Cer; lane 4, C12-NBD-phytoCer. (B) TLC analysis of transglycosylation products using various GSLs as donors. Lane 1, GalCer; lane 2, CDS; lane 3, CTS; lane 4, CTeS. The reaction mixture containing 10 μU of EGALC, 2 nmol of TGC (panel A) or GSLs indicated (panel B) and 500 pmol fluorescent ceramides indicated (panel A) or C6-NBD-Cer (panel B) in 10 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 was incubated at 37°C for 1 h. Incubation was carried out with boiled enzyme (lane a) or with enzyme (lane b). (C) MALDI-TOF MS analysis of the transglycosylation product, C5-BODIPY-TGC. (D) Proposed structure of C5-BODIPY-TGC and C12-NBD-TGC. (E) Hydrolysis of fluorescent TGCs by EGALC. Lane 1, C5-BODIPY-TGC; lane 2, C6-NBD-TGC; lane 3, C12-NBD-TGC. The reaction mixture containing 0.1 mU of EGALC and 20 pmol fluorescent TGCs in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 was incubated at 37°C for 1 h. Incubation was carried out with boiled enzyme (lane a) or with enzyme (lane b).

TGC to the primary hydroxyl group of Triton X-100 ($n = 4$–8) generating Triton X-100-TGC-oligosaccharide as shown in Fig. 3C. However, it should be noted that the generation of Triton X-100-TGC-oligosaccharide disappeared when the amount of EGALC was increased by 4 times (0.1 mU) (Fig. 1A, lane 3), possibly because the product was hydrolysed by EGALC.

Transglycosylation Reaction of EGALC for Fluorescent Ceramides—A fluorescent TGC could be useful for measuring the activities of TGC-metabolizing enzymes. Thus, we tested whether or not fluorescent ceramides are utilized by EGALC in the transglycosylation reaction as an acceptor. As shown in Fig. 4A, fluorescent products were generated in the reaction of EGALC with TGC in the presence of C5-BODIPY-Cer (lane 1b), C6-NBD-Cer (lane 2b), C12-NBD-Cer (lane 3b) and C12-NBD-phytoCer (lane 4b). However, these fluorescent products were not generated in the reaction with boiled EGALC (Fig. 4A, lanes 1a, 2a, 3a and 4a). The generation of fluorescent TGC containing C12-NBD-phytoCer was much slower than that containing other fluorescent ceramide, indicating C12-NBD-phytoCer was a poor acceptor substrate for EGALC under the conditions used. Next, various GSLs were examined as donor substrates for the transglycosylation reaction with EGALC using C6-NBD-Cer as an acceptor. As a result,

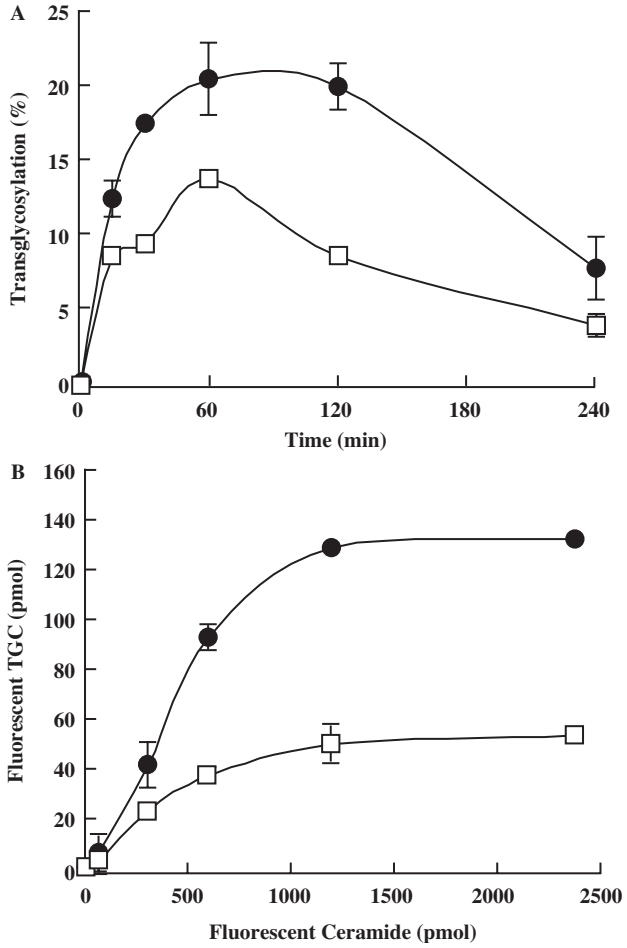

Fig. 5. **Time course of the generation of NBD-TGC by EGALC (A) and effect of the concentration of NBD-Cer on the transglycosylation activity of EGALC (B).** The reaction mixture containing 10 μU of EGALC, 2 nmol of TGC and 500 pmol (A) or the indicated amount (B) of NBD-Cer in 10 μl of 50 mM sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 was incubated at 37°C for the indicated periods (A) or 1 h (B). (A) Closed-circle, C6-NBD-Cer; open square, C12-NBD-Cer. (B) Closed-circle, C6-NBD-Cer; open square, C12-NBD-Cer.

it was found that GalCer (Galβ1-1Cer), CDS (Galβ1-6Galβ1-1'Cer), CTS (Galα1-6Galβ1-6Galβ1-1'Cer) and CTeS (Galα1-6Galα1-6Galβ1-6Galβ1-1'Cer) were good donor substrates for the transglycosylation reaction generating corresponding fluorescent GSLs (Fig. 4B). On the other hand, GlcCer, sulfatide, LacCer, GM1a and Gb3Cer were not utilized as donor substrates by the enzyme (data not shown). These results are well consistent with the specificity of the hydrolytic reaction of EGALC except for GalCer (*16*). The hydrolysis of GalCer by EGALC was very slow in compared to CDS, TGC, CTS and CTeS (*16*), while GalCer was appeared to be a good acceptor for the transglycosylation activity. C6-NBD-GalCer was hydrolysed very slowly and thus the fluorescent GalCer generated was likely to accumulate in the reaction mixture.

The fluorescent product was purified from the reaction of TGC with the enzyme in the presence of C5-BODIPY-Cer and then analysed by MALDI-TOF MS. As a result, a pseudo-molecular ion, $[M+Na]^+$, of C5-BODIPY-TGC was detected at *m/z* 1111.4 (Fig. 4C). The proposed structures of C5-BODIPY-TGC and C12-NBD-TGC are shown in Fig. 4D. Fluorescent ceramides seem to be coupled to the oligosaccharide via a β-, but not α-, galactosidic linkage in the C5-BODIPY-TGC, C6-NBD-TGC and C12-NBD-TGC because the synthesized fluorescent GSLs were completely hydrolysed by EGALC which is an endo-type 1,1′-β-galactohydrolase (EC.3.2.1.123) generating corresponding fluorescent ceramides (Fig. 4E).

Figure 5A shows the time course for the apparent generation of fluorescent TGC using C12-NBD-Cer or C6-NBD-Cer as an acceptor. The apparent generation of C6-NBD-TGC was much faster than that of C12-NBD-TGC. The maximal generation of fluorescent TGC was observed at around 60 min at which time 20 and 13% of the C6-NBD-Cer and C12-NBD-Cer added were converted to the fluorescent TGCs, respectively, under the conditions used. The yield of fluorescent TGC decreased gradually beyond 120 min, possibly due to the hydrolysis of the fluorescent product by EGALC. The effects of the acceptor's concentration on the transglycosylation activity of EGALC are shown in Fig. 5B. The maximal generation of the fluorescent TGCs was found to be ~125 μM for both C6-NBD-Cer and C12-NBD-Cer.

DISCUSSION

EGCase is an endo-type glycohydrolase belonging to GH family 5. Among EGCases reported so far, EGALC is the only enzyme capable of degrading GSLs containing R-Galβ1-1'Cer (Fig. 6A). The three-dimensional model of EGALC and site-directed mutagenesis have clearly demonstrated that Glu-234 at the end of β–strand 4 and Glu-341 at the end of β-strand 7 function as an acid/base catalyst and a nucleophile, respectively (*16*). In this study, these two glutamate residues were also found to be essential for the transglycosylation activity of EGALC (Fig. 1A). Thus, the transglycosylation reaction with EGALC is thought to proceed in the manner shown in Fig. 6B, in which R_1 is a ceramide moiety and R_2 is an acceptor moiety such as alkanols, etc. On the other hand, R_2 is H for the hydrolysis reaction.

In the present study, we found that EGALC could transfer the intact sugar chain of TGC to the primary hydroxyl group of alkanols, non-ionic detergents and fluorescent ceramides. The transglycosylation activity has been also found in EGCases (ceramide glycanase) from the leech (*20*), bacteria (*21*) and jellyfish (*19*). The most striking difference in the transglycosylation reaction between EGALC and other EGCases/ceramide glycanases is in the specificity for donor substrates, i.e. GSLs containing the R-Galβ1-1'Cer can be utilized as a donor substrate by EGALC but not other enzymes. On the other hand, the acceptor specificity of the transglycosylation activity of EGALC was much broader, i.e. not only methanol but also ethanol, 1-propanol,

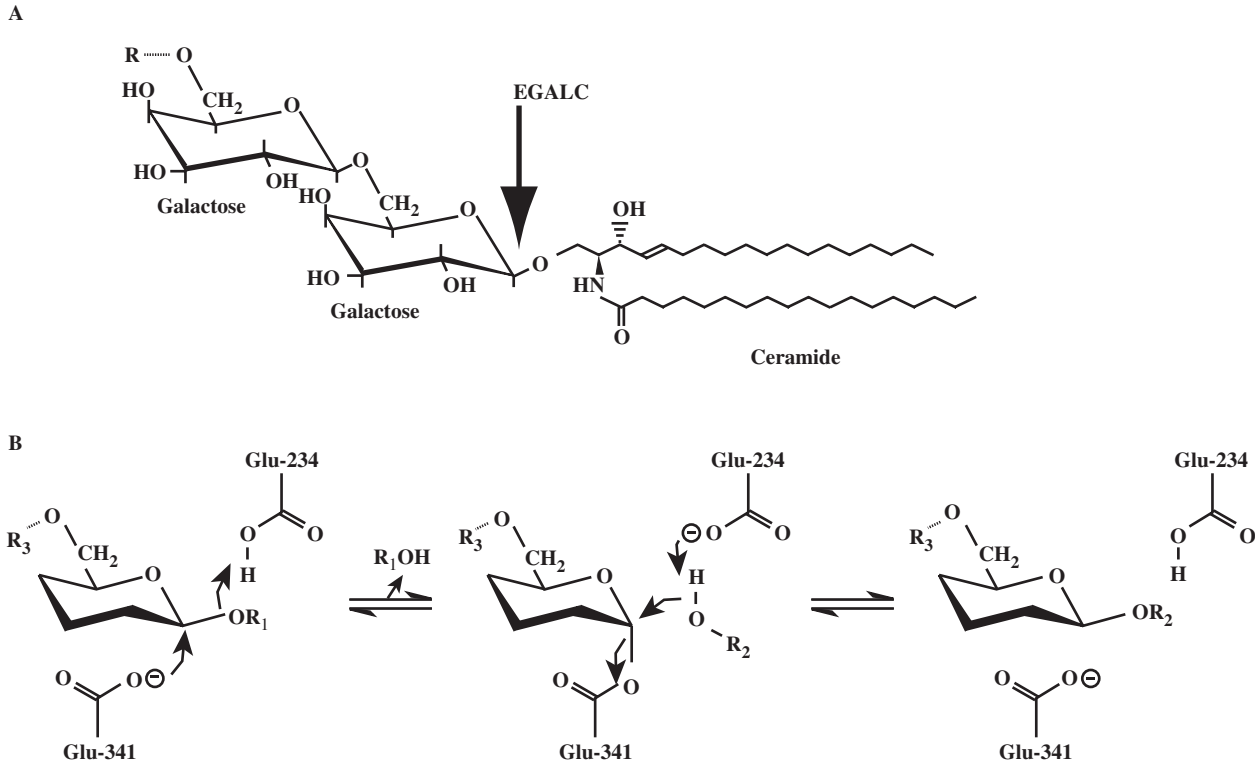

Fig. 6. **The point where EGALC acts on 6-gala GSLs (A) and transglycosylation mechanism of EGALC (B).** (A) The point where EGALC acts on the 6-gala GSLs which contain the common structure of R-Galβ1-6Galβ1-1'Cer. The enzyme hydrolyses the *O*-glycosidic linkage between the oligosaccharide and the ceramide of 6-gala GSLs. (B) EGALC, which is a GH5 glycosidase, catalyses hydrolysis or transglycosylation via a double displacement mechanism involving a covalent glycosyl-enzyme intermediate. In this figure, R_1 is a ceramide moiety of TGC and R_2 is an acceptor moiety such as an alkanol, non-ionic detergent or fluorescent ceramide, respectively.

1-butanol, 1-pentanol and 1-hexanol were found to be good acceptors for the reaction with EGALC. Among them, methanol is the most preferable acceptor and 80% TGC was converted to methyl-TGC-oligosaccharide under the conditions used (Fig. 2B). EGALC was also able to utilize Triton X-100 and C6-NBD-Cer as acceptor substrates but not monosaccharides. The acceptor specificity of EGALC seems to be quite different from that of leech and coryneform enzymes. Because, the leech enzyme was strongly inhibited by short chain 1-alkanols and no transglycosylation products were observed when methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol were used as an acceptor (*20*). On the other hand, methanol was a pretty good acceptor for coryneform enzyme, although ethanol, 1-propanol and 1-butanol were poor acceptors (*21*). The difference in substrate specificity between EGALC and other EGCases (ceramide glycanase) will be disclosed when X-ray crystal structures of EGALC–substrate complex are solved.

EGALC can catalyse the transglycosylation using GalCer as an acceptor and C6-NBD-Cer as a donor, generating C6-NBD-GalCer. Thus we have examined whether EGALC can catalyse the condensation reaction using galactose and C6-NBD-Cer. As a result, the C6-NBD-GalCer was not produced under the conditions used (data not shown), indicating EGALC hardly catalyses the condensation reaction.

Recently, it was shown that long chain (C5–C8)-alkyl-glucosides completely inhibit ultrasound-induced cytolysis, possibly because these alkyl-glucosides efficiently quench cytotoxic radicals and/or their precursors at the gas/solution interface of collapsing cavitation bubbles (*22*). These protective effects by alky-glucosides could be used for ultrasound-assisted tumour therapy, drug delivery and gene therapy. EGALC could transfer the trigalactooligosaccharide from TGC to various alkanols (C1∼C6) and non-ionic detergents such as Triton X-100. It is expected that the addition of oligosaccharides to alkanols and detergents would provide some new features, for instance, solubility, affinity for biomembranes, quenching of free radicals and solubilizing ability for membrane proteins. The functions of alkyl-TGC-oligosaccharides and Triton X-100-TGC-oligosaccharides prepared in the present study remain to be elucidated.

EGALC was able to transfer the trigalactooligosaccharide from TGC to fluorescent ceramides, C5-BODIPY-Cer and C6-/C12-NBD-Cer, generating corresponding fluorescent TGCs (Fig. 4A). These fluorescent TGCs were easily purified with Sep-Pak silica and plus C18 cartridges. It is worth noting that these fluorescent

TGCs were hydrolysed by EGALC to produce trigalactooligosacccharides and corresponding fluorescent ceramides (Fig. 4E). Thus, these fluorescent TGCs would be useful for the development of a sensitive assay of EGALC and possibly other enzymes which are involved in the metabolism of 6-gala series GSLs that are physiologically relevant in some pathogenic fungi and parasites.

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REFERENCES

1. Hakomori, S. (1981) Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. *Annu. Rev. Biochem.* **50**, 733–764
2. Matsubara, T. and Hayashi, A. (1981) Structural studies on glycolipid of shellfish. III. Novel glycolipids from *Turbo cornutus*. *J. Biochem.* **89**, 645–650
3. Noda, N., Tanaka, R., Miyahara, K., and Tsukamoto, T. (1996) Six trigalactosylceramides from the leech (*Hirudo nipponica*). *Chem. Pharm. Bull.* **44**, 895–899
4. Sugita, M., Fujii, H., Dulaney, J. T., Inagaki, F., Suzuki, M., Suzuki, A., and Ohta, S. (1995) Structural elucidation of two novel amphoteric glycosphingolipids from the earthworm, *Pheretima hilgendorfi*. *Biochim. Biophys. Acta* **1259**, 220–226
5. Persat, F., Bouhours, J.F., Mojon, M., and Petavy, A.F. (1992) Glycosphingolipids with Gal beta 1-6Gal sequences in metacestodes of the parasite *Echinococcus multilocularis*. *J. Biol. Chem.* **267**, 8764–8769
6. Dennis, R.D., Baumeister, S., Geyer, R., Peter-Katalinic, J., Hartmann, R., Egge, H., Geyer, E., and Wiegandt, H. (1992) Glycosphingolipids in cestodes. Chemical structures of ceramide monosaccharide, disaccharide, trisaccharide and tetrasaccharide from metacestodes of the fox tapeworm, *Taenia crassiceps* (Cestoda: Cyclophyllidea). *Eur. J. Biochem.* **207**, 1053–1062
7. Kawakami, Y., Nakamura, K., Kojima, H., Suzuki, M., Inagaki, F., Suzuki, A., Sonoki, S., Uchida, A., Murata, Y., and Tamai, Y. (1993) A novel fucosylated glycosphingolipid with a Gal beta 1-4Glc beta 1-3Gal sequence in plerocercoids of the parasite, *Spirometra erinacei*. *J. Biochem.* **114**, 677–683
8. Aoki, K., Uchiyama, R., Yamauchi, S., Katayama, T., Itonori, S., Sugita, M., Hada, N., Yamada-Hada, J., Takeda, T., Kumagai, H., and Yamamoto, K. (2004) Newlydiscovered neutral glycosphingolipids in aureobasidin A-resistant zygomycetes: Identification of a novel family of Gala-series glycolipids with core Gal alpha 1-6Gal beta 1-6Gal beta sequences. *J. Biol. Chem.* **279**, 32028–32034
9. Ito, M. and Yamagata, T. (1986) A novel glycosphingolipid-degrading enzyme cleaves the linkage between the oligosaccharide and ceramide of neutral and acidic glycosphingolipids. *J. Biol. Chem.* **261**, 14278–14282
10. Li, S.-C., DeGasperi, R., Muldrey, J.E., and Li, Y.-T. (1986) A unique glycosphingolipid-splitting enzyme (ceramide-glycanase from leech) cleaves the linkage between the oligosaccharide and the ceramide. *Biochem. Biophys. Res. Commun.* **141**, 346–352
11. Li, Y.T., Ishikawa, Y., and Li, S.C. (1987) Occurrence of ceramide-glycanase in the earthworm, Lumbricus terrestris. *Biochem. Biophys. Res. Commun.* **149**, 167–172
12. Ashida, H., Yamamoto, K., Kumagai, H., and Tochikura, T. (1992) Purification and characterization of membrane-bound endoglycoceramidase from *Corynebacterium* sp. *Eur. J. Biochem.* **205**, 729–735
13. Horibata, Y., Okino, N., Ichinose, S., Omori, A., and Ito, M. (2000) Purification, characterization, and cDNA cloning of a novel acidic endoglycoceramidase from the jellyfish, *Cyanea nozakii*. *J. Biol. Chem.* **275**, 31297–31304
14. Horibata, Y., Sakaguchi, K., Okino, N., Iida, H., Inagaki, M., Fujisawa, T., Hama, Y., and Ito, M. (2004) Unique catabolic pathway of glycosphingolipids in a hydrozoan, *Hydra magnipapillata*, involving endoglycoceramidase. *J. Biol. Chem.* **279**, 33379–33389
15. Ito, M. and Yamagata, T. (1989) Purification and characterization of glycosphingolipid-specific endoglycosidases (endoglycoceramidases) from a mutant strain of *Rhodococcus* sp. *J. Biol. Chem.* **264**, 9510–9519
16. Ishibashi, Y., Nakasone, T., Kiyohara, M., Horibata, Y., Sakaguchi, K., Hijikata, A., Ichinose, S., Omori, A., Yasui, Y., Imamura, A., Ishida, H., Kiso, M., Okino, N., and Ito, M. (2007) A novel endoglycoceramidase hydrolyzes oligogalactosylceramides to produce galactooligosaccharides and ceramides. *J. Biol. Chem.* **282**, 11386–11396
17. Tani, M., Kita, K., Komori, H., Nakagawa, T., and Ito, M. (1998) Enzymatic synthesis of ω-amino-ceramide: preparation of a sensitive fluorescent substrate for ceramidase. *Anal. Biochem.* **263**, 183–188
18. Taki, T., Kasama, T., Handa, S., and Ishikawa, D. (1994) A simple and quantitative purification of glycosphingolipids and phospholipids by thin-layer chromatography blotting. *Anal. Biochem.* **223**, 232–238
19. Horibata, Y., Higashi, H., and Ito, M. (2001) Transglycosylation and reverse hydrolysis reactions of endoglycoceramidase from the jellyfish, *Cyanea nozakii*. *J. Biochem.* **130**, 263–268
20. Li, Y.-T., Zhou, B., Rao, B.N.N., Schweinggruber, H., and Li, S.-C. (1991) Synthesis of neoglycoconjyugates using oligosaccharide transferring activity of ceramide glycanase. *J. Biol. Chem.* **266**, 10723–10726
21. Ashida, H., Tsuji, Y., Yamamoto, K., Kumagai, H., and Tochikura, T. (1993) Transglycosylation activity of endoglycoceramidase from *Corynebacterium* sp. *Arch. Biol. Biophys.* **305**, 559–562
22. Sostaric, J.Z., Miyoshi, N., Riesz, P., DeGraff, W.G., and Mitchell, J.B. (2005) *n*-Alkyl glucopyranosides completely inhibit ultrasound-induced cytolysis. *Free Radic. Biol. Med.* **39**, 1539–1548