Synthesis of a novel photoactivatable glucosylceramide cross-linker

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Abstract The biosynthesis of glucosylceramide (GlcCer) is a key rate-limiting step in complex glycosphingolipid (GSL) biosynthesis. To further define interacting partners of GlcCer, we have made a cleavable, biotinylated, photoreactive GlcCer analog in which the reactive nitrene is closely apposed to the GlcCer head group, by substituting the native fatty acid with 2-aminohexadecanoic acid. Two amino-GlcCer diastereomer cross-linkers (XLA and XLB) were generated. XLB proved an effective lactosylceramide (LacCer) synthesize substrate while XLA was inhibitory. Both probes specifically bound and cross-linked the GlcCer binding protein, glycolipid transfer protein (GLTP), but not other GSL binding proteins (Shiga toxin and cholera toxin). GlcCer inhibited GLTP cross-linking. Both GlcCer cross-linkers competed with microsomal nitrobenzoxadiazole (NBD)-GlcCer anabolism to NBD-LacCer. GLTP showed marked, ATP-dependent enhancement of cell-free intact microsomal LacCer synthesis from endogenous or exogenous liposomal GlcCer, supporting a role in the transport/membrane translocation of cytosolic and extra-Golgi GlcCer. GLTP was specifically labeled by either XLA or XLB GlcCer with a small subset of microsomal proteins. These cross-linkers will serve to probe physiologically relevant GlcCer-interacting cellular proteins.—Budani, M., M. Mylvaganam, B. Binnington, and C. Lingwood. Synthesis of a novel photoactivatable glucosylceramide cross-linker. J. Lipid Res. 2016. 57: 1728–1736.

Glycosphingolipid (GSL) accumulation is the basis of the pathology of lysosomal GSL storage diseases (1). In addition, aberrant GSL synthesis plays a key cofactor role in the pathology of many other human diseases (1), and in models of such disease, GSL blockade ameliorates symptoms (2–4). Understanding the synthesis of complex GSL is therefore crucial in generating the means for selective therapeutic correction of GSL levels.

Glycosyltransferase knockout studies in mice identify central roles for GSLs in embryology and differentiation, particularly in the peripheral and central nervous system (5). However, differences are observed for the same deletion in different studies (6–8), indicating that other factors in the regulation of GSL biosynthesis remain to be determined. One such factor is the relationship between the synthesis of the acidic and the several neutral GSL subclasses from lactosylceramide (LacCer) (9). GSL synthesis is complicated by the fact that the common precursor, glucosylceramide (GlCer), is made on the outer membrane of the Golgi (10, 11), while complex GSL synthesis occurs within the Golgi luminal membrane. The mechanism by which GlCer translocation is achieved is still largely a matter of conjecture (12). Phosphatidylinositol-4-phosphate adapter protein 2 (FAPP2)-facilitated cytosolic GlCer traffic is implicated in neutral GSL synthesis, while vesicular GlCer traffic is involved in ganglioside biosynthesis (13). We have proposed the Golgi located MDR1 (multidrug resistance protein 1) pump as a potential mechanism for flipping GlCer into the Golgi (14), but its role remains ill-defined and is unlikely the only mechanism. Furthermore, GlCer is emerging as an important factor in intra-cellular membrane traffic (15) and membrane order (16).

Supplementary key words gangliosides • glycolipids • Golgi apparatus • lipids/chemistry • lipid transfer proteins • flippase • glycosphingolipid anabolism • glycolipid transport

Abbreviations: 2A-GlCer, 2-aminohexadecanoyl glucosyl sphingosine; AEBSE, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; BOC anhydride, di-t-tert-butyl dicarbonate; BOP, benzotriazole-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate; CBE, conduri- tol B epoxide; CTB, cholera toxin B subunit; DCM, dichloromethane; DMF, dimethylformamide; Gal, galactose; Gb₂, globotriaosylceramide; GlCer, glucosylceramide; GLTP, glycolipid transfer protein; GSL, glycosphingolipid; LacCer, lactosylceramide; LCS, LacCer synthase; NBD, nitrobenzoxadiazole; SA-HRP, streptavidin horseradish peroxidase conjugate; sulfo-BSA, sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(pazido benzamido)-hexanoamido) ethyl-1,3′-dithiopropionate; t-BOC, tert-butyloxycarbonyl; TFA, trifluoroacetic acid; VTB, verotoxin-1 B subunit.

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Published, JLR Papers in Press, July 13, 2016
DOI 10.1194/jlr.D069609

Manuscript received 24 May 2016.

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GlcCer synthesis and trafficking are, in addition, regulated by statin-sensitive prenylation mechanisms (17).

As a means to address the mechanism by which GlcCer is trafficked intracellularly and translocated into the Golgi lumen, we have designed a novel GlcCer-based photoaffinity probe, using a 2-amino fatty acid derivative (18). The cross-linker is converted to LacCer and competes with nitrobenzoxadiazole (NBD)-GlcCer for GSL synthesis in cell free studies and thus provides a potential means to define GlcCer binding proteins, which should include any GlcCer flippase.

MATERIALS AND METHODS

Reagents

Sulfo-N-hydroxysuccinimidyld-2-[(biotinamido)2-[(p-azido benzamido)-hexanoamido) ethyl-1,3′-dithiopropionate (sulfo-SBEd) biotin label transfer reagent (no-weigh format) and streptavidin horseradish peroxidase conjugate (SA-HRP) were purchased from Thermo Scientific. GlcCer (glucocerebrosides) was purchased from Matreya LLC. 2-Aminoheptadecanoic acid, di-t-butyl dicarbonate (BOC anhydride), Mg(OAc)2, UDP-galactose (UDP-Gal), pyridine, ethyl acetate, trifluoroacetic acid (TFA), NaOH, HCl, triethylamine (TEA), acetic acid, acetic anhydride, dimethylformamide (DMF), dichloromethane (DCM), benzotriazole-1-yl-oxytris(dimethylamino)hexadecanoic acid (NBD-X SE) was purified by silica gel column chromatography (80% yield).

t-BOC deprotection

The t-BOC protected 2A-GlcCer analog was dried, lyophilized overnight, dissolved in TFA/water (1:1, v/v), and then stirred at room temperature for 7 h (21). HCl was added to the reaction products to protonate the trifluoroacetate salt and then dried under nitrogen without heat. Products 2-aminoheptadecanoyl glucosyl sphingosine (2A-GlcCer) isomers A and B were purified by silica gel column chromatography, and purified products (59% A and B combined yield) were compared by TLC CHCl3/CH3OH/water (65:25:4, v/v/v). The two products were analyzed by positive TOF mass spectrometry.

Acetylation of 2A-GlcCer amino analog

Both 2A-GlcCer A and B analogs, were dissolved in acetic anhydride-pyridine (1:1, v/v), incubated at 37°C for 2 h, and dried (23). To deacetylate the sugar only, products were dissolved in TEA/methanol/water (1:1:1, v/v/v) and incubated at 37°C overnight (23). Starting materials and N-acetyl products were compared by TLC in CHCl3/CH3OH/water (65:25:4, v/v/v) and then purified by silica gel column chromatography. Each product was analyzed by positive TOF mass spectrometry.

2A-GlcCer analog coupling to trifunctional photoactivatable cross-linker, sulfo-SBEd

Both 2A-GlcCer A (1.3 mg) and 2A-GlcCer B (0.6 mg) analogs were dissolved separately in DMF and incubated in the dark at room temperature with 1 mg sulfo-SBEd per analog for 3 h (supplier’s protocol). Reaction products, termed 2A-GlcCer XLA and 2A-GlcCer XLB (abbreviated XLA and XLB) were desalted using C18 Sep-Pak, purified by silica gel column chromatography (33% and 31% yield for XLA and XLB, respectively), and characterized by positive TOF mass spectrometry.

Preparation of crude microsomes

The DU145 prostate cancer cell line was chosen to study because these cells express a full complement of neutral and acidic GSLs. Near-confluent DU145 cells, maintained with MEM supplemented with 10% FBS, were washed with D-PBS, detached by trypsin, collected in an equal volume of FBS to inhibit trypsin, and washed twice with ice-cold D-PBS (360 g, 4°C), and pellets were stored at −80°C. Cell pellets were suspended in one volume of ice-cold homogenization buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.5 M sucrose), and homogenized with...
30 strokes of a Dounce homogenizer. Nuclei and debris were pelleted at 1,000 g for 10 min at 4°C. The supernatant was collected and centrifuged at 10,000 g for 10 min at 4°C. The supernatant (crude microsomes) was collected, and protein concentration measured. The protease inhibitor AEBSF was added to 0.1 mM, and 200 µg aliquots were stored at −80°C. This microsome preparation method is designed to retain cytosolic factors and minimize their dilution and is a modification of that used by De Rosa et al. (9); DTT was omitted to avoid reduction of the cleavable disulfide bond in XLA and XLB.

**LacCer synthase (LCS) assays**

**Detergent-containing assay to measure LCS activity.** Triton X-100 (0.3%) and 20 µg C16:0 GlcCer, XLA or XLB were dried under nitrogen, and sonicated, and incubated in 20 mM cacodylate, pH 6.8, 10 mM MnCl2, and 1 mM MgCl2 and incubated with 130 µg DU145 microsomes and 0.5 µCi 3H-UDP-Gal at 37°C for 3 h (24).

**Detergent-free assay to assess GlcCer translocation.** The detergent-free radiolabeled LCS assay is a modification of that of Chatterjee and Castiglione (24). Bovine GlcCer (20 µg) was dried and sonicated in water, incubated with 20 mM cacodylate, pH 6.8, 10 mM MnCl2, and 1 mM MgCl2, 2 µM GLTP, 5 µM ATP, 120 µg intact DU145 microsomes, and 0.5 µCi 3H-UDP-Gal (100 µl final volume) at 37°C for 3 h.

For both LCS assays, phospholipids were saponified at room temperature for 2 h with 1 M NaOH in methanol. Each reaction was neutralized with equal normal HCl and 23 mM NH4OAc. Chloroform was added to form a Folch partition (CHCl3/CH3OH/water, 2:1:0.6, v/v/v), the GSL-containing lower phase was washed with theoretical upper phase (CHCl3/CH3OH/water, 1:47:48), and the lower phase was extracted and dried under nitrogen. Samples were dissolved in 2 µl of ethanol (0.75 µM final concentration), incubated with cross-linker at room temperature for 1 h. For microsomal protein cross-linking, XLA or XLB were dried in 1.5 ml microtubes under air, dissolved in water, sonicated, and stored at −20°C until use. Thawed cross-linker micelles (0.1 µg) were preincubated with 2 mM GLTP and 15 µg GlcCer micelles or water of equal volume at 37°C for 1 h. GLTP, cross-linkers, and GlcCer samples were incubated with 1 mM MnCl2, 1 mM Mg(OAc)2, 20 mM cacodylate pH 6.8, protease inhibitor cocktail, 0.5 mM UDP-Gal, 0.25 mM CBE, and 100 µg DU145 microsomes at 37°C for 1 h. Purified and microsomal proteins were cross-linked with Spectroline Model EB-280C UV (302 nm) from a distance of 5 cm for 15 min. Samples were analyzed by SDS-PAGE, stained with Coomassie blue, or by Western blot using SA-HRP.

**RESULTS**

**Design and synthesis of 2A-GlcCer XL (XLA and XLB) photoactivatable cross-linkers**

The 2A-GlcCer XL was designed to closely mimic native GlcCer with two hydrophobic chains for membrane association, a cleavable disulfide bond, photoactivatable aryl azide in the proximity to the head group, and a biotin tag for affinity isolation. An overview of 2A-GlcCer XL synthesis is shown in Fig. 1. GlcCer was decetylated to lyso-GlcCer, desalted on a C-18 reverse phase silica gel column, and purified. The amino function of 2-aminohexadecanoic acid was protected with t-BOC, which increases yield of the subsequent BOP reaction by preventing polymerization of the 2-aminofatty acid. TLC staining with ninhydrin was used to monitor t-BOC protection reaction progression (Fig. 2A). 2-Aminohexadecanoic acid contains both t and l forms, which appears to run as two distinct bands when separated by TLC. After t-BOC protection the t, l isomers may have closely resolved to appear as one band. Product was purified on silica column and was confirmed by negative TOF mass spectrometry to have the expected mass of t-BOC protected 2-aminohexadecanoic acid [2-((t-butoxycarbonyl) amino)hexadecanoic acid], 371.3 amu. Lyso-GlcCer was coupled to 2-((t-butoxycarbonyl)amino)hexadecanoic acid to produce the product t-BOC-protected 2A-GlcCer. Reaction progression was monitored by TLC compared with C16:0 GlcCer analog and lyso-GlcCer by orcinol staining (Fig. 2B). t-BOC-protected 2A-GlcCer was deprotected, the two products (A and B) were purified and compared by TLC (Fig. 2C), and 2A-GlcCer A and B were confirmed as isomers by positive TOF mass spectrometry with a mass of 714.6 amu.

Diastereomers are not usually so well resolved by TLC (Fig. 2C). Therefore, the amino group on the fatty acid moiety of the 2A-GlcCer analogs was acetylated, to probe whether the TLC separation of the two isomers is due to differential intramolecular hydrogen bonding. After acetylation, the separation of the two isomers was much reduced, suggesting that polarity of isomer A is due to an NH intramolecular hydrogen bond (Fig. 2D). Both acetylated isomers A and B had an expected mass of 756.6 amu. 2A-GlcCer A and B analogs were separately coupled to sulfo-SBED to synthesize the biotinylated cross-linkers.
Fig. 1. Overview of 2A-GlcCer XL (XLA and XLB) synthesis. GlcCer was deacylated in 1 M NaOH/methanol at 70°C for 4 days (i). The amino function of 2-aminohexadecanoic acid was protected with t-BOC (ii). Mole ratio of 1:1.5:2 of 2-aminohexadecanoic acid, BOC anhydride, and sodium bicarbonate dissolved in CH₃OH/water (3.4:1, v/v) stirred for 2 days at room temperature. Lyso-GlcCer was coupled to t-BOC-protected 2-aminohexadecanoic acid (iii). BOP reagent (27.0 µmol) and t-BOC-protected 2-aminohexadecanoic acid (32.4 µmol) were dissolved in 5:5:1 DMF/DCM/TEA and incubated at -60°C for 10 min under nitrogen, and lyso-GlcCer (10.8 µmol) in 5:5:1 DMF/DCM/TEA was added to the reaction and incubated for 1.5 h at -60°C under nitrogen. t-BOC-protected 2A-GlcCer was deprotected (iv). t-BOC-protected 2A-GlcCer was dissolved in TFA/water (1:1, v/v) and stirred at room temperature for 7 h. 2A-GlcCer isomers A and B were purified. The 2A-GlcCer analogs were coupled to sulfo-SBED (v). Both 2A-GlcCer A and B analogs were dissolved separately in DMF and incubated in the dark at room temperature with 1 mg sulfo-SBED per analog for 3 h.

2A-GlcCer XLA and 2A-GlcCer XLB (abbreviated XLA and XLB). XLA and XLB were purified (Fig. 2E) and confirmed by positive TOF mass spectrometry to have the expected mass of 1,376.8 amu (Fig. 3).

Cross-linker characterization

GLTP binding. To determine the selectivity of XLA and XLB, cross-linking to known GlcCer binding protein GLTP was compared with non-GSL binding proteins VTB and CTB, and non-GSL binding protein gelatin. Selectivity was established by incubating 0.75 µM XLA or XLB with an equal mass of GLTP, VTB, CTB, and gelatin. Western blot with SA-HRP shows GLTP (25 kDa) was preferentially cross-linked by both XLA or XLB compared with gelatin and other GSL binding proteins (Fig. 4A, left panel). SDS-PAGE gel stained with Coomassie blue shows an equal quantity of GLTP, VTB, CTB, and gelatin present in cross-linking reactions (Fig. 4A, right panel). XLA and XLB cross-linking competition for GLTP was also investigated by addition of excess GlcCer. Western blot with SA-HRP shows GLTP cross-linking was reduced by half in the presence of excess GlcCer for both XLA and XLB compared with cross-linker alone, confirming GlcCer competes with both cross-linkers as ligands for GLTP binding (Fig. 4B).

LCS substrates. XLA and XLB were assessed as LCS substrates, in a ³H-UDP-Gal LCS assay with DU145 microsomes in detergent (Fig. 5A). Exogenous C16:0 GlcCer standard was used as a positive substrate control, which generated a tritiated product coincident with the lower band of the LacCer standard. XLB gave a product which comigrated with the upper band of the LacCer standard. The orcinol-stained TLC plate confirmed the putative substrate XLA was not degraded during the incubation period (Fig. 5B).

LCS competition. A detergent-free substrate competition LCS assay was used to determine whether NBD-GlcCer and XLA or XLB compete for LCS and putative GlcCer flipases. NBD-LacCer synthesis was reduced in the presence of either XLA or XLB, consistent with substrate competition (Fig. 5C). Competition with NBD-GlcCer breakdown by β-glucocerebrosidase was not completely avoided, even in the presence of the inhibitor, CBE. Because the LCS is in the Golgi lumen and this assay retains an intact (Golgi) membrane, it provides a potential index of Golgi luminal access to exogenous GlcCer, including translocation.
protein at 70 kDa present in GLTP only control (also seen in Fig. 4B).

DISCUSSION

XLA and XLB were designed with two alkyl chains to closely mimic native GlcCer. The 2NH₂ fatty acid moiety of 2A-GlcCer provides a primary amine for coupling to the biotin-labeled cross-linker sulfo-SBED. The position of the amine maintains the hydrophilicity of the polar head group of the analog once coupled to sulfo-SBED and is appropriately positioned to cross-link head group binding proteins.

Methods involved in synthesizing XLA/XLB are versatile and can easily be interchanged with lyso forms of different GSLs, different fatty acid chain lengths, and different cross-linkers.

After coupling the protected fatty acid to lyso-GlcCer, TLC separated two products. These are isomers resulting from the α and β forms of 2-aminohexadecanoic acid used in the coupling reaction with lyso-GlcCer. The two isomers of protected 2A-GlcCer analog were deprotected in the same reaction, which resulted in two compounds that ran very differently on TLC but with identical mass, which has not been previously observed. This effect is likely the result of an intramolecular hydrogen bond between the primary amine and the hydroxyl group of the sphingosine moiety in one of the isomers. To address this possibility, the primary amines of the two products were acetylated. Acetylation of the amine function considerably reduced the TLC separation, indicating an intramolecular H-bond from the amino group of isomer A was responsible for the TLC separation. Both isomers were coupled to sulfo-SBED.

Fig. 2. XLA and XLB synthesis reactions were monitored by TLC. A: t-BOC protection of 2-aminohexadecanoic acid. TLC of 2-aminohexadecanoic acid, and reaction products were stained with ninhydrin. B: Lyso-GlcCer coupling reaction to t-BOC-protected 2-aminohexadecanoic acid. TLC of reaction products compared with C16:0 GlcCer analog and lyso-GlcCer stained with orcinol. C: TLC stained with orcinol of purified products from t-BOC deprotection reaction. D: 2A-GlcCer acetylation suggests intramolecular hydrogen bonding in isomer A. 2A-GlcCer A and B compared with acetylated products by TLC, stained with orcinol. Discrepancy of resolved acetylated products is significantly reduced. E: TLC stained with orcinol of purified biotinylated cross-linkers 2A-GlcCer XLA and XLB compared with precursors and C16:0 GlcCer.

GLTP delivery of GlcCer into microsomal membranes

Because GLTP has been used to transfer GSLs to model membranes to modify glycolipid composition (26), we rationalized that GLTP could be used to insert XLA and XLB into microsomal membranes. However, first we needed to establish whether GLTP delivery of GlcCer could increase LacCer synthesis. We used 3H-UDP-Gal incorporation in detergent-free microsomes to monitor LacCer and globo-triaosylceramide (Gb₃) synthesis from endogenous (Fig. 6A) or exogenous (Fig. 6B) GlcCer, in the presence and absence of GLTP and ATP. GLTP markedly increased LacCer synthesis (and subsequent Gb₃ synthesis) both from endogenous and exogenous GlcCer. This effect was significantly enhanced in the presence of ATP. ATP alone had little effect on LacCer synthesis but reduced the observed GLTP-increased radiolabeling of GlcCer. LacCer and Gb₃ synthesis were significantly increased by exogenous GlcCer supply. In the absence of ATP, however, exogenous GlcCer had no LacCer/Gb₃ stimulatory effect.

XLA and XLB cross-linking to microsomal proteins

XLA and XLB cross-linking was assessed in DU145 microsomes ± GLTP to deliver and insert the cross-linker into the microsomal membranes (Fig. 7). XLA or XLB were preincubated ± GLTP and then incubated with microsomes for 1 h before cross-linking with UV light for 15 min. Western blot with SA-HRP shows GLTP did not enhance cross-linking of microsomal proteins compared with XLA or XLB alone. Several protein bands of interest near 45, 50, 55, 100, and above 170 kDa were cross-linked by both XLA and XLB. GLTP was also cross-linked during the reaction. GLTP cross-linking was reduced in the presence of microsomes, as well as a cross-linked protein at 70 kDa present in GLTP only control (also seen in Fig. 4B).

XLA and XLB were designed with two alkyl chains to closely mimic native GlcCer. The 2NH₂ fatty acid moiety of 2A-GlcCer provides a primary amine for coupling to the biotin-labeled cross-linker sulfo-SBED. The position of the amine maintains the hydrophilicity of the polar head group of the analog once coupled to sulfo-SBED and is appropriately positioned to cross-link head group binding proteins. The methods involved in synthesizing XLA/XLB are versatile and can easily be interchanged with lyso forms of different GSLs, different fatty acid chain lengths, and different cross-linkers.

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to synthesize XLA and XLB, which show interesting differential activity in vitro.

The specificity of XLA and XLB cross-linking was shown using the known GlcCer binding protein GLTP. Recombinant GLTP was preferentially cross-linked by both cross-linkers as compared with GSL binding proteins VTB and CTB. Cross-linking GLTP with XLA and XLB decreased in the presence of GlcCer, which supports a common binding site on GLTP (for XLA/B and GlcCer) and also suggests GLTP can be used to transfer cross-linker into membranes.

The \(^3\)H-LCS assay in detergent showed only XLB was converted into its LacCer form, not XLA. However, XLA still decreased NBD-LacCer synthesis when incubated with NBD-GlcCer in a detergent-free LCS assay, showing retained competition for LCS or putative flippases. Without detergent, the intact microsomal membrane remains a barrier, requiring substrate membrane translocation prior to luminal LacCer synthesis. Microsomal conversion of exogenous liposomal GlcCer to LacCer thus provides an index of GlcCer “flippase” activity. NBD-LacCer synthesis in intact

Fig. 3. Positive TOF of purified XLA and XLB. A: XLA with mass of 1,376.79 Da. B: XLB with mass of 1,376.79 Da.
This provides a dramatic example of lipid modulation of GSL function (28, 29). The role of GLTP in GSL synthesis was clearly shown in the cell-free microsomal system. In the absence of detergent, GLTP strongly promoted LacCer synthesis from both endogenous and exogenous GlcCer. This is consistent with the correlation between GLTP and cellular GlcCer levels (30). The GLTP-dependent increase we observed was amplified by ATP, but ATP alone did not affect LacCer synthesis. Conversion to Gb3 was similarly stimulated by GLTP/ATP.

GLTP does not appear to be ATP dependent, suggesting an

cell microsomes similarly suggests NBD-GlcCer is translocated by a GlcCer flippase prior to conversion by LCS. NBD-GlcCer has been shown to be a substrate for MDR1-mediated membrane flipping (27). Interestingly, the microsomal cytosolic β-glucosidase-mediated breakdown to NBD-Cer was less effectively blocked, particularly by XLA. The altered orientation of the acyl chain isoform in XLA may restrict access to the 4′OH of the glucose moiety required for LCS action. XLB is a very good substrate for LCS, more effective than C16:0 GlcCer in our in vitro assay; in contrast, XLA acts as an LCS inhibitor, rather than an active substrate.

Fig. 4. XLA and XLB preferentially cross-links and shows substrate competition for GLTP. A: XLA or XLB (0.75 µM) was incubated with an equal mass of GLTP (2 mM), VTB, CTB, and gelatin. XLA and XLB were cross-linked to interacting proteins with UV light for 15 min. Western blot with SA-HRP shows GLTP was preferentially cross-linked, gel stained with Coomassie blue shows the presence of equal amounts of GLTP, VTB, CTB, and gelatin. B: GLTP, XLA, or XLB were incubated with or without GlcCer for 1 h at 37°C before cross-linking with UV light for 15 min. Representative Western blot with SA-HRP shows GlcCer reduces GLTP cross-linking for both XLA and XLB.

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Fig. 5. Evaluation of GlcCer cross-linker substrate activity for LCS. A: DU145 microsomes were incubated with no exogenous GlcCer, semisynthetic C16:0 GlcCer, XLA or XLB in the presence of Triton X-100 and [3H]-UDP-Gal. Analysis of reaction products by TLC followed by fluorography. TLC shows formation of a product consistent with LacCer from C16:0 GlcCer and XLB but not XLA. [3C]-Gal-labeled GSLs from vero cells are in “Std” lane. B: A portion of each sample from A was analyzed by orcinol-stained TLC to confirm XLA was not degraded during the incubation period. C: NBD-LacCer synthesis in DU145 crude microsomes; NBD-GlcCer and XLA or XLB were incubated with intact microsomes for 3 h at 37°C. TLC analysis of fluorescent NBD products revealed some glucocerebrosidase (GBA) 2/GBA3-mediated (32) breakdown to NBD ceramide. Conversion of NBD-GlcCer into NBD-LacCer was reduced in the presence of excess XLA or XLB, suggesting competition for GlcCer translocase or LCS or both.
synthesis. This suggested that using GLTP to deliver XLA and XLB to microsomal membranes would enhance cross-linking to GlcCer binding proteins and putative flippases.

XLA and XLB were cross-linked in DU145 microsomes using GLTP to deliver and insert the cross-linker into the microsomal membranes, as GLTP has been previously used to insert and extract GSLs in model membranes (26, 31), and our studies show GLTP enhancement of microsomal GSL synthesis. Recombinant GLTP was cross-linked by both XLA and XLB in DU145 microsomes, but cross-linking of microsomal proteins was not enhanced by GLTP delivery. This would suggest translocation is not the rate-limiting step. Cross-linking of the 70 kDa species was reduced in micromosomes compared with GLTP alone, suggesting that this could be a GlcCer-donating species. Several microsomal proteins were consistently cross-linked in both micelle- and GLTP-delivered cross-linking. Our future studies are directed toward the characterization of these putative GlcCer binding proteins and flippases and their potential role in GSL synthesis.

The authors thank Li Zhang, Jonathan Krieger, and Paul Taylor from SickKids Proteomics, Analytics, Robotics & Chemical Biology Centre, Hospital for Sick Children for mass spectrometry services, and Dr. Thorsten Lang from Department of Membrane Biochemistry at the LIMES Institute, University of Bonn, Germany, for kindly providing recombinant GLTP.

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