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Metabolically incorporated photocrosslinking sialic acid covalently captures a ganglioside-protein complex

Michelle R. Bond^{a,b,*}, Chad M. Whitman^{a,b,*}, and Jennifer J. Kohler^{a,†}

^a Division of Translational Research, Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9185

^b Department of Chemistry, Stanford University, Stanford, CA 94305-5080

Abstract

When photoirradiated, an unnatural sialic acid analog can covalently capture the complex formed by ganglioside GM1 and cholera toxin subunit B.

Cell surface glycoconjugates mediate essential intra- and inter-cellular interactions. Many of these recognition events are regulated by the presence or absence of sialic acids,¹ a family of nine carbon α -keto acids attached to glycoproteins and glycosphingolipids. While sialylated molecules serve important regulatory roles for mammalian cells, they are also exploited by many pathogens.² For example, influenza viruses utilize hemagglutinin to bind sialylated cell surface receptors, while *Trypanosoma cruzi*'s trans-sialidase transfers sialic acids from host glycoconjugates to the parasite.² Sialylated glycosphingolipids, known as gangliosides (Fig. 1a), are of particular interest because they often serve as the principal recognition elements for the invasion of host cells by viruses, bacteria, and their secreted toxins.³

Explicitly identifying ganglioside interaction partners and deciphering the molecular details of these complexes are challenging tasks. Most glycan-mediated complexes have low affinities and their transient natures make traditional purification techniques impractical, as the unstable complexes cannot withstand the washing steps. Furthermore, the amphiphilicity of gangliosides poses additional constraints on purification. Methods to capture ganglioside-protein complexes in their native settings would represent a powerful tool for characterizing these labile complexes. Our strategy is to introduce photoreactive groups into glycan components of cellular glycoconjugates. We accomplish this using metabolic oligosaccharide engineering, a technique that has been highly successful at introducing a wide range of chemical modifications into various monosaccharides, including sialic acid (Fig. 1b).⁴ Diazirine modification of cellular sialosides is achieved by culturing mammalian cells with a diazirine-containing *N*-acetylmannosamine analog, Ac₄ManNDaz, compound **2** (Fig. 1c), which is metabolized by cells and displayed on the cell surface as the sialic acid analog, SiaDaz, **4** (Fig. 1c). Upon UV irradiation, the diazirine can be activated to a carbene. If positioned appropriately, the highly reactive carbene can form a covalent bond between sialic acid and the sialoside binding partner(s). The covalently-linked complex can then be analyzed and the components identified. We recently demonstrated the incorporation of a diazirine into the glycans attached to cell surface sialic acid binding lectin CD22 and used the diazirine's crosslinking capacity to capture CD22 oligomers.⁵

[†]To whom correspondence should be addressed. Phone: +1 (214) 648-1214; Fax: +1 (214) 648-4156; jennifer.kohler@utsouthwestern.edu.

*These authors contributed equally to this work.

Encouraged by reports of the incorporation of unnatural sialic acid analogs into gangliosides,⁶ we investigated whether mammalian cells are capable of producing SiaDAz-containing gangliosides. Jurkat cells, which have been used previously in metabolic oligosaccharide engineering,^{6f,7} were cultured with **2** (Ac₄ManNDaZ) for 72 hours. As a control, cells were also cultured with the 2-epimer of Ac₄ManNDaZ, Ac₄GlcNDaZ – compound **3** (Fig. 1c), which is not expected to be metabolized to SiaDAz.⁸ Additionally, Jurkat cells were cultured with Ac₄ManNAc to determine whether exogenous addition of a normal metabolite in the sialic acid biosynthesis pathway affects ganglioside production. Acetylated sugars⁹ were added to the media at a final concentration of 100 μM. After harvesting the cultured cells, the gangliosides were extracted using established methods¹⁰ (described in supplementary information) and resolved using high performance TLC (HPTLC) plates. To visualize gangliosides, the HPTLC plates were stained with resorcinol, which, upon heating, reacts specifically with sialic acid-containing molecules.¹¹

By resorcinol staining, we observed that untreated Jurkat cells synthesize multiple gangliosides (Fig. 2a). These data were corroborated by mass spectrometry data, which indicated the presence of gangliosides GM3, GM2, GM1, and GD1a, each present in two forms that arise from different fatty acid chain lengths (Fig. 2b, S4 and Table S1). While cells cultured with **1** produced gangliosides whose profile appeared identical to that of untreated cells, cells that were cultured with **2** produced novel ganglioside products (Fig. 2a). We interpret these new bands to correspond to diazirine-modified gangliosides, consistent with the prediction that introduction of the diazirine side chain should increase the hydrophobicity of gangliosides and their mobility in the TLC eluent. In addition to these new molecules, the **2**-treated cells continue to generate their natural gangliosides. We confirmed production of diazirine-modified gangliosides by MALDI-TOF-MS (Fig. 2c, S5, and Table S2). Gangliosides from cells cultured with **3** were indistinguishable from those from untreated cells (Fig. 2a), suggesting that **3** is not efficiently metabolized to **4** in Jurkat cells.

To determine if SiaDAz-containing gangliosides can still be recognized as specific receptors, we used a fluorescently-labeled GM1-binder, cholera toxin subunit B-Alexa Fluor 488 conjugate (CTxB-Alexa488), to probe HPTLC plates (Fig. 2d).¹² As expected, CTxB-Alexa488 binds GM1 in all of the Jurkat cell extracts. Importantly, CTxB-Alexa488 also recognizes additional species in extracts from cells cultured with **2**; we assign these additional bands to diazirine-modified GM1 (GM1-SiaDAz). These results provide evidence that Jurkat cells cultured with **2** produce gangliosides that contain **4** and, further, that CTxB recognition of GM1 is maintained upon installation of the unnatural diazirine side chain on sialic acid.

With the observation that GM1-SiaDAz is recognized by CTxB, we next investigated whether we could covalently capture the ganglioside-protein complex in a cellular setting using photoirradiation. In brief, Jurkat cells were cultured with no additional sugar, **1**, **2**, or **3** for 70 hours, followed by the addition of CTxB for 45 min to allow cell surface binding. Cells were exposed to 365 nm light for 45 min to initiate photocrosslinking. After cell lysis and SDS gel electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, which was then probed for the presence of CTxB (Fig. 3a and S6) by standard immunoblotting techniques. When we analyzed lysates from Jurkat cells cultured with **2** and photoirradiated, we observed both unmodified CTxB and an additional band whose mobility was consistent with the addition of a GM1 molecule to CTxB. In contrast, untreated, **1**-treated, and **3**-treated cells displayed a single band corresponding to unmodified CTxB (Fig. 3a). In complementary experiments where we probed the membrane for GM1, multiple bands were observed only when cells were cultured with **2**, consistent with the formation of a covalent GM1-CTxB complex (Fig. S6a). Experiments performed without photoirradiation confirmed that formation of the putative GM1-CTxB complex was UV-dependent (reported in supplemental information, Fig. S6).

To confirm that the novel complex consisted of CTxB crosslinked to a ganglioside, we performed cell culturing and crosslinking in the presence of a glycosphingolipid biosynthesis inhibitor, *N*-butyldeoxygalactonojirimycin (NB-DGJ).¹³ When the inhibitor was added to cultured cells, we observed a marked decrease in GM1 expression (Fig. S6d and f) as well as a decrease in CTxB binding (Fig. 3b and S6e). Importantly, the intensity of the band corresponding to the putative GM1-CTxB species decreases dramatically. Taken together, these results demonstrate that Jurkat cells cultured with Ac₄ManNDaZ produce a diazirine-modified ganglioside that can be efficiently photocrosslinked to CTxB.

In summary, we demonstrate the use of metabolic oligosaccharide engineering to enable a mammalian cell line to produce photoactivatable gangliosides. Metabolically-produced GM1-SiaDaZ is recognized by a GM1 binding partner, CTxB, and photoirradiation can be used to efficiently capture this ganglioside-protein complex. These results show promise for using this photoreactive tool to capture and characterize ganglioside-mediated interactions. The ganglioside GM1 is of special interest because it is highly enriched in membrane microdomains commonly known as lipid rafts.¹⁴ The ability to metabolically introduce a photocrosslinker into GM1 and to form covalent complexes with neighboring proteins represents a method for gaining information about the composition of lipid rafts with minimal disruption to the native organization of membrane molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Strategy to incorporate diazirine into gangliosides. (a) Glycosphingolipid GM1. The sialic acid is highlighted in red. (b) Enzymes in the sialic acid metabolic pathway convert ManNAc and analogs to activated CMP-sialic acids, which are added to glycoconjugates by sialyltransferases. Unnatural substituents are indicated by -R. (c) Synthesized sugar analogs Ac₄ManNAc (**1**), Ac₄ManNDaz (**2**), Ac₄GlcNDaz (**3**). Ac₄ManNDaz is expected to be metabolized to SiaDaz (**4**).

Fig. 2. Production of diazirine-modified gangliosides. (a) Resorcinol-stained HPTLC plate reveals the profile of gangliosides produced by Jurkat cells. Ganglioside standards are shown in the first and last lanes. In untreated Jurkat cells, we observe multiple bands for each ganglioside, which we interpret as corresponding to different fatty acid chain lengths. Additional species are observed when cells are cultured with **2**. (b) MALDI-TOF-MS analysis identified GM3, GM2, GM1, and GD1a gangliosides in untreated Jurkat cells; peak identity reported in supplemental information (Table S1). (c) MALDI-TOF-MS analysis identified gangliosides containing a naturally occurring sialic acid (*N*-acetyl neuraminic acid, NeuAc) or **4** (SiaDAz) in **2**-treated cells; peak identity reported in supplemental information (Table S2). Ionization resulted in some loss of N₂ from diazirines (*). (d) GM1 gangliosides, including GM1-SiaDAz, are recognized by CTxB-Alexa488.

Fig. 3.

Photocrosslinking of GM1-SiaDAz to CTxB. All blots were probed for CTxB and β -actin. (a) A new CTxB-containing species appears in cells cultured with **2** and exposed to UV irradiation, indicative of crosslinking to GM1-SiaDAz. (b) Jurkat cells cultured with the glycosphingolipid biosynthesis inhibitor NB-DGJ demonstrate decreased production of GM1 (Fig. S6), decreased CTxB binding, and diminished levels of the crosslinked GM1-CTxB complex.