Bio-orthogonal Chemistry

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Selective Exo-Enzymatic Labeling of N-Glycans on the Surface of Living Cells by Recombinant ST6Gal I**

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The bio-orthogonal chemical reporter strategy is an emerging technology for the visualization and isolation of glycoconjugates in living cells and model organisms.[1] It exploits the promiscuity of the biosynthetic machinery, which makes it possible to incorporate monosaccharides that have a unique chemical functionality (the reporter) into glycans in living cells. The chemical reporter can then be reacted with a probe linked to a complementary bio-orthogonal functional group. The mutually selective chemical reactivity of the two functional groups ensures that only the metabolically labeled glycans are detected. Azides are particularly versatile reporters because of their small size and virtual absence in biological systems.[2] They can be tagged by Staudinger ligation using modified phosphines,[3] by copper(I)-catalyzed cycloaddition with terminal alkynes (CuAAC),[4] or by strain-promoted alkyne–azide cycloaddition (SPAAC).[5]

Metabolic labeling with azide-containing monosaccharides results in the tagging of different classes of glycans, including N- and O-linked glycoproteins, proteoglycans and glycolipids, because they are derived from a common pool of nucleotide sugars, which can be functionalized with an azide group for labeling purposes.[5a] A growing body of data supports the idea that various types of glycolipids and glycoproteins localize and recycle differently in the context of diseased cells, as evidenced by their accumulation within distinct intracellular compartments and vesicles.[6] Thus, there is a need to augment the bio-orthogonal chemical reporter strategy with technologies that allow selective labeling of specific classes of glycoconjugates. Here, we show that the inherent substrate specificity of recombinant ST6Gal I sialyltransferase[7] can be exploited for the selective labeling, on living cells, of N-linked glycans with azide-modified sialic acid. This selective exo-enzymatic labeling (SEEL) strategy will offer unique opportunities to track, capture, and identify subsets of cell surface glycoconjugates in the context of healthy and diseased cells.

It is known that sialyltransferases tolerate modifications at C-5 and C-9[8], and therefore cytidine monophosphate (CMP)–sialic acid derivative **4** was prepared with an azide at C-9 of the sialic acid moiety. This compound was easily synthesized from the methyl ester of sialic acid (**1**) through selective tosylation of the C-9 primary hydroxy group to give **2**, which was treated with sodium azide in a mixture of acetone and water, and heated under reflux to afford **3** in an almost quantitative yield (Scheme 1). Surprisingly, the methyl ester was removed under these conditions to give the required free carboxylic acid. Condensation of **3** with CTP in the presence of recombinant CMP–sialic acid synthetase from *Neisseria meningitis* [EC 2.7.7.43][9] and inorganic pyrophosphatase from *Saccharomyces cerevisiae* [EC 3.6.1.1] gave, after purification by size-exclusion column chromatography, CMP-Neu5Ac9N_3 (**4**). Incubation of *N*-acetyllactosamine and **4** in the presence of ST6Gal I led to an almost quantitative formation of Neu5Ac9N_3α(2,6)Galβ(1,4)GlcNAc (Scheme S1 in the Supporting Information), highlighting the fact that the C-9 azido moiety of **4** is tolerated by the enzyme. Kinetic analysis of the enzymatic transformation showed that the azide moiety of **4** had only marginally impacted the K_m value (0.39 mM for **4** vs. 0.18 mM for CMP-Neu5Ac), with no appreciable influence on the V_{max} value (Figure S1 in the Supporting Information).

Human skin fibroblasts were incubated with ST6Gal I in the presence of either CMP-Neu5Ac9N_3 (**4**) or CMP-Neu5Ac (2 mM) for 2 h at 37 °C. In addition, cells were metabolically labeled through feeding with peracetylated *N*-α-azidoacetylmannosamine (Ac_4ManNAz) or peracetylated *N*-acetylmannosamine (Ac_4ManNAc), which can be incorporated into glycoproteins and gangliosides as *N*-azidoacetyl sialic acid (SiaNAz) or sialic acid (Neu5Ac), respectively.[3a] The cells were exposed to sulfated dibenzocyclooctynylamide (S-DIBO) modified with biotin (**5**, 30 μM)[10] for 1 h followed by staining with avidin-fluorescein isothiocyanate (FITC) for 15 min at 4 °C. The efficiency of the two-step cell surface labeling was determined by measuring the fluorescence intensity of the cell lysates. Both enzymatic and metabolic labeling approaches resulted in robust staining, whereas the

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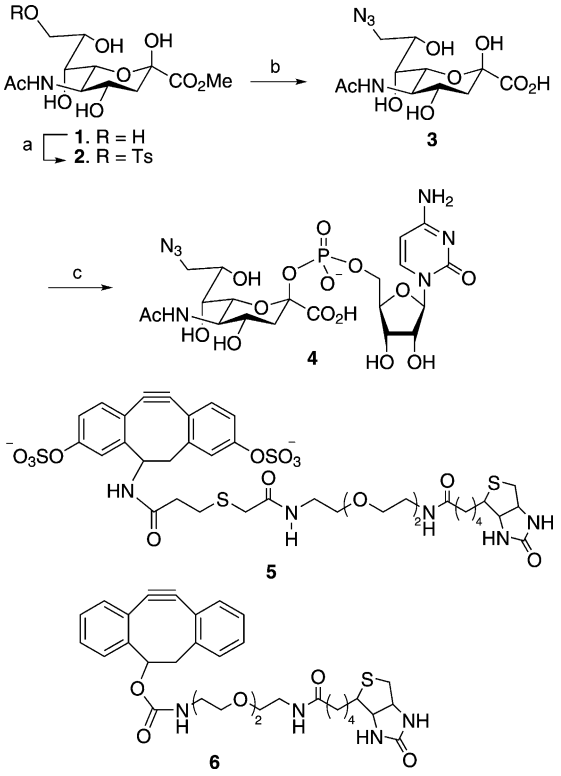

Scheme 1. Chemical synthesis of sialic acid analogues modified by a chemical reporter and chemical structures of **5** and **6**. Reagents and conditions: a) tosyl chloride (TsCl), pyridine (80%); b) NaN_3, acetone/H_2O, reflux (80%); c) CTP, CMP-NeuAc synthetase, 100 mM Tris-HCl buffer, pH 8.92, 20 mM $MgCl_2$ (62%).

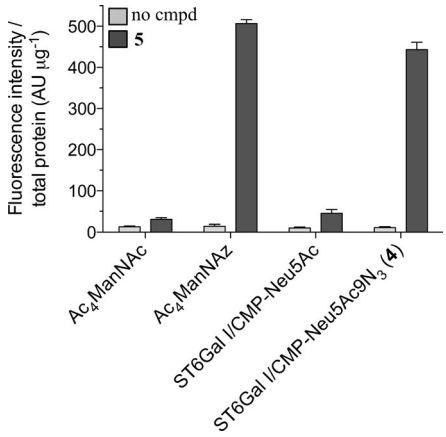

Figure 1. Comparison of metabolic and enzymatic cell surface labeling. For metabolic labeling, fibroblasts were grown for 2 days in the presence of Ac_4ManNAc or Ac_4ManNAz (100 μM). For enzymatic labeling, fibroblasts grown for 2 days were incubated with CMP-Neu5Ac9N_3 (**4**) or CMP-Neu5Ac in the presence of ST6Gal I for 2 h at 37 °C. Next, the cells were incubated with or without **5** (30 μM) for 1 h at RT and then incubated with avidin-FITC for 15 min at 4 °C, after which cell lysates were assessed for fluorescence intensity. AU indicates arbitrary fluorescence units. Data ($n = 3$) are presented as mean ± SD. Compound **5** was selected because it does not pass through the cell membrane and therefore only labels cell surface glycoconjugates, making it possible to compare labeling by the exogenously administered sialyltransferase, which only labels cell surface glycoconjugates, with metabolic labeling, which results in the modification of both intra- and extracellular glycoconjugates.

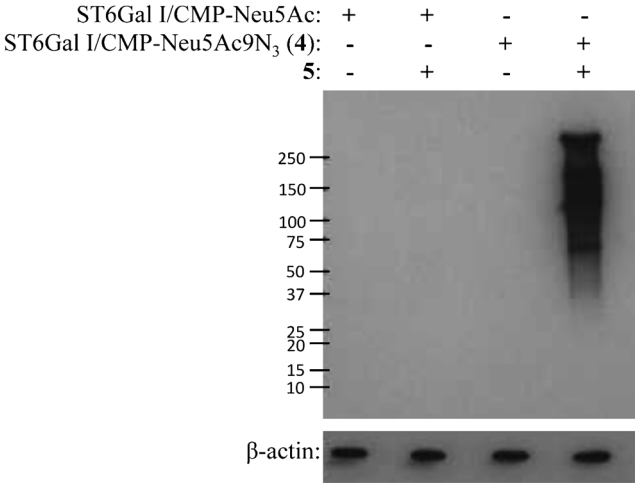

Figure 2. Determination of the labeling specificity of recombinant ST6Gal I. Cell lysates from enzymatically labeled fibroblasts were resolved by SDS-PAGE, and the blot was probed with an HRP-conjugated anti-biotin antibody. Total protein loading was confirmed through β-actin staining.

control treatments gave very low responses (Figure 1). Analysis of cell lysates by SDS-PAGE and Western blotting further confirmed the requirement for enzyme ST6Gal I and compound **4** for cell labeling (Figure 2). A concentration of 100 μM of **4** was sufficient for robust labeling (Figure S2 in the Supporting Information). Furthermore, robust labeling could be achieved in other cell types, such as CHO-K1. As expected, the staining was enhanced when the CHO glycosylation mutant Lec2 was employed, because these cells exhibit greatly reduced sialic acid incorporation as a result of an inactivated CMP-sialic acid transporter (Figure S3 in the Supporting Information).

Next, the selectivity for N-glycans of the cell surface labeling by ST6Gal I was evaluated by SDS-PAGE of cell lysates that were either untreated or treated with peptide-N-glycosidase F (PNGase F), followed by blotting and probing with a horseradish peroxidase (HRP)-conjugated anti-biotin antibody (Figure 3). Treatment with PNGase F,[11] which is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose in hybrid and complex oligosaccharides of N-linked glycoproteins, completely abolished staining, demonstrating that ST6Gal I had only modified the N-linked glycans on cell surface glycoproteins. A similar experiment using a lysate obtained from cells metabolically labeled with ManNAz showed residual staining (Figure S4 in the Supporting Information), which demonstrates that this approach tags other classes of glycoconjugates in addition to N-linked glycans. As an additional demonstration of ST6Gal I selectivity, glycolipids were isolated from cells labeled by ST6Gal I and **4** and analyzed by mass spectrometry.[12] No glycolipids that had been modified by azide-containing sialic acid were detected (Figure S5 in the Supporting Information).[13] To further support the selectivity

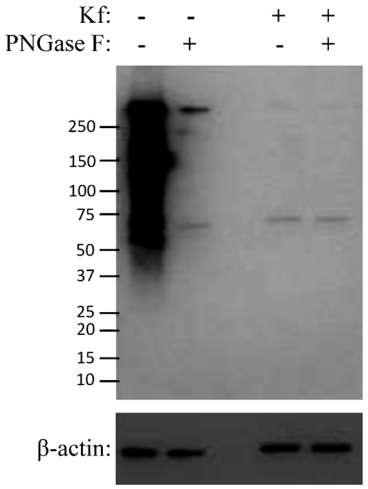

Figure 3. Determination of the labeling specificity of recombinant ST6Gal I. Fibroblasts grown for 2 days either without treatment or in the presence of Kf (10 μM) were incubated with CMP-Neu5Ac9N_3 (**4**, 2 mM) in the presence of ST6Gal I for 2 h at 37 °C. Next, the cells were incubated with **6** (30 μM) for 1 h at RT. Cell lysates (either untreated or PNGase F treated) were resolved by SDS-PAGE and the blot was probed with an HRP-conjugated anti-biotin antibody. Total protein loading was confirmed through β-actin staining.

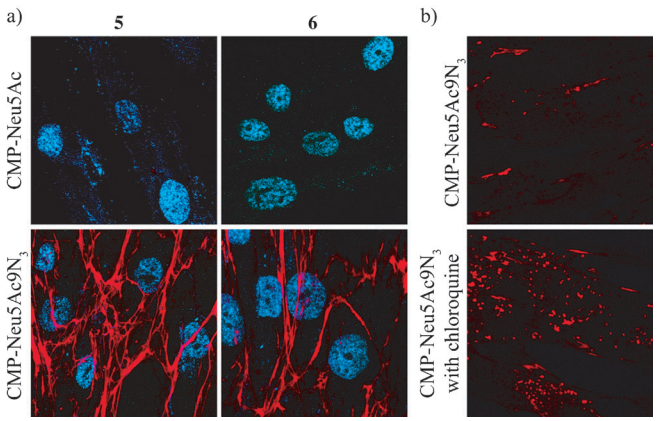

Figure 4. Localization and trafficking of ST6Gal I-tagged glycoproteins. a) Enzymatic labeling of fibroblasts. Human skin fibroblasts were incubated with CMP-Neu5Ac or CMP-Neu5Ac9N_3 (**4**) in the presence of ST6Gal I for 2 h at 37 °C. Next, live cells were incubated with **5** or **6** for 1 h at RT and stained with streptavidin-Alexa Fluor 568 for 15 min at 4 °C. After washing, fixing, and staining for the nucleus with the far-red-fluorescent dye TO-PRO-3 iodide, cells were visualized by using confocal microscopy. Images of cells labeled with Alexa Fluor 568 (568 nm) and TO-PRO iodide (633 nm) are merged and shown in red and blue, respectively. b) Effect of chloroquine on enzymatic labeling. Fibroblasts were incubated with CMP-Neu5Ac9N_3 (**4**) in the presence of ST6Gal I for 2 h at 37 °C. Next, live cells were incubated with **6** for 4 h at 37 °C in the absence or presence of chloroquine (50 μM). After incubation with streptavidin-Alexa Fluor 568, the cells were visualized by confocal microscopy. Images of cells labeled with Alexa Fluor 568 are shown in red.

of staining by ST6Gal I, labeling efficiencies were measured in fibroblasts grown in the presence of kifunensine (Kf; 10 μM). This compound inhibits Golgi mannosidase I, thereby causing a complete shift in the structure of N-linked oligosaccharides, from complex chains to $Man_9(GlcNAc)_2$ structures that can not be modified by sialosides.[14] This treatment completely abolished staining (Figure 3).

Next, we investigated the use of the SEEL technology for the visualization of trafficking of N-linked glycoconjugates. Fibroblasts were enzymatically labeled by using ST6Gal I and CMP-Neu5Ac9N_3 (**4**), and the resulting azide-modified N-linked glycans were visualized by confocal microscopy after cycloaddition with S-DIBO derivative **5**, and treatment with streptavidin-Alexa Fluor 568 (Figure 4a). As expected, robust staining of the cell surface and fibrillar network was observed. Parallel experiments with DIBO derivative **6**, which can pass through the cell membrane,[10,15] also showed no staining of intracellular structures (e.g. Golgi), thus confirming that exogenously administered ST6Gal I only labels cell surface glycans (Figure 4a and S6 in the Supporting Information). The cell surface glycans of CHO-K1 cells could be readily labeled, and as expected, more robust staining was observed in the CHO glycosylation mutant Lec2 (Figure S7 in the Supporting Information). Next, cells were treated with chloroquine to raise the lysosomal pH and prevent efficient catabolism within this compartment (Figure 4b). Under these conditions, labeled glycoconjugates localized within intracellular vesicles resembling late endosomes/lysosomes, thereby highlighting the fact that this technology can be employed to study the trafficking of cell surface glycoconjugates.

Metabolic labeling of azide-containing monosaccharides has been successfully employed for the detection and isolation of sialylated, fucosylated and O-GlcNAc modified proteins in mammalian cells. Recently, the metabolic labeling strategy has been expanded to plants[16] and bacteria[17] by feeding alkyne-derivatized fucose and azide-modified 3-deoxy-D-manno-oct-2-ulosonic acid (KDO), respectively. In an alternative approach, azide-modified glycoconjugates have been formed by exposing cells or cell lysates to an azide-modified sugar nucleotide and a glycosyltransferase. For example, an engineered galactosyltransferase can selectively transfer UDP-*N*-α-azidoacetylgalactosamine (UDP-GalNAz) to O-GlcNAc modified proteins.[18] Furthermore, it has been shown that GDP-6-azidofucose is readily accepted by a recombinant *H. pylori* α(1,3)-fucosyltransferase, and this has been successfully employed to image glycans in the enveloping layer of zebrafish embryos.[19] The attraction of enzymatic labeling is that it affords near quantitative labeling, does not perturb signaling pathways, and is amenable to all cell types.

We have demonstrated for the first time that the inherent substrate specificities of glycosyltransferases can be exploited for the tagging of a specific subset of cell surface glycoconjugates. In particular, it has been shown that rat ST6Gal I can readily accept a CMP–sialic acid analogue modified at C-9 by an azide moiety. It was found that cells have sufficient acceptor sites for ST6Gal I to achieve robust staining without the need for treatment with a sialidase. The exogenously administered sialyltransferase only modified N-linked glycans at the cell surface and extracellular matrix. The tagged glycoconjugates could, however, be internalized, and the accumulation of glycoconjugates in vesicular structures could

easily be detected following chloroquine-induced disruption of lysosomal function. Many inherited human diseases are caused by defects in the proteins involved in retrograde transport through the endosomal network, or in enzymes responsible for the lysosomal catabolism of glycosylated compounds.[20] The SEEL strategy described here will provide a unique opportunity to track, capture, and identify subsets of cell surface glycoconjugates. It will make it possible to dissect the mechanisms that underlie altered glycoconjugate recycling and storage, and identify those molecules, the cell surface localization or secretion of which are most affected. Many sialyltransferases exhibit unique substrate specificities[21] and it is thus expected that SEEL could be extended to other types of glycoconjugates.

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