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Selective Exo-Enzymatic Labeling (SEEL) of *N*-Glycans of Living Cells by Recombinant ST6Gal I**

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Abstract

SEEL cell surface glycans—*N*-Glycans of living cells could be selectively tagged by exogenously administered recombinant ST6Gal I sialyltransferase and azido-modified CMP-Neu5Ac followed by a strain promoted cycloaddition using biotin modified dibenzylcyclooctynol (DIBO). The methodology will make it possible to dissect the mechanisms that underlie altered glycoconjugate recycling and storage in disease and identify the glycoconjugates whose cell surface localization or secretion are affected.

Keywords

click chemistry; glycosyltransferase; *N*-glycans; sialic acid; trafficking

The bio-orthogonal chemical reporter strategy is an emerging technology for the visualization and isolation of glycoconjugates of living cells and model organisms.^[1] It exploits promiscuity of the biosynthetic machinery, which makes it possible to incorporate monosaccharides that have a unique chemical functionality (the reporter) into glycans of 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 due to their small size and virtual absence in biological systems.^[2] They can be tagged by Staudinger ligation using modified phosphines,^[3] copper(I)-catalyzed cycloaddition with terminal alkynes (CuAAC),^[4] or by strain-promoted alkyne-azide cycloaddition (SPAAC).^[5]

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Metabolic labeling with azido-containing monosaccharides results in the tagging of different class of glycans including *N*- and *O*-linked glycoproteins, proteoglycans and glycolipids because they share a common pool of azide-modified nucleotide-sugar.^[5a] A growing body of data supports that various types of glycolipids and glycoproteins localize and recycle differently in the context of disease 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 demonstrate that the inherent substrate specificity of recombinant ST6Gal I sialyltransferase^[7] can be exploited for the selective labeling of *N*-linked glycans of living cells with azido-modified sialic acid. This Selective Exo-Enzymatic Labeling (SEEL) strategy will offer unique abilities 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 CMP-sialic acid derivative **4** was prepared having an azide at C-9 of the sialic acid moiety. This compound was easily synthesized starting from the methyl ester of sialic acid (**1**) by selective tosylation of the C-9 primary hydroxyl to give **2**, which was treated with sodium azide in a mixture of acetone and water that was heated under reflux to afford **3** in an almost quantitative yield (Scheme 1). Surprisingly, the methyl ester of **3** was removed under these conditions to give the required free carboxylic acid. Condensation of **3** with CTP in the presence of the recombinant CMP-sialic acid synthetase from *Neisseria meningitis* [EC 2.7.7.43]^[9] and the inorganic pyrophosphatase from *Saccharomyces cerevisiae* [EC 3.6.1.1] gave, after purification by size exclusion column chromatography, CMP-Neu5Ac9N₃ (**4**). Incubation of *N*-acetylactosamine and CMP-Neu5Ac9N₃ (**4**) in the presence of ST6Gal I led to almost quantitative formation of Neu5Ac9N₃α(2,6)Galβ(1,4)GlcNAc (Scheme S1) highlighting that the C-9 azido moiety of **4** is tolerated by the enzyme. Kinetic analysis of the enzymatic transformation showed that the azido 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}* (Figure S1).

Fibroblasts were incubated with ST6Gal I in the presence of CMP-Neu5Ac9N₃ (**4**) or CMP-Neu5Ac (2 mM) for 2 h at 37 °C. In addition, cells were metabolically labeled by feeding peracetylated *N*-α-azidoacetylmannosamine (Ac₄ManNAz) or peracetylated *N*-acetylmannosamine (Ac₄ManNAc), 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 modified with biotin (S-DIBO **5**, 30 μM)^[10] for 1 h followed by staining with avidin-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. As can be seen in Figure 1, both enzymatic- and metabolic labeling approaches resulted in robust staining, whereas the control treatments gave very low responses. Analysis of cell lysates by SDS-PAGE and Western blotting further confirmed the requirement of compound ST6Gal I and compound **4** for cell labeling (Figure 2). A concentration of 100 μM of **4** was sufficient for robust labeling (Figure S2). Furthermore, robust labeling could be achieved in other cell types such as CHO-K1. As expected, the staining was enhanced when the glycosylation CHO mutant Lec2 was employed as these cells exhibit greatly reduced sialic acid incorporation due to an inactivated CMP-sialic acid transporter (Figure S3).

Next, the selectivity of the cell surface labeling by ST6Gal I for *N*-glycans was evaluated by SDS-PAGE of cell lysates that were untreated or exposed to peptide-*N*-glycosidase F (PNGase F), followed by blotting and probing with an anti-biotin antibody conjugated to HRP (Figure 3). Treatment with PNGase F,^[11] which is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex

oligosaccharides of *N*-linked glycoproteins, completely abolished staining demonstrating that ST6Gal I had only modified *N*-linked glycans of cell surface glycoproteins. A similar experiment using a lysate obtained from cells metabolically labeled with ManNAz showed residual staining (Figure S4) demonstrating that this approach tags other classes of glycoconjugates in addition to *N*-linked glycans. As additional demonstration of ST6Gal I selectivity, glycolipids were isolated from cells labeled with ST6Gal I and **4** and analyzed by mass spectrometry.^[12] No glycolipids were detected modified by azido-containing sialic acid (Figure S5).^[13] To further support the selectivity of staining by ST6Gal I, labeling efficiencies were measured in fibroblasts grown in the presence of kifunensine (Kf; 10 μ M). This compound inhibits Golgi mannosidase I thereby causing a complete shift in the structure of the *N*-linked oligosaccharides from complex chains to Man₉(GlcNAc)₂ structures that can not be modified by sialosides.^[14] This treatment completely abolished staining (Figure 3).

Next, attention was focused on the use of the SEEL technology for the visualization of trafficking of *N*-linked glycoconjugates. Thus, fibroblasts were enzymatically labeled with ST6Gal I and CMP-Neu5Ac9N₃ (**4**) and the resulting azido-modified *N*-linked glycans were visualized by confocal microscopy after cycloaddition with biotin-modified S-DIBO (**5**) and treatment with streptavidin-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 the cell membrane,^[10, 15] also showed no staining of intracellular structures (*e.g.* Golgi), confirming that exogenously administered ST6Gal I only labels cell surface glycans (Figures 4a and S6). Cell surface glycans of CHO-K1 cells could be readily labeled, and as expected, more robust staining was observed in the glycosylation CHO mutant Lec2 (Figure S7). Next, cells were treated with chloroquine to disrupt lysosomal pH and prevent efficient catabolism within this compartment (Figure 4b). Under these conditions, labeled glycoconjugates localized within intracellular vesicles resembling late endosomes/lysosomes, highlighting that the technology can be employed to study trafficking of cell surface glycoconjugates.

Metabolic labeling of azido-containing monosaccharides has been successfully employed for the detection and isolation of sialylated, fucosylated and *O*-GlcNAc modified proteins of mammalian cells. Recently, the metabolic labeling strategy has been expanded to plants^[16] and bacteria^[17] by feeding azido-modified KDO or alkyne derivatized fucose, respectively. In an alternative approach, azido-modified glycoconjugates have been formed by exposing cells or cell lysates to an azido-modified sugar nucleotide and a glycosyltransferase. For example, an engineered galactosyltransferase can selectively transfer UDP-*N*- α -azidoacetyl galactosamine (UDP-GalNAz) to *O*-GlcNAc modified proteins.^[18] Furthermore, it has been shown that GDP-6-azide-fucose is readily accepted by a recombinant *H. pylori* α (1,3)-fucosyltransferase and was successfully employed to image glycans of 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 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 analog modified at C-9 by an azido moiety. It was found that native cells have sufficient acceptor sites for ST6Gal I to achieve robust staining without a 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 for example 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 proteins involved in retrograde transport through the endosomal network or in enzymes responsible for the lysosomal catabolism of glycosylated compounds.^[20] The Selective Exo-Enzymatic Labeling (SEEL) strategy described here will provide a unique ability 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 the molecules whose cell surface localization or secretion are most affected. Many sialyltransferases exhibit unique substrate specificities^[21] and thus it is the expectation that SEEL can be extended to other types of glycoconjugates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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-Neu5Ac9N₃ (4) or CMP-Neu5Ac in the presence of ST6Gal I for 2 h at 37 °C. Next, the cells were incubated with S-DIBO (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 $\pm$ SD. S-DIBO (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 that results in the modification of intra- and extracellular glycoconjugates.

Figure 2.

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

Figure 3.

Determination of the labeling specificity of recombinant ST6Gal I. Fibroblasts grown for 2 days without any drug treatment or in the presence of Kf (10 μ M) were incubated with CMP-Neu5Ac9N₃ (**4**, 2 mM) in the presence of ST6Gal I for 2 h at 37 °C. Next, the cells were incubated with DIBO (**6**, 30 μ M) for 1 h at RT. Cell lysates (untreated or PNGase F treated) were resolved by SDS-PAGE and the blot was probed with an anti-biotin antibody conjugated to HRP. Total protein loading was confirmed with β -actin.

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-Neu5Ac9N₃ (4) in the presence of ST6Gal I for 2 h at 37 °C. Next, live cells were incubated with S-DIBO (5) or DIBO (6) for 1 h at RT and streptavidin-Alexa Fluor 568. After washing, fixing and staining for the nucleus with the far-red-fluorescent dye TO-PRO-3 iodide, cells were visualized by confocal microscopy. Images of cells labeled with Alexa Fluor (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-Neu5Ac9N₃ (4) in the presence of ST6Gal I for 2 h at 37 °C. Next, live cells were incubated with DIBO (6) for 4 h at 37 °C in the absence or presence of chloroquine (50 μM). After incubation with streptavidin-Alexa Fluor 568 nm, cells were visualized by confocal microscopy. Images of cells labeled with Alexa Fluor are shown in red.

Scheme 1.

Chemical synthesis of sialic acid analogs modified by a chemical reporter. Chemical structures of S-DIBO (**5**) and DIBO (**6**)