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Communication

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Chemoenzymatic Probes for Detecting and Imaging Fucose-α(1-2)-galactose Glycan Biomarkers

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Supporting Information

ABSTRACT: The disaccharide motif fucose-α(1-2)-galactose (Fucα(1-2)Gal) is involved in many important physiological processes, such as learning and memory, inflammation, asthma, and tumorigenesis. However, the size and structural complexity of Fucα(1-2)Gal-containing glycans have posed a significant challenge to their detection. We report a new chemoenzymatic strategy for the rapid, sensitive detection of Fucα(1-2)Gal glycans. We demonstrate that the approach is highly selective for the Fucα(1-2)Gal motif, detects a variety of complex glycans and glycoproteins, and can be used to profile the relative abundance of the motif on live cells, discriminating malignant from normal cells. This approach represents a new potential strategy for biomarker detection and expands the technologies available for understanding the roles of this important class of carbohydrates in physiology and disease.

Defects in glycosylation are a hallmark of many human diseases, including autoimmune disorders, neurodegenerative diseases, and cancer.[1] As part of a broader program to understand the role of protein glycosylation in disease, we are investigating the glycan motif fucose-α(1-2)-galactose (Fucα(1-2)Gal). Fucα(1-2)Gal is found on the nonreducing terminus of a large family of important glycans, including blood group H1 and H2, Globo H, Fuc-GM1, Lewis B, and Lewis Y. These glycans play roles in learning and memory[2] and contribute to asthma, inflammation, and tumorigenesis.[3] However, the size and structural complexity of Fucα(1-2)Gal glycans, which range from simple linear to large branched structures, has posed a significant challenge to their detection and study.

Antibodies or lectins are typically used to detect glycans, but these methods often suffer from weak binding affinity and limited specificity, displaying cross-reactivity toward multiple glycan epitopes.[4] An alternative method, metabolic labeling, provides a powerful and versatile approach to the detection of glycans.[5] However, metabolic labeling requires the uptake of non-natural monosaccharide analogues into biosynthetic pathways, which allows for their incorporation into numerous glycans. As a consequence, disaccharide or trisaccharide motifs of specific sugar composition and glycosidic linkage, such as Fucα(1-2)Gal, cannot be uniquely detected. Also, the non-natural sugar must compete with natural sugars and thus is often incorporated substoichiometrically into glycoconjugates, reducing detection sensitivity. Given the diversity of carbohydrate structures at the cell surface, there is an urgent need to develop new technologies for the specific detection of complex glycans.

In this communication, we report the first strategy for the rapid, sensitive, and selective detection of Fucα(1-2)Gal glycans. Our approach capitalizes on the substrate tolerance of a bacterial glycosyltransferase to covalently tag specific glycans of interest with a non-natural sugar analogue. As the reaction proceeds in quantitative yield, stoichiometric addition of the non-natural sugar can be achieved, affording higher detection sensitivity relative to antibodies, lectins, and metabolic labeling. Although chemoenzymatic approaches have been reported for two saccharides, *O*-linked-β-*N*-acetylglucosamine(*O*-GlcNAc)[6] and *N*-acetyllactosamine (LacNAc),[7] this study demonstrates the first direct detection of complex oligosaccharides, opening up the potential to track a broad range of physiologically important glycans.

We exploited the bacterial homologue of the human blood group A antigen glycosyltransferase (BgtA), which transfers *N*-acetylgalactosamine (GalNAc) from UDP-GalNAc onto the C-3 position of Gal in Fucα(1-2)Gal structures.[8] We reasoned that BgtA might tolerate substitution at the C-2 position of GalNAc, allowing for the selective tagging of Fucα(1-2)Gal with an azido or ketone functionality (Figure 1A). To test the approach, Fucα(1-2)Gal substrate **1** was synthesized via reductive amination of 2′-fucosyllactose with *p*-nitrobenzylamine and sodium cyanoborohydride (Figures 1B and S1, Supporting Information (SI)). Indeed, treatment of **1** with BgtA and either UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz, **2**) or UDP-2-deoxy-2-(acetonyl)-β-D-galactopyranoside (UDP-ketoGal, **3**) led to complete conversion to the desired products **4** and **5**, respectively, after 12 h at 4 °C, as determined by liquid chromatography−mass spectrometry (LC−MS; Figures 1B, S2, and S3, SI). Kinetic analysis revealed an apparent k_{cat}/K_m value of 5.7 nM^{-1} min^{-1} for UDP-GalNAz, approximately 7-fold lower than the value of 40.4 nM^{-1} min^{-1} obtained for the natural UDP-GalNAc substrate (Figure S4, SI). Subsequent reaction with an aza-dibenzo-cyclooctyne-biotin derivative (ADIBO-biotin, **6**; Figure S2, SI) using

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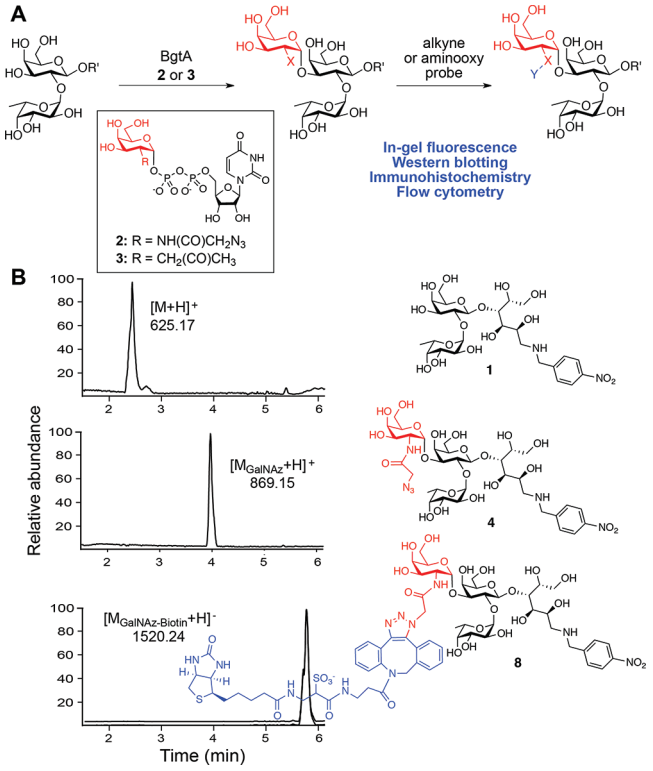

Figure 1. (A) Chemoenzymatic strategy for the detection of Fucα(1-2)Gal glycans. (B) Labeling of substrate **1**. LC−MS traces monitoring the reaction progress at time 0 (top), 12 h after the addition of BgtA and **2** (middle), and 3 h after the addition of ADIBO-biotin **6** (bottom). See SI for details.

copper-free click chemistry (3 h, rt) or with the aminooxy-biotin derivative **7** (Figure S2, SI; 24 h, rt) afforded the biotinylated products **8** and **9**, respectively, in quantitative yield (Figures 1B, S2, and S3, SI).

Having demonstrated that BgtA accepts non-natural substrates, we profiled the glycans detected by BgtA using carbohydrate microarrays from the Consortium for Functional Glycomics.[9] Glycosylation reactions with BgtA and UDP-GalNAz were performed on 611 different glycans simultaneously at three different time points. Following reaction with ADIBO-biotin, biotinylated glycans were detected using Cy5-conjugated streptavidin. Strong fluorescence labeling of Fucα-(1-2)Gal structures was observed within 0.5 h (Figure 2A). Notably, the top 26 glycans labeled contained terminal Fucα(1-2)Gal structures, highlighting the specificity of the chemoenzymatic approach. Moreover, ~91% of the terminal Fucα(1-2)Gal containing glycans were labeled on the array, including the H1 (68, 69) and H2 antigens (76, 77), the ganglioside Fuc-GM1 (65), and the Globo H antigen (60), a hexasaccharide overexpressed on breast, lung and prostate tumors[3b,c] and associated with poor prognosis (Figures 2A and S5, SI).[3d,e] A wide variety of linear (e.g., 501, 75, and 60) and branched structures (e.g., 450, 362, and 457) containing the Fucα(1-2)Gal motif were efficiently labeled (Figures 2A and S6, SI). Modifications of the core disaccharide, such as replacing Gal with GlcNAc, or changing the α(1-2) linkage to an α(1-3), α(1-4), or β(1-3) linkage eliminated the enzymatic labeling by BgtA (e.g., 80, 81, and 82; Figure S6, SI).

Consistent with a previous report,[8] BgtA exhibited more relaxed specificity toward structures appended to the reducing end of the Gal residue. Specifically, glycans containing a β(1-3)GalNAc, β(1-3)GlcNAc, β(1-4)GlcNAc, or β(1-4)Glc in this position were efficiently labeled (e.g., 62, 66, 74, and 78, respectively; Figures 2A and S5, SI). Although moderate structural substitutions of the GlcNAc were tolerated such as 6-*O*-sulfation (e.g., 501 and 222; Figure 2A), branching at this position via α(1-3) or α(1-4) fucosylation led to weak labeling,

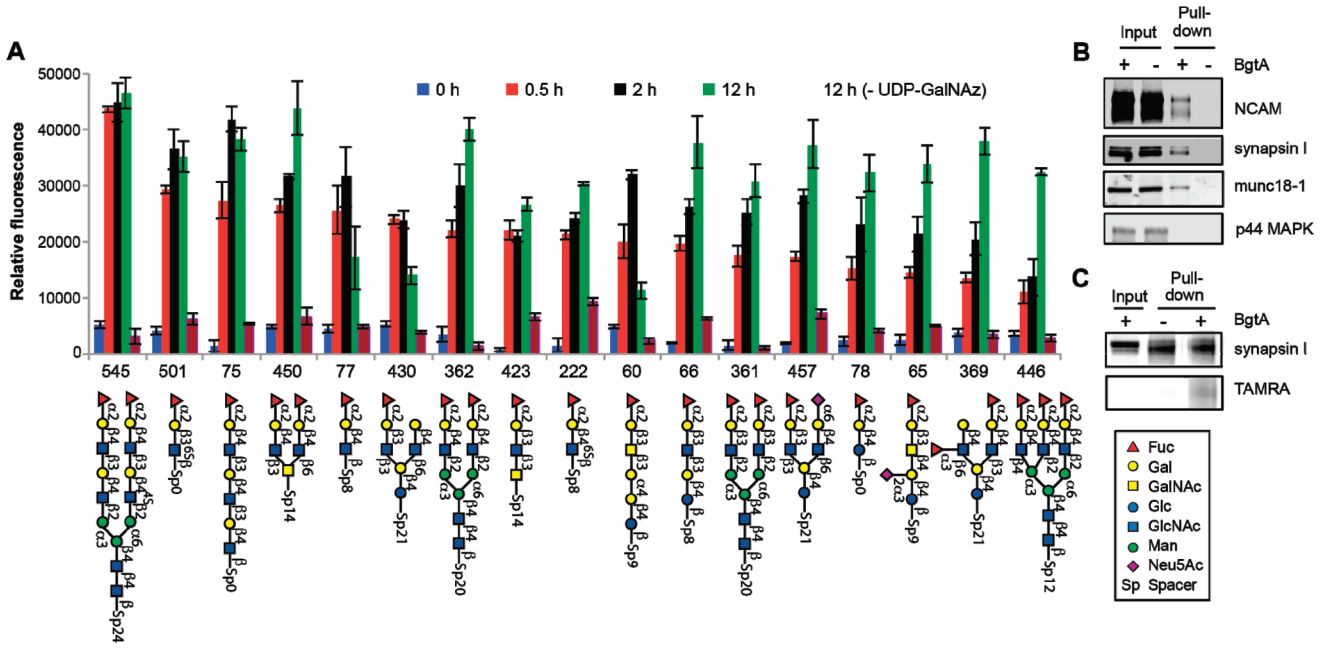

Figure 2. (A) Time course analysis using glycan microarrays. Representative structures from the top 26 glycans with the highest relative fluorescence intensity after 0.5 h are plotted, all of which represent terminal Fucα(1-2)Gal structures. UDP-GalNAz was omitted from some of the reactions as a control (12 h, -UDP-GalNAz). (B) Chemoenzymatic detection of endogenous Fucα(1-2)Gal glycoproteins from neuronal lysates. (C) Chemoenzymatic detection of Flag-tagged synapsin I expressed in HeLa cells. See SI for experimental details.

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as in the case of the Lewis B (61) and Lewis Y (72, 73) antigens, or no appreciable labeling (e.g., 71, and 363; Figures S5 and S7, SI). Interestingly, we also observed weak labeling of Galβ(1-4)GlcNAc structures on the glycan array (Figure S7, SI). However, these structures also exhibited high background signal even in the absence of UDP-GalNAz, and BgtA failed to label *p*-nitrophenyl 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-β-D-glucopyranoside (Galβ(1-4)GlcNAc-pNP) in solution (2 h, 25 °C), suggesting that Galβ(1-4)GlcNAc structures are not covalently labeled by BgtA. Together, these studies demonstrate the strong specificity of BgtA for Fucα(1-2)Gal structures and the power of glycan microarrays to rapidly profile the specificities of glycosyltransferases for the development of chemoenzymatic detection strategies.

To determine whether the approach could be used to track Fucα(1-2)Gal glycoproteins in complex cell lysates, we labeled proteins from rat brain extracts with BgtA and UDP-GalNAz, followed by the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction with tetramethyl-6-carboxyrhodamine dye **10** (alkyne-TAMRA; Figure S2, SI). We observed strong fluorescence labeling of Fucα(1-2)Gal glycoproteins, with minimal nonspecific labeling in the absence of BgtA, UDP-GalNAz, or alkyne-TAMRA (Figure S8, SI). To confirm further the specificity of the reaction, we labeled the lysates with the alkyne-biotin derivative **11** (Figure S2, SI), captured the biotinylated proteins using streptavidin resin, and immunoblotted for the presence of known Fucα(1-2)Gal glycoproteins.[10] Neural cell adhesion molecule (NCAM), synapsin I, and munc18-1 were all chemoenzymatically labeled and detected in the presence, but not in the absence, of BgtA (Figure 2B). In contrast, p44 mitogen-associated protein kinase (p44 MAPK), a protein that has not been shown to be fucosylated, was not detected. Glycosylated synapsin I was also readily observed following overexpression of Flag-tagged synapsin I in HeLa cells, chemoenzymatic labeling of the lysates with alkyne-TAMRA, synapsin immunoprecipitation, and visualization using an anti-TAMRA antibody (Figure 2C). Importantly, UEAI lectin affinity chromatography failed to pull-down and detect glycosylated synapsin I when performed on the same scale (Figure S9, SI). Moreover, previous studies have reported that the Fucα(1-2)Gal-specific antibody A46-B/B10 does not immunoprecipitate glycosylated synapsin I from the same neuronal lysates.[10a] Thus, our chemoenzymatic approach enables the highly sensitive detection of glycoproteins and provides a variety of different enrichment strategies and readouts for the Fucα(1-2)Gal motif.

We next investigated whether the chemoenzymatic strategy could be used to image Fucα(1-2)Gal glycans in cells. HeLa cells overexpressing Flag-tagged synapsin I were fixed, permeabilized, and chemoenzymatically labeled on coverslips with BgtA and UDP-GalNAz. CuAAC chemistry was then performed using an alkyne-functionalized Alexa Fluor 488 dye (**12**; Figure S2, SI) to install a fluorescent reporter onto the Fucα(1-2)Gal glycans. Strong fluorescence labeling was observed in cells transfected with synapsin I, and the labeling showed excellent colocalization with intracellular synapsin I expression (Figure 3A). No labeling of cells was observed in the absence of BgtA, and only weak labeling of endogenous Fucα(1-2)Gal glycoproteins was seen in the absence of synapsin I overexpression (Figures 3A and S10, SI), confirming the specificity of the in situ chemoenzymatic reaction.

As the Fucα(1-2)Gal epitope has been reported to be a useful biomarker for cancer progression and prognosis,[3d,e] the ability to detect Fucα(1-2)Gal glycan levels on the surface of cancer cells would facilitate investigations into Fucα(1-2)Gal as a diagnostic or prognostic marker and a therapeutic target for cancer vaccines. However, antibodies and lectins that bind Fucα(1-2)Gal have been shown to cross-react with other sugar epitopes[4a,b] such as β-linked Fuc[4a] or recognize an incomplete subset of Fucα(1-2)Gal glycans,[4c] indicating the need for more selective, yet comprehensive, high-affinity detection methods. We therefore applied our chemoenzymatic approach to the detection of Fucα(1-2)Gal glycans on live cancer cells. Cells from the human breast adenocarcinoma cell line MCF-7 were chemoenzymatically labeled with BgtA and UDP-GalNAz for 1 h at 37 °C. After reaction with ADIBO-biotin (1 h, rt), Fucα(1-2)Gal glycans were detected using streptavidin conjugated to Alexa Fluor 488 dye. Membrane-associated fluorescence was observed for cells treated with both BgtA and UDP-GalNAz, whereas no labeling was detected for control cells labeled in the absence of BgtA (Figure 3B).

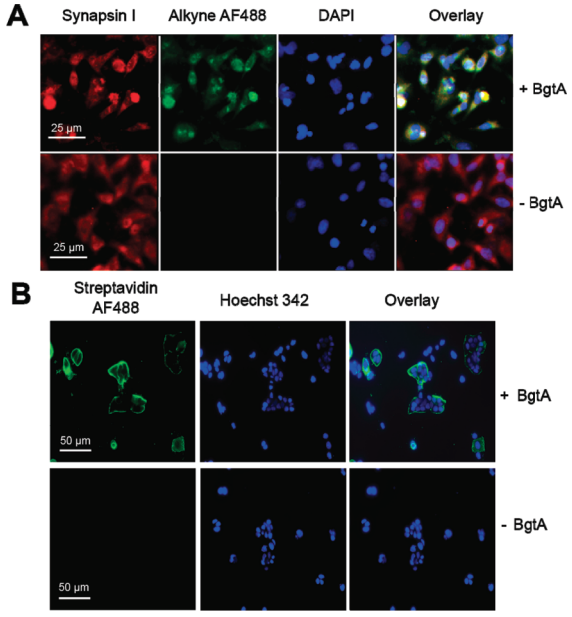

Figure 3. (A) Fluorescence detection of Fucα(1-2)Gal glycans (green) in HeLa cells shows excellent colocalization (yellow) with Flag-tagged synapsin I (red). No labeling was observed in the absence of BgtA. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). (B) Fluorescence detection of Fucα(1-2)Gal glycans (green) on live MCF-7 cells. Nuclei were stained with Hoechst 342 (blue).

We next compared the expression levels of Fucα(1-2)Gal glycans across different cancer and noncancer cell lines. MCF-7 (breast cancer), MDA-mb-231 (highly invasive breast cancer), H1299 (lung cancer), LnCAP (prostate cancer), and primary prostrate epithelial cells (PrEC) cells were chemoenzymatically labeled in suspension with BgtA and UDP-GalNAz (2 h, 37 °C), reacted with ADIBO-biotin (1 h, rt), and stained with the streptavidin–Alexa Fluor 488 conjugate (20 min, 4 °C). As shown by flow cytometry analysis, MDA-mb-231, MCF-7, and LnCaP cells displayed the highest levels of fluorescence (Figure 4), consistent with reports of high Globo H expression on mammary and prostate tumors.[3b,c] H1299 cells, a model for nonsmall cell lung carcinoma and also reported to express Globo H,[3f] showed lower Fucα(1-2)Gal expression. Importantly, flow cytometry analysis revealed a 53% increase in Fucα(1-2)Gal expression on the surface of LnCAP cells compared to noncancerous PrEC cells. These results

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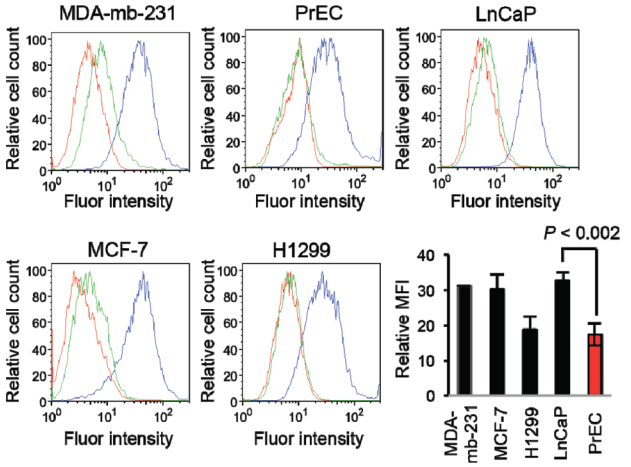

Figure 4. Flow cytometry analysis of the relative expression levels of Fucα(1-2)Gal glycans across various cancer cell lines, with comparison to noncancerous PrEC cells. Cells were untreated (red) or chemoenzymatically labeled in the presence (blue) or absence (green) of BgtA. Quantification of the mean fluorescence intensity (MFI) relative to cells labeled in the absence of BgtA is shown on the right. Error bars represent data from duplicate (MCF-7, MDA-mb-231, H1299) or triplicate (LnCAP, PrEC) experiments.

demonstrate that our chemoenzymatic labeling approach can readily discriminate cancerous cells from normal cells, providing a new potential strategy for biomarker detection. The method could be particularly useful for the detection of prostate cancer from tissue biopsies, as the current standard of PSA detection to diagnose prostate cancer has a significant false-positive rate, leading to overtreatment.[11] In addition to histological detection, our chemoenzymatic approach could potentially provide a new strategy to distinguish normal PSA from tumorigenic PSA, which is reported to have higher levels of Fucα(1-2)Gal glycosylation.[12]

In conclusion, we have developed a new chemoenzymatic strategy that detects Fucα(1-2)Gal glycans with improved efficiency and selectivity over existing methods. Our strategy detects a variety of complex Fucα(1-2)Gal glycans and glycoproteins and permits living cells or complex tissue extracts to be rapidly interrogated. We anticipate that the strategy will accelerate both the discovery of new Fucα(1-2)Gal glycoproteins and advance an understanding of the biological roles of this important sugar in neurobiology and cancer. Moreover, this study represents a proof-of-concept that chemoenzymatic labeling strategies can be extended to more complex oligosaccharides. Future studies will expand chemoenzymatic detection approaches to a broad range of glycans to provide a powerful new set of tools for glycomics research.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures and detailed experimental procedures, including the synthesis of **1**, chemoenzymatic labeling reactions, LC−MS analyses, detection of glycoproteins, immunostaining, flow cytometry analysis, and complete refs 9 and 11. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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