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A Tandem Enzymatic Approach for Detecting and Imaging Tumor-Associated Thomsen–Friedenreich Antigen Disaccharide

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Supporting Information

ABSTRACT: The disaccharide galactose-β1,3-*N*-acetylgalactosamine (Galβ1,3-GalNAc) attached to serine and/or threonine residues of proteins, also known as the Thomsen–Friedenreich (TF) antigen, is highly expressed in various types of human carcinomas. It has been shown to contribute to tumor development, progression, and metastasis. However, current methods have limited power in detecting and imaging TF antigens among a variety of complex cell-surface glycans. Here we describe a tandem enzymatic strategy to detect and label TF antigen disaccharide with high sensitivity and selectivity. We demonstrate that this strategy enables detection of TF antigens on proteins, profiling and identification of unknown TF antigen-modified glycoproteins, and simultaneous labeling of multiple forms of complex glycan motifs on the same cell. This approach expands the capability of glycan labeling to probe the functional role of TF antigens in cancer biology.

Abnormal changes in cell-surface glycosylation are generally considered as a hallmark of cancer.[1] These changes include both the altered expression of naturally occurring glycans and the expression of novel glycans. Studies have identified a number of glycans whose expressions have been used as reliable indicators of cell malignancy (also termed tumor-associated glycan antigens).[2] Among these glycan antigens, the disaccharide galactose-β1,3-*N*-acetylgalactosamine (Galβ1,3-GalNAc) attached to serine and/or threonine residues of proteins, also known as the Thomsen–Friedenreich (TF) antigen, is highly expressed in about 90% of primary human carcinomas.[3] Increasing experimental evidence has shown that the occurrence of TF antigens is functionally important in cancer development, metastasis, and angiogenesis.[4] Its broad expression and strong correlation with cancer have attracted increased exploration into its potential use as a cancer diagnostic marker and in cancer immunotherapy.[5]

The ability to detect and image tumor-associated glycans on the cellular level offers a powerful tool to understand the functional role of glycans in cancer biology. On the other hand, because of the complexity of glycans, increasing the sensitivity and selectivity of glycan detection remains a persistent challenge. Current methods typically rely on antibodies or lectins, which have laid the foundation of functional glycomics to date. However, because of the inherent properties of glycans, these methods often suffer from relatively low glycan binding affinity and cross-reactivity toward multiple glycan structures.[6] Alternatively, Bertozzi and others developed an elegant bioorthogonal chemical reporter strategy.[7] In this strategy, monosaccharides with bioorthogonal functionalities compete with their natural counterparts for incorporation into glycans. Successful incorporation followed by covalent conjugation with different probes allows for sensitive detection of cell-surface glycans. However, the use of monosaccharides as chemical reporters has generally precluded the detection of high-order glycans with specific sugar compositions and glycosidic linkages. Thus, this strategy has limited power in labeling complex tumor-associated glycan structures.

In this communication, we report a novel tandem enzymatic strategy for sensitive and selective detection of TF antigen disaccharide. We take advantage of the exquisite substrate specificity of two bacterial glycosyltransferases that act in a tandem manner to convert TF antigen disaccharide to a tetrasaccharide containing a bioorthogonal functionality. The resulting glycoconjugates can then be detected with fluorescent and/or affinity probes through copper(I)-mediated azide–alkyne cycloaddition (CuAAC) or copper-free click chemistry. Although chemoenzymatic strategies for the detection of several glycans have been reported,[8] strategies for the detection of tumor-associated glycans such as TF antigens have not been explored.

In our previous studies, we characterized two bacterial glycosyltransferases: the fucosyltransferase WbwK and the *N*-acetylgalactosaminyltransferase BgtA. We demonstrated that WbwK possesses strict substrate specificity toward glycans containing Galβ1,3-GalNAc-α (TF antigen disaccharide) structures and converts them to Fucα1,2-Galβ1,3-GalNAc-α structures (Table S1 in the Supporting Information).[9] In addition, BgtA was shown to display a strong substrate preference for Fucα1,2-Galβ structures to form GalNAcα1,3-(Fucα1,2)-Galβ, with much more relaxed specificity toward structures appended to the Gal residue.[8b,10] More importantly, BgtA can readily transfer GalNAc analogues containing bioorthogonal functionalities that permit sensitive detection

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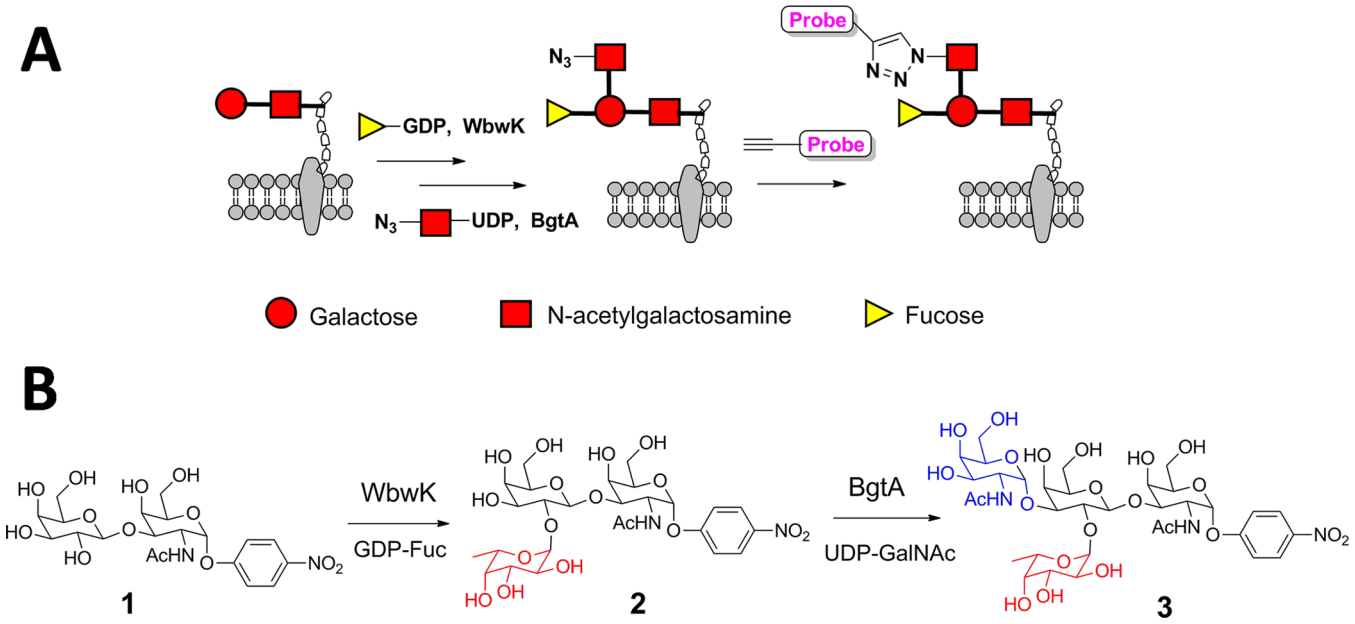

Figure 1. (A) Labeling of TF antigens with a tandem enzymatic approach. (B) Validation of the strategy with an in vitro tandem enzymatic reaction.

of Fucα1,2-Gal-containing glycans in cell lysates and in live cells.[8b] Given the unique properties of these two glycosyltransferases, we reasoned that they could work in a tandem manner to install fluorescent and/or affinity probes for sensitive and selective detection of TF antigen disaccharide (Figure 1A). Furthermore, we could possibly achieve simultaneous labeling of both TF antigen disaccharide- and Fucα1,2-Gal-containing glycans in one experimental setting. To test our hypothesis, we first carried out an in vitro tandem enzymatic reaction in which TF antigen disaccharide **1** was used as the starting substrate (Figure 1B). WbwK and BgtA were expressed and affinity-purified as previously reported.[9,10] Notably, both enzymatic reactions proceeded quantitatively to yield the desired products **2** and **3**, respectively, after 2 h at 37 °C, as determined by liquid chromatography and mass spectrometry (Figure S1 in the Supporting Information).

Having validated the reactions carried out by the two enzymes, we tested whether this approach could be used to label known TF antigen-expressing glycoproteins. Two such glycoproteins are the transmembrane mucin protein MUC1, which is overexpressed in many epithelial cancers and strongly associated with metastasis, and the high-molecular-weight splicing variant of the cell-surface adhesion molecule CD44v6, whose expression is upregulated in a majority of squamous cell carcinomas.[3] To eliminate the possible labeling of Fucα1,2-Gal glycans, we performed a blocking step by treating cell lysates with BgtA and the natural substrate UDP-GalNAc. In the subsequent labeling with the WbwK–BgtA enzymatic reaction, UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz) was used to introduce the bioorthogonal functionality. This was followed by the CuAAC reaction with alkyne–biotin, capture of the biotinylated proteins with streptavidin resins, and subsequent immunoblotting for the presence of the glycoproteins of interest. Control experiments conducted in the absence of UDP-GalNAz or GDP-Fuc showed comparable minimal labeling, indicating that blocking of the existing Fucα1,2-Gal glycans was complete (Figure 2). Both MUC1 and CD44v6 were readily detected after the treatment. Corresponding signals were absent in the control samples, indicating the

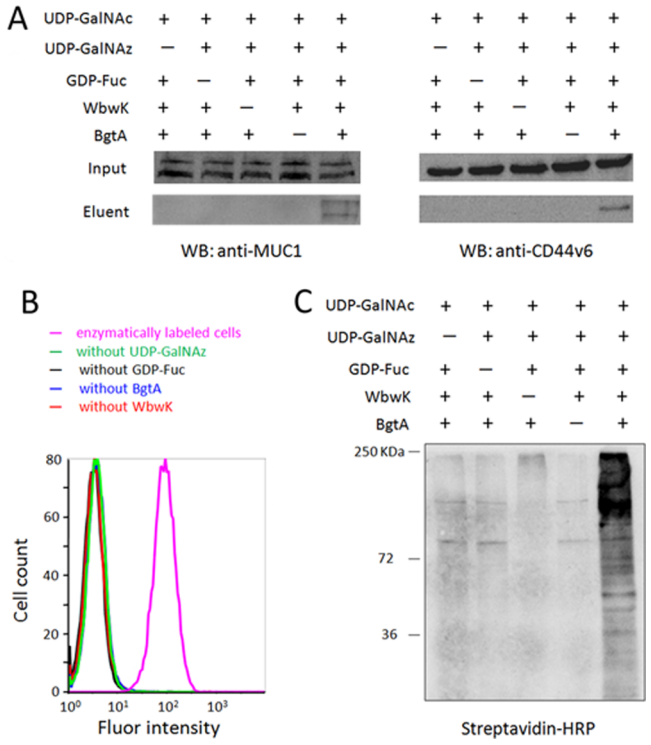

Figure 2. Labeling of TF antigen disaccharide with the tandem enzymatic strategy. Possible interference of Fucα1,2-Gal glycan labeling was eliminated by a blocking step with the natural substrate UDP-GalNAc by BgtA. (A) Labeling of known endogenous TF antigen-containing glycoproteins from cell lysates. (B) Flow cytometry analysis of Jurkat cell surface TF antigens that were chemoenzymatically labeled with AF647 DIBO-alkyne. (C) Labeling and detection of TF antigen-containing glycoproteins from MCF-7 cell lysates.

specificity of the reaction (Figure 2A). Notably, TF antigen-specific antibody failed to immunoprecipitate MUC1 when performed at the same scale, suggesting that the tandem enzymatic approach is able to achieve higher sensitivity (Figure S2).

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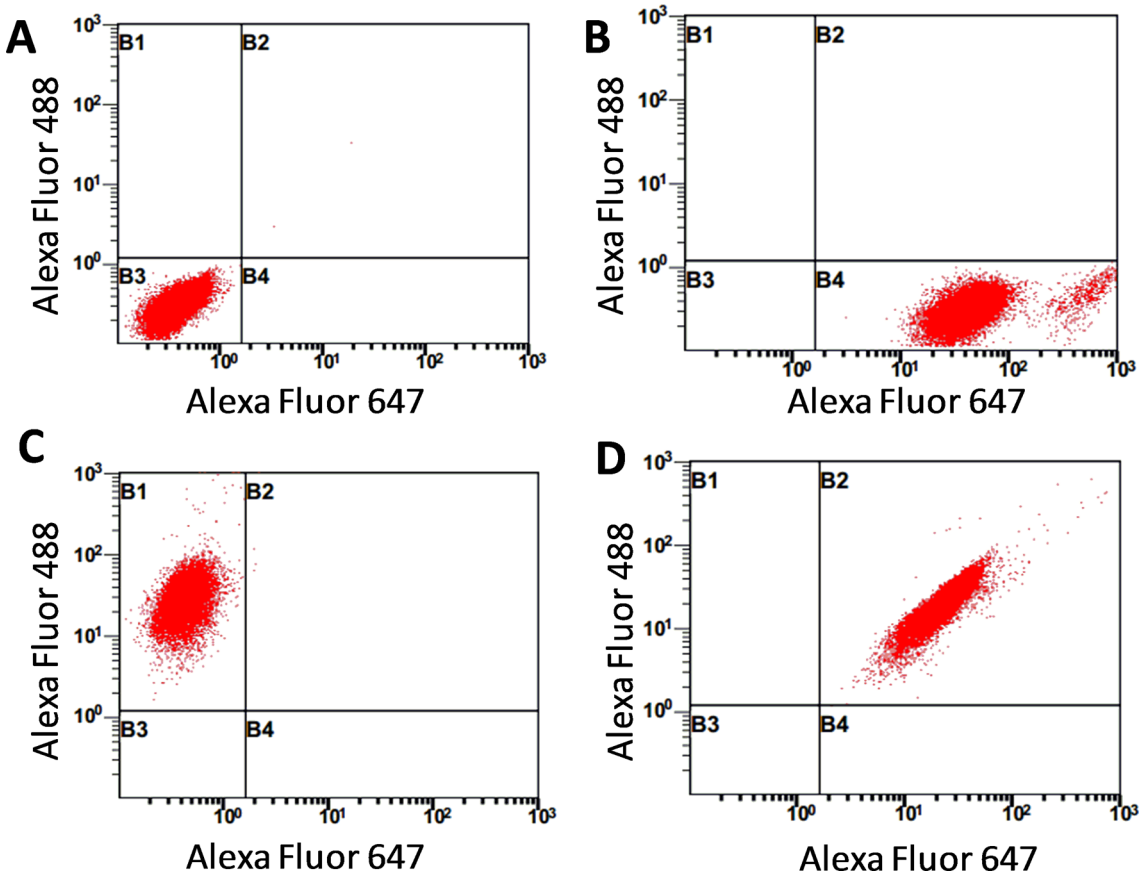

Figure 3. Dual labeling of TF antigen disaccharide and Fucα1,2-Gal glycans on live Jurkat cells. Incorporation of two different bioorthogonal functionalities (keto and azido) was achieved by tandem enzymatic reactions. The fluorescence of each sample was measured by flow cytometry. (A) − UDP-ketoGal, − UDP-GalNAz. (B) − UDP-ketoGal, + UDP-GalNAz. (C) + UDP-ketoGal, − UDP-GalNAz. (D) + UDP-ketoGal, + UDP-GalNAz.

Next we investigated whether the tandem enzymatic strategy could be used to label TF antigen disaccharides in cells. Human leukemia Jurkat cells were subjected to the tandem enzymatic reaction as described above to install GalNAz onto TF antigen disaccharides. After further conjugation using copper-free click chemistry with Alexa Fluor 647 (AF647) DIBO-alkyne (1 h, room temperature), the cells were analyzed by flow cytometry. Robust fluorescent labeling was detected in cells subjected to enzymatic treatments, whereas no labeling was observed for control cells labeled in the absence of various reaction components (Figure 2B). As a further confirmation, cell lysates of human breast adenocarcinoma MCF-7 cells were subjected to the tandem enzymatic labeling followed by the CuAAC reaction with alkyne−biotin and then detected with streptavidin-linked horseradish peroxidase (HRP). We observed a strong labeling signal from TF antigen-containing glycoproteins with minimal nonspecific labeling in the controls (Figures 2C and S3).

Although increased TF antigen expression has frequently been observed in many human carcinomas, there has been relatively little study of profiling the cellular proteins that carry the TF antigen. The ability to tag the TF antigen disaccharide with biotin permits efficient isolation and enrichment of TF antigen-containing glycoproteins for identification by mass spectrometry. Cell lysates of MCF-7 cells treated with enzymatic labeling were captured on alkyne resins using CuAAC. The alkyne resins containing the covalently bound glycoproteins were subjected to stringent washing and subsequent on-resin trypsin digestion to release peptides for nano-LC/MS analysis. The data acquisition and database searching methodologies are detailed in the Supporting Information. In total, we identified 788 putative TF antigen-modified proteins (Table S2). These proteins are involved in signal transduction, cell communication, intracellular transport, cell organization, and metabolism, suggesting that TF antigens likely play important roles in regulating these biological processes. The selectivity of this approach was further confirmed by probing three protein hits (epithelial cell adhesion molecule, integrin β1, and insulin-like growth factor 1 receptor) that are strongly associated with cancer progression and metastasis with the specific anti-TF antigen antibody (Figure S4). Thus, this tandem enzymatic approach enabled the first detailed profiling of TF antigen-modified proteins, which provides a foundation for further exploration of the functional role of TF antigens.

During the enzymatic labeling of TF antigen disaccharide, we performed an initial blocking step with the natural substrate UDP-GalNAc to eliminate the possible labeling of Fucα1,2-Gal glycans. We reasoned that if UDP-GalNAc were substituted with its unnatural analogue UDP-ketoGal in the blocking step, we could achieve simultaneous labeling of both TF antigen disaccharide and Fucα1,2-Gal glycans with two different fluorescent probes in one experimental setting. Jurkat cells were enzymatically labeled to incorporate ketone and azido functionalities, reacted with AF488-hydrazine and AF647 DIBO-alkyne in one pot, and then subjected to flow cytometry

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analysis. As shown in Figure 3, cells treated with UDP-ketoGal or UDP-GalNAz alone exhibited significant labeling relative to the background, indicating labeling of Fucα1,2-Gal glycans and TF antigen disaccharides, respectively (Figure 3B,C). As expected, cells treated with both UDP-ketoGal and UDP-GalNAz showed dual labeling with both fluorescent dyes (Figure 3D). Thus, TF antigen disaccharide and Fucα1,2-Gal glycans could be simultaneously visualized on the same cells.

In conclusion, we have developed a new tandem enzymatic strategy for the labeling and imaging of TF antigen disaccharide. Our strategy enables sensitive and selective detection of TF antigen disaccharide at different cellular levels, including proteins, complex cell lysates, and live cells. With this strategy, we have identified a number of new TF antigen-modified proteins. In addition, this strategy can be readily adapted for dual labeling of two different types of glycans on the same cells, further illustrating its powerful utility. This study significantly expands the scope of current glycan detection and will likely provide a powerful tool for functional glycomics research.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting figures and tables (XLSX) and detailed experimental procedures, including LC−MS analyses, comparative detection of TF antigens on MUC1 proteins, enzyme substrate analysis, and glycoprotein profiling. 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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