Differential Acceptance Specificity of Human Fucosyltransferases toward GDP-azidofucose and GDP-alkynylfucose as Glycosylation Probes

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KEYWORDS: Human fucosyltransferases, Fucosylation probes, Azide and alkyne, Acceptance specificity

ABSTRACT: Fucosylated glycans on glycoproteins and glycolipids play critical roles in functional regulations, significantly impacting human health and disease. Fucosylated glycans in humans are categorized into three types: core fucose, terminal fucose, and O-linked fucose, with each type contributing uniquely to physiological and pathological processes. Fucosylation is associated with various conditions, including cancer, autoimmune diseases, and developmental disorders, and it can also affect the efficacy of therapeutic antibodies. To investigate aberrant fucosylation in disease progression, azido- and alkynyl-fucose analogs have been utilized as orthogonal clickable probes. However, it remains unclear whether all human fucosyltransferases (FUTs) can accept these probes. In this study, we evaluated the utilization of GDP-fucose analogs, including GDP-6-azidofucose (GDP-6-Az-Fuc), GDP-6-alkynyl fucose (GDP-6-Alk-Fuc), and GDP-7-alkynyl fucose (GDP-7-Alk-Fuc) as donor substrates and natural N-glycans as acceptors and compared their specificity with GDP-fucose for nine human FUTs (FUT1-9) that are involved in the biosynthesis of glycoproteins. We determined key kinetic parameters and catalytic efficiencies of individual FUTs for their fucosylation of specific biantennary N-glycan acceptors to assess their incorporation of these analogs into glycoprotein N-glycans. Compared to GDP-fucose, all analogs were much weaker substrates for FUTs except FUT4 which exhibited better tolerance toward the analogs especially GDP-7-Alk-Fuc. Notably, GDP-7-Alk-Fuc was better accepted than GDP-6-Alk-Fuc and GDP-6-Az-Fuc as substrates for these nine human FUTs. These findings reveal the variability in the acceptance specificity and catalytic efficiency of the human FUT family toward the probes and emphasize the potential bias in identifying fucosylated glycans as therapeutic targets.

INTRODUCTION

Fucosylation is an important glycosylation reaction in the co-translational and post-translational modification processes that significantly impact protein function, cellular signaling, and the progression of diseases. By modifying glycans on glycoproteins, fucosylation enhances functional diversity, influencing enzymatic activity, proteolytic processing, cellular trafficking, and molecular interactions.[1] Even small changes in glycan composition, such as the addition or removal of individual sugar units, can lead to pathological conditions that are associated with diseases like Alzheimer's disease, cancer, and diabetes.[2–4]

The process of fucosylation is mediated by fucosyltransferases (FUTs), which use GDP-fucose as donor substrate and glycans as acceptors. GDP-fucose is synthesized through both *de novo* and *salvage* pathways and transported into the lumen of the endoplasmic reticulum (ER) and Golgi apparatus, where FUTs catalyze the transfer of fucose from GDP-fucose to specific acceptors to generate different fucosylated glycans with different glycosidic linkages.[5] The incorporation of terminal fucose residues to glycans, including structures such as Lewis and globo-series antigens, is catalyzed by various types of FUTs. In humans, there have been 13 FUTs identified, with different substrate preferences.[6,7] In general, FUTs1-9 are involved in the fucosylation of N- and O-glycans structures, whereas FUTs10-13 function as protein O-fucosyltransferases, modifying specific protein domains rather than general O-glycans.[6–14] The structural and functional impacts of N- and O-fucosylations on glycans and glycoconjugates demonstrate the multifaceted importance of fucosylation in maintaining physiological homeostasis and its potential as a target for therapeutic intervention.[15]

To identify the glycans through aberrant glycosylations that are associated with disease progression, metabolic glycan labeling has been developed as a powerful technique that allows for the visualization and tracking of the glycosylation process in living cells.[16–22] By introducing chemically modified sugars into cellular systems as a glycosylation probe, one can visualize the incorporation of the probe

into glycoconjugates.[16-25] Of many sugar probes used for the study of fucosylation, the clickable fucose analogs like 6-azidofucose (6-Az-Fuc)[16,19] or the less toxic and more cell permeable 6-alkynylfucose (6-Alk-Fuc)[19] and 7-alkynylfucose (7-Alk-Fuc)[24] have been used to enable selective labeling of glycans in living systems without disrupting native biological processes. This approach takes advantage of the high specificity and biocompatibility of the orthogonal [3+2] cycloaddition reactions between azides and alkynes pioneered by Sharpless, Meldal and Bertozzi[25] , allowing for visualization of labeled glycan structures with high sensitivity and low cytotoxicity in cellular environments. However, the substrate preferences of individual FUTs toward these analogs are still unclear, making it difficult to interpret their labeling selectivity and enzymatic incorporation. Characterizing these preferences is thus crucial for optimizing probe design and for ensuring accurate depiction of glycosylation patterns.[19,26] A deeper understanding of substrate specificity, supported by kinetic analyses, would thus help clarify these interactions and reduce off-target effects or bias interpretations. To address this issues, we and the Taniguchi/Kizuka group recently introduced the 7-Alk-Fuc analog to increase its acceptance and incorporation into glycoconjugates and evaluated the specificity in cell-based assays.[24] Subsequently, the Kizuka group further investigated the azido- and alkynyl fucose analogs and their GDP-derivatives as glycosylation probes in cell-based and enzymatic assays respectively with mouse and human FUTs and revealed differential acceptance of these probes[23]. Inspired by this work, we sought to evaluate these probes with all human FUTs, particularly those involved in protein N-glycosylation in order to understand their differential specificity and enzymatic incorporation efficiency and to assess the accuracy of such labeling study for identification of aberrant N-glycosylation in human diseases.[19,26] We focus our study on nine human fucosyltransferases (FUT1-9) and investigate their specificity toward GDP-fucose and the clickable GDP-fucose analogs, including GDP-6-Az-Fuc, GDP-6-Alk-Fuc and GDP-7-Alk-Fuc as donors and synthetic N-glycans as acceptors[24] to assess their differential catalytic efficiency of incorporating the probes into protein N-glycans.

RESULT AND DISCUSSION

The linkage specificity and functional roles of individual FUTs have been investigated to some extent previously.[6,7,9,10,12] For example, FUT1 is an α1,2-fucosyltransferase that transfers fucose to the terminal galactose of N-or O-glycans including type 2 chain (Galβ1-4GlcNAc-R) to create the H antigens. FUT2 is also an α1,2-fucosyltransferase that adds fucose to the terminal galactose of O-glycans or type 1 chains (Galβ1-3GlcNAc-R) and participate in the synthesis of N-glycans on glycoproteins and complex series of Lewis antigen epitopes with distinct acceptor specificity, influencing the diversity of N-glycan and Lewis antigen structures.[8]. FUT3-7 and FUT9 are α1,3/4-fucosyltransferases, involved in the addition of fucose to N-acetylglucosamine (GlcNAc) residues in different types of sugar chains, such as type 1 and type 2 lactosamine chains. While FUT3 and FUT5 add fucose in both α1,3 and α1,4 linkages, FUT4, FUT6 and FUT9 mainly create α1,3 linkage, with FUT9 involved in cell adhesion, embryonic development, and mature granulocytes. Additionally, FUT3, FUT5, and FU6 can add fucose to the internal GlcNAc of non-sialylated lactosamine repeats, FUT6 can also add fucose to the internal GlcNAc of alpha-2,6-sialylated lactosamine repeats, and FUT7 specifically adds fucose to the terminal GlcNAc of sialylated lactosamine repeats to create sialyl Lewis x structure. FUT8 is a unique α1,6-fucosyltransferase responsible for adding fucose to the innermost N-acetylglucosamine (GlcNAc) residue of N-linked glycans, a process known as core fucosylation. This modification plays a crucial role in regulating the structural and functional properties of glycoproteins, influencing protein folding, stability, receptor binding, and signal transduction. Core fucosylation has been implicated in various physiological processes, including immune modulation, cell growth, and development, and its disregulation is linked to cancer and inflammation. Therefore, detecting the core fucose level on the cell surface using molecular probes is a valuable approach for assessing glycosylation-related changes in glycoprotein function, cellular signaling, and disease-associated glycosylation.[9,10] Overall, N-fucosylations play significant roles in immune regulation, and their expression patterns often serve as biomarkers in cancer diagnostics.[11] On the other hand, O-fucosylations are often formed by directly attaching fucose to glycolipids or the serine- or threonine-linked glycan of glycoproteins and are associated with various functions, including Notch signaling, a pathway critical for cell differentiation and developmental processes,[12] and extracellular protease secretion[13] through O-fucosylation of Thrombospondin Type 1 Repeats (TSRs). FUTs10-13 have been identified as protein O-fucosyltransferases. Among them, FUT12 (POFUT1) and FUT13 (POFUT2) catalyze O□fucosylation on EGF and TSR domains, respectively, whereas FUT10 (POFUT3) and FUT11 (POFUT4) specifically modify the EMI domain.[6]

The diverse fucosylation patterns as described highlight the complexity and significance of glycan-mediated regulation in biology and cancer progression. In particular, the presence of cancer-specific antigens as terminal fucosylated structures has proven valuable in cancer diagnostics, providing molecular signatures for early detection and classification of tumors.[14] We believe that development of efficient and unbiased fucosylation probes will facilitate the discovery of more disease-associated fucosylated glycans.

Enzymatic Synthesis of Glycan Acceptors as Substrates for FUTs. To investigate the substrate specificity and catalytic efficiency of FUTs, glycan acceptors were enzymatically prepared from sialyl glycopeptide (SGP) from eggs as described previously[27] and used in kinetic assays with GDP-fucose and its clickable analogs (Figure 1).

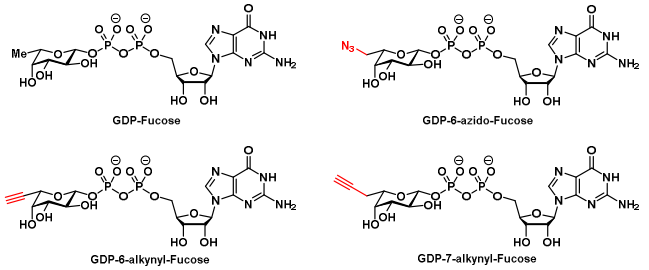

Figure 1. Structures of GDP-Fuc, GDP-6-AZ-Fuc, GDP-6-Alk-Fuc and GDP-7-Alk-Fuc probes.

Initially, SGP was subjected to pronase digestion to truncate the peptide backbone down to the asparagine (Asn) residue. This was followed by treatment with sialidase to remove terminal sialic acids, yielding the G2 glycan, which was used as the acceptor substrate for FUT1-6 and FUT9. To obtain the G0 glycan as the preferred substrate for FUT8, G2 glycan was further treated with galactosidase to remove terminal galactose residues. For FUT7, the G0 glycan was enzymatically sialylated with α2,3-sialyltransferase to generate the S2G2 glycan (Figure 2). All these glycan substrates were confirmed by LC/MS analysis (Supplementary Information Figure S1) and used as acceptors for individual FUTs.

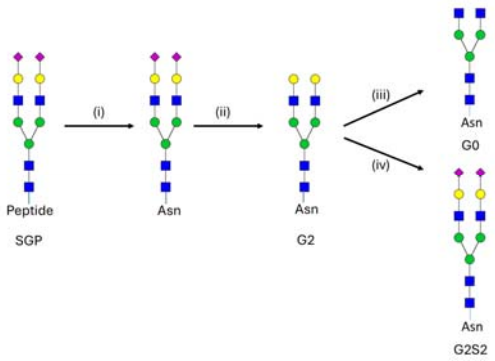

Figure 2. Preparation of acceptor substrates. The acceptor substrates were derived from SGP through pronase digestion followed by sialidase treatment to give the G2 glycan as substrate for FUT1–6 and FUT9. To generate the glycan with terminal GlcNAc as substrate for FUT8, G2 glycan was enzymatically converted to G0 glycan using galactosidase. For FUT7, S2G2 glycan was produced from G2 with α2,3-sialyltransferase to add α2,3-linked sialic acids to terminal Gal.

Michaelis-Menten Profiling Reveals Distinct Specificity and Catalytic Efficiency Among FUTs toward GDP-Fucose and N-glycan Acceptors. The enzymatic activity was quantified using the GDP-Glo™ Glycosyltransferase Assay Kit (Promega), with biantennary glycans linked to Asn as acceptor substrates. The resulting Michaelis-Menten kinetics, illustrated in Figure 3, revealed distinct substrate affinities and turnover rates among the FUTs, highlighting differences across FUTs. The α1,2-fucosyltransferases FUT1 and FUT2 were directly compared, with FUT1 exhibiting superior catalytic efficiency toward G2 substrate. FUT3-7 and FUT9 are classified as 3/4-fucosyltransferases and are primarily involved in the synthesis of Lewis antigens, which are important for cell-cell interactions. The Km values were successfully determined for all enzymes and catalytic efficiencies (Kcat/Km) were summarized in Table 1 with GDP-fucose as donor and specific N-glycans as acceptors. Based on our result, FUT9 exhibited the highest enzymatic activity toward GDP-fucose and G2, achieving a Kcat/Km of 4,547.5 $min^{-1}\cdot mM^{-1}$. However, attempts to utilize GDP-fucose analogs by FUT9 have shown low efficiency. In contrast, FUT8, responsible for core fucosylation, demonstrated a lower but still significant catalytic efficiency of 1,110.3 $min^{-1}\cdot mM^{-1}$ toward G0 and GDP-fucose. These findings highlight the functional diversity of FUTs and their differential utilization of GDP-fucose and N-glycans, offering insights into their potential roles in glycan remodeling and disease pathology.

Enzymatic Activity and Substrate Preferences of FUTs toward GDP-Fucose Analogs. To further investigate the utilization of fucose analogs by FUTs, we conducted comparative studies using GDP-6-Az-Fuc, GDP-6-Alk-Fuc, and 7-Alk-Fuc as donor substrates for various human FUTss. These three analogs have been used as probes for tracking fucosylation, particularly through bioorthogonal click reactions that enable precise labeling and detection.

In our comparative studies utilizing the three GDP-fucose analogs as donor substrates, and among the various FUTs capable of catalyzing 1,3-linked fucosylation, FUT4 was shown to be an effective enzyme due to its higher catalytic efficiency across a broad range of substrates based on initial velocity (Figure 4). Although FUT4 exhibited lower enzymatic activity toward GDP-fucose analogs in comparative studies with GDP-fucose, it interestingly showed a broad substrate tolerance compared to other FUTss. This broader substrate acceptance makes FUT4 an ideal choice for glycoengineering strategies such as antibody-drug conjugates (ADCs) or metabolic glycan labelling. To further illustrate substrate preference, a hotspot map of glycosylation activity is shown in Supplementary Information Figure S2. Moreover, FUT4 and FUT9 are both α1,3-fucosyltransferases that primarily catalyze the synthesis of Lewis x antigens and are expressed in distinct tissue types. However, their efficiency in utilizing fucose analogs and their enzymatic activity profiles differ significantly. Despite this shared catalytic function, the two enzymes exhibit markedly different substrate preferences and enzymatic efficiencies toward GDP-fucose analogs. FUT4 is capable of efficiently utilizing all three GDP-fucose analogs, with particularly high activity toward GDP-7-Alk-Fuc. In contrast, FUT9 showed limited activity, with a slight preference for 6-Az-Fuc.

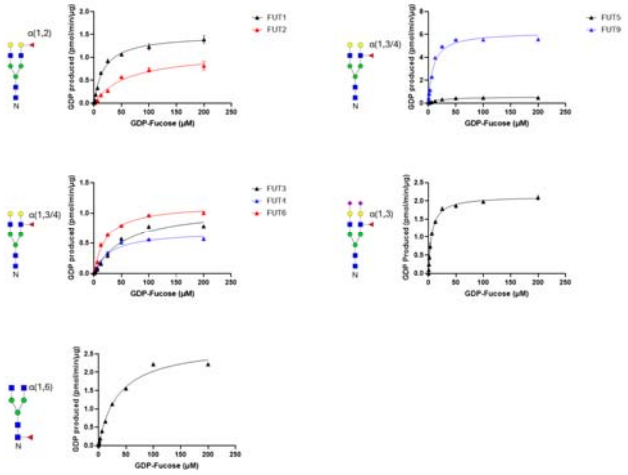

Figure 3. Michaelis-Menten kinetics of FUTs with GDP-Fucose as Donor Substrate and N-glycans as acceptors. Michaelis-Menten curves were generated for various FUTs using GDP-fucose as the donor substrate. Enzymatic activity was quantified by measuring GDP production via the GDP-Glo™ Glycosyltransferase Assay Kit (Promega) in the presence of biantennary glycan-Asn acceptor at different concentrations.

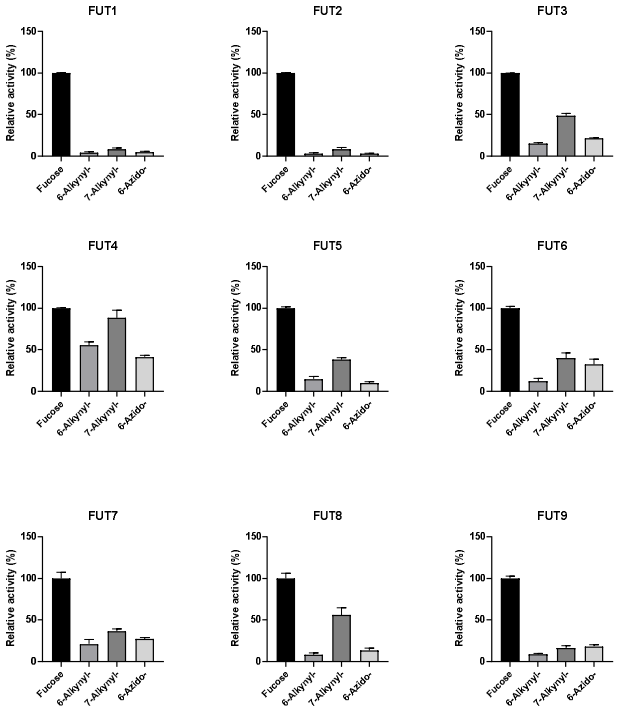

Figure 4. Comparative initial enzymatic activity of FUTs toward GDP-fucose analogs. FUTs were incubated with biantennary glycan-Asn and various GDP-fucose analogs at concentrations five-fold higher than their respective Km values. Enzymatic activity was normalized to GDP-fucose (set as 100%). FUT4 accepted all GDP-fucose analogs, with a pronounced preference for GDP-7-Alk-Fuc. FUT3-8 also demonstrated notable activity toward GDP-7-Alk-fucose.

Table 1. Glycosylation kinetics of FUTs toward GDP-fucose and Asn-linked N-glycan acceptors.

Enzyme	Substrate	K_m(μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\cdot mM^{-1}$)
FUT1	G2	18.46 ± 3.34	4.86	263.2
FUT2	G2	62.35 ± 5.36	1.08	17.32
FUT3	G2	56.67 ± 3.72	1.16	20.48
FUT4	G2	29.28 ± 2.44	1.44	49.18
FUT5	G2	22.42 ± 1.21	1.49	66.45
FUT6	G2	22.3 ± 3.46	3.10	139.01
FUT7	G2S2	5.96 ± 0.48	21.45	3,598.9
FUT8	G0	36.52 ± 5.47	40.54	1,110.3
FUT9	G2	9.05 ± 2.43	41.16	4,547.5

As described above, among the FUTs, FUT4 can accept all clickable probes and the enzyme plays a significant role in catalyzing the α1,3-fucosylation of N-acetyllactosamine structures to generate specific Lewis antigen subtypes and modulate cellular processes such as epithelial-mesenchymal transition (EMT), immune cell interactions, and tumor progression. In lung adenocarcinoma, FUT4 reshapes the glycoproteome to promote metastatic behavior, whereas in osteosarcoma, silencing FUT4 activates the tumor suppressor FOXO1, leading to inhibited tumor growth.[28,29] Given its strong correlation with cancer aggressiveness, FUT4 expression serves as a valuable biomarker for monitoring tumor progression. The broad acceptance specificity of FUT4 toward the three GDP-fucose analogs underscores the importance of fucose-targeting molecular probes, which can be used to detect FUT4 activity in real time and support early diagnosis, prognosis, and treatment planning in oncology.

Most FUTs, including FUT4, still lack experimentally resolved structures, which limits our understanding of their substrate specificity and boarder tolerance of analog substrates within its active site. However, our findings of different substrate specificity and efficiency of FUTs toward donor substrate analogs provide new opportunities for glycan-specific conjugation and development of more precise glycosylation probes.

CONCLUSION

Despite recent studies of clickable glycosylation probes, the outcome may not accurately reflect the enzymatic processes that occur in humans under normal physiological or diseased conditions, which exhibit substrate specificities and catalytic properties toward unnatural substrates. This study systematically examines all currently known human FUTs involved in N□glycan modification, providing a more biologically relevant comparison and more reliable representation of the enzymatic characteristics of human

FUTs. Although fucose analogs have already been used as glycosylation probes, the differential acceptance specificity and catalytic efficiency of various human FUTs toward these probes may complicate the interpretation of metabolic labeling experiments, as multiple FUTs may accept the probes differentially and act on different acceptor molecules with different affinities, leading to a misinterpretation of result. While the azido and alkynyl fucose probes can be delivered into cellular systems and generate glycoproteins with modified fucose residues, such approaches may introduce bias due to the complexity of biological processes involving multiple FUTs acting simultaneously with different efficiency and specificity. Compared to GDP-fucose, all clickable probes used in this study were much weaker substrates for FUTs except FUT4 which exhibited better tolerance toward all probes especially GDP-7-Alk-Fuc, consistent with previous study demonstrating that 7□Alk□Fuc is more acceptable and possesses lower cytotoxicity than other analogs.[23,24]

Given the strong potential of clickable fucosylation probes, this study provides a better understanding of their biological processing by employing all currently known human FUTs involved in N□glycan modification, comparing their relative activities toward natural GDP□Fuc, and generating a unified dataset that minimizes uncertainty, reduces experimental bias and misinterpretation. The comprehensive evaluation their specificity toward GDP-fucose analogs revealed the variability in the acceptance of fucosylation probes among the human FUT family with potential bias in identifying fucosylated glycans as disease targets.

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Author Contributions

Y.-F.Z., T.-H.T. and C.-H.W. designed the study. Y.-F.Z. and T.-H.T. performed experiments and wrote the manuscript. R.L. and P.C. prepared glycan analogs. C.-H.W. supervised the project and wrote the manuscript. All authors reviewed and approved the final manuscript.

Acknowledgment

This research was supported by the NIH (AI-130227).

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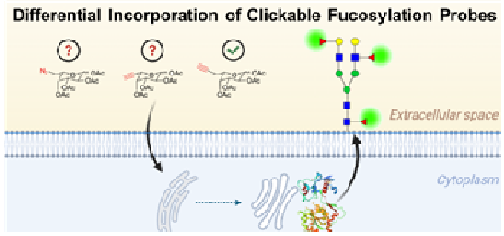
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