Enzymatic synthesis of a fluorogenic reporter substrate and a high-throughput assay for fucosyltransferase VIII provide a toolkit to probe and inhibit core fucosylation

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Abstract

We report a straight-forward enzymatic synthesis of the 4-methylumbelliferyl glycoside of a complex-type oligosaccharide substrate for core-fucosylation. We demonstrate the use of this synthetic glycoconjugate in a newly developed enzyme assay to probe the activity and inhibition of fucosyltransferase VIII, which catalyzes the core fucosylation of N-glycans on eukaryotic glycoproteins. In this fucosyltransferase assay, we use the fluorogenic probe and a specific glycospidase in a sequential coupled enzyme reaction to distinguish an unmodified 4-methylumbelliferyl oligosaccharide probe from a fucosylated probe. Our findings show that this strategy is very sensitive and very specific in its detection of enzyme activity and can even be used for analyzing impure tissue lysate samples.

Introduction

Glycosylation is an important post-translational modification that occurs on more than half of eukaryotic proteins. This type of modification plays many key roles in the function of glycoproteins and is involved in their folding, stability, localization, trafficking, secretion, and molecular recognition [1-3]. N-linked glycosylation occurs on proteins through the attachment and subsequent tailoring of glycans to the amide nitrogen of an asparagine side-chain [4]. A common—and very important—modification on N-glycans is core fucosylation, catalyzed by the enzyme fucosyltransferase VIII (FUT8) [5]. This α1,6-fucosyltransferase acts on the complex-type N-glycans, transferring a fucose sugar unit from guanosine 5’-diphospho-β-L-fucose (GDP-fucose) onto the 6-hydroxyl group of the glycan’s innermost N-acetylglucosamine (GlcNAc) residue, which is N-linked to asparagine. The activity of this enzyme is critical in mammalian development as demonstrated by knock-out studies in mice which showed that 80% of mice born as FUT8-null pups do not live past three days and the remaining survivors have severe growth defects [6, 7]. Several of these adverse phenotypes can be attributed to the absence of core fucosylation resulting in the failure to activate cell-surface growth factor receptors. These include transforming growth factor beta (TGFβ) receptor [6], epidermal growth factor (EGF) receptor [7], and the vascular endothelial growth factor (VEGF) receptor [8]. FUT8-catalyzed core fucosylation also influences cell adhesion and cell migration processes by its effect on the turnover and expression of E-cadherin [9], L1CAM [10], and α3β1 integrin [11]. Furthermore, misregulation of FUT8 resulting in abnormal core fucosylation patterns has been identified as both a marker and a driver of various cancers (e.g. by promoting tumour growth and proliferation through its influence on the aforementioned receptors and cell adhesion molecules) [9, 10, 12, 13].

Many of the molecular mechanisms by which core fucosylation effects the biological function of glycoproteins have yet to be determined. Further insight into the downstream effects of fucosylation catalyzed by FUT8 would be aided by the use of potent and specific small molecule inhibitors that influence the activity of the enzyme. FUT8 inhibitors also have potential as targeted therapeutics, however it remains a challenge to identify or design such molecules [14, 15]. Meeting this challenge would also be very beneficial in efforts towards producing therapeutic glycoproteins on which core fucosylation can have a considerable effect. For example, monoclonal antibodies (mAbs) raised for cancer immunotherapy can be used in treatments to selectively kill cancer cells through antibody-directed cell-mediated cytotoxicity (ADCC), which is greatly enhanced in cancer-targeting mAbs devoid of core fucosylation since they bind Fcγ receptor IIA (FcγRIIA; CD16) on natural killer (NK) cells more tightly than those with core-fucosylated N-glycans [16-20].
In this study, we have developed the tools to efficiently assay FUT8 activity in high-throughput and identify inhibitor molecules. Our approach is built upon a strategy that we have previously developed to probe the activity and inhibition of glycosyltransferases [21]. This strategy consists of a sensitive fluorescence-based assay that uses fluorigenically labeled oligosaccharide probes and specific glycosidases as coupling enzymes to distinguish unmodified probes from those modified by a glycosyltransferase. Based on this concept, we have enzymatically synthesized a 4-methylumbelliferyl oligosaccharide, MU-G0, as a substrate and fluorogenic probe for FUT8. The MU-G0 probe can also be a substrate for digestion by chitinase, releasing fluorescent 4-methylumbelliferone, but not if it is modified by FUT8-catalyzed addition of a fucose unit (Fig. 1). Using this method, we can detect FUT8 activity in assays using only picomoles of the probe and enzyme. The specificity of our assay is exquisite, allowing the detection of FUT8 activity in complex mixtures consisting of impure tissue lysate without noticeable background interference.

Results

Chemoenzymatic synthesis of a 4-methylumbelliferyl asialo-, agalacto-, biantennary complex-type N-glycan as a probe for FUT8 activity

Core fucosylation is catalyzed by FUT8 in the Golgi and occurs during a defined stage of N-glycan assembly, and thus FUT8 displays selectivity for its N-glycan acceptor substrate with preference towards specific glycoforms over others. Recent studies have shown that the optimal substrate for FUT8 is the complex-type asialo-, agalacto-, biantennary heptasaccharide known as G0 [22]. We therefore decided to synthesize the 4-methylumbelliferyl glycoside of the G0 heptasaccharide (MU-G0) as a probe for FUT8 activity. We opted for an enzymatic approach to obtain this compound (Fig. 2) following recent reports detailing both the efficient extraction of sialoglycopeptide (SGP) as a starting material from chicken egg yolk [23-26] and the use of engineered glycosidases as synthetic tools [27-29]. SGP consists of a complex-type disialylated biantennary undecasaccharide—sometimes referred to as the G2S2 glycan—N-linked to the asparagine side-chain of a hexapeptide. Glycosidases can be used to trim down the G2S2 undecasaccharide to the G0 heptasaccharide structure while the N-glycosidic linkage to the peptide can be replaced with an O-glycosidic linkage to a 4-methylumbelliferyl group by enzymatic transglycosylation, producing MU-G0. The 4-methylumbelliferyl group of this glycoconjugate serves as a fluorogenic reporter that, when cleaved from the heptasaccharide as 4-methylumbelliferyl, gives a fluorescence signal with excitation at 372 nm and emission at 445 nm, yet it also serves an additional purpose in facilitating purification of MU-G0 by reverse phase chromatography through its interaction with the hydrophobic C18-silica resin.

We performed a multi-step, one-pot enzymatic synthesis to produce our MU-G0 probe (Fig. 2). The first step uses a mutant endo-β-N-acetylglucosaminidase from *Mucor hiemalis* (EndoM N175Q, engineered for reduced hydrolytic activity and improved transglycosidase activity [29]), to convert starting materials SGP and 4-methylumbelliferyl β-N-acetylglucosamine (MU-
βGlcNAc) into the 4-methylumbelliferyl glycoside of the G2S2 complex-type glycan (MU-G2S2) with the release of a β-N-acetylglucosaminyl hexapeptide (GlcNAc-hexapeptide). The hydrolytic activity of the wild-type EndoM, which breaks the β-1,4-glycosidic bond in the N,N'-diacetylchitobiose core of N-glycans, is significantly diminished in the N175Q mutant [29]. In place of water (as in hydrolysis), the mutant enzyme can catalyze reaction with the N-acetylglucosaminyl group of a suitable acceptor to form a new β-1,4-glycosidic linkage. We carried out a reaction with SGP (5 mM) and MU-βGlcNAc (25 mM) in a buffered solution (50 mM sodium phosphate + 10% DMSO, pH 7) containing EndoM (0.5 mg/mL) and after overnight incubation at 30 °C observed formation of the transglycosylation product, MU-G2S2, by TLC and mass spectrometry. Since 4-methylumbelliferyl glycosides are difficult to separate from one another with the low-resolution C18-silica solid-phase extraction cartridges that we used for subsequent chromatography, in order to facilitate the downstream purification, excess MU-βGlcNAc (a relatively inexpensive starting material) was digested (into GlcNAc and 4-methylumbelliferone) by the addition of an exo-β-N-acetylhexosaminidase from Streptomyces plicatus (SpHex) [30]. After a one-hour incubation with SpHex (0.07 mg/mL) at 37 °C, the reaction was heat inactivated at 75 °C for 10 minutes.

For the conversion of the MU-G2S2 intermediate into our MU-G0 probe, we added an exo-α-neuraminidase from Clostridium perfringens (Nanl) [31] and an exo-β-galactosidase from Streptococcus pneumoniae (BgaA) [32] to the reaction mixture (0.4 μg/mL and 0.15 mg/mL, respectively) to cleave sialic acid (5-acetylneuraminic acid; Neu5Ac) and galactose (Gal) units from each branch of the biantennary glycan. After overnight incubation at 37 °C, the digestion was complete (as determined by mass spectrometry). Purification involved liquid-liquid extraction with ethyl acetate to remove free 4-methylumbelliferone leaving MU-G0 in the aqueous phase, and reverse phase chromatography using a C18-silica solid-phase extraction cartridge (with a stepwise gradient between 0 and 30% aqueous methanol) to separate the product from other contaminants (e.g. buffer salts, enzymes, glycopeptides, and cleaved monosaccharides) resulting in an overall 32% yield from the multi-step reaction.

**Validation and optimization of a fluorescence based FUT8 activity assay**

Having the MU-G0 compound in hand, we then tested its utility as a probe for FUT8 activity. To be effective in the strategy that we devised for an activity assay, our probe must be a substrate for fucosylation by FUT8 and it must also be a substrate for hydrolysis by the endo-glycosidase (chitinase) used in the downstream fluorescence development reaction, yet the product of FUT8-

![Chemoenzymatic synthesis of MU-G0 starting from SGP and MU-βGlcNAc using wild-type and engineered glycosidases.](image-url)
catalyzed fucosylation must not be hydrolyzed by the endo-glycosidase. If these conditions are met, then from sequential reactions involving first FUT8-catalyzed fucosylation followed by glycosidase-catalyzed hydrolysis, we expect to see decreased fluorescence in assays with higher FUT8 activity compared to controls where there is none.

In recent work wherein Peng George Wang and coworkers surveyed the substrate specificity of FUT8 [22], which normally acts on N-glycans to catalyze fucosylation on the 6-hydroxyl group of the innermost GlcNAc attached to an asparagine side-chain by an N-glycosidic bond, it was shown that the enzyme can also act upon free glycan substrates that have an anomeric hydroxyl group at the reducing-end GlcNAc. This demonstrated that the N-glycosidic linkage to asparagine is unnecessary for substrate recognition by FUT8. However, it had remained to be shown experimentally whether or not FUT8 would recognize the MU-G0 glycan, which bears an O-glycosidic bond between the innermost GlcNAc and a 4-methylumbelliferyl group. We therefore carried out reactions with MU-G0 (10 µM), GDP-fucose (100 µM), and varying amounts of FUT8 in buffered solution (50 mM sodium phosphate, pH 7). Conversion of MU-G0 to the fucosylated product, MU-G0F, was observed by mass spectrometry. In 30-minute reactions incubated at 37 °C, the degree of conversion was greater with increasing FUT8 concentration between 0 and 17.5 µg/mL (Fig. 3A). We also tested if chitinase from S. griseus [33] could selectively hydrolyze MU-G0, leaving MU-G0F undigested. Aliquots of the same FUT8 reaction samples, which had been heat-inactivated (95 °C for 5 minutes), were then treated with chitinase (0.125 mg/mL for 30 minutes at 37 °C) and we saw that the remaining MU-G0 in the samples had been completely hydrolyzed, releasing 4-methylumbelliferone (which could be detected by mass spectrometry or fluorescence) while MU-G0F was left intact (Fig. 3B). This confirmed to us that chitinase was able to recognize the N,N'-diacetylchitobiose core of a complex-type glycan and cleave an O-linked 4-methylumbelliferyl group (as had been previously demonstrated by Lai-Xi Wang and co-workers [34]), and that fucosylation of the N,N'-diacetylchitobiase moiety abolishes this recognition. We therefore could use this to our advantage in a chitinase-dependent FUT8
activity wherein fluorescence signal is inversely proportional to the degree of FUT8-catalyzed fucosylation.

In further tests, we varied incubation time and donor substrate (GDP-fucose) concentration in our chitinase-dependent FUT8 assay, keeping the MU-G0 acceptor substrate concentration fixed at 10 µM and FUT8 concentration fixed at 8 µg/mL. Assay mixtures contained between 10 to 200 µM of GDP-fucose and were incubated at 37 °C for varying lengths of time before stopping the reactions by heat-inactivation (95 °C for 5 minutes). Once FUT8 was inactivated, fluorescence was developed by adding chitinase (0.125 mg/mL) and incubating the mixture at 37 °C for 30 minutes. We observed that when using only 1 equivalent (10 µM) of GDP-fucose, the FUT8 reaction required more than 3 hours to go to 90% completion (Fig. 4). At the 30-minute mark of the FUT8 reaction, assays containing 10 µM GDP-fucose had gone to about 50% completion, whereas those containing 40, 100, and 200 µM GDP-fucose had gone to more than 70%, 80%, and 90% completion, respectively.

**Inhibition assays with GDP and GDP-2F-Fuc**

We focused next on evaluating the assay for detecting inhibition of FUT8. In inhibition assays, we chose to perform the fucosylation reaction using 8 µg/mL of FUT8, 10 µM of MU-G0 and 100 µM GDP-fucose in 30-minute incubations at 37 °C (prior to heat-inactivation). Under these conditions, in the absence of inhibitor the reaction goes to near-completion, and any significant decrease in activity due to inhibition should produce a measurable difference in the fluorescence signal when developed with chitinase. Since blocking fucosylation would allow hydrolysis of MU-G0 by chitinase, inhibition of FUT8 should correspond to an increase in fluorescence intensity compared to controls performed in absence of inhibitor.

Previously reported fucosyltransferase inhibitors include GDP [35, 36] and guanosine 5’-diphospho-2-deoxy-2-fluoro-β-L-fucose (GDP-2F-Fuc) [36, 37]. Using our assay system, we carried out the FUT8 reaction with varying inhibitor concentrations (from 0 to 1 mM of either GDP or GDP-2F-Fuc) and after developing fluorescence with chitinase we took the intensity of the signal as a measure of FUT8 inhibition. We observed a response curve (Fig. 5) that gave an IC50 of 12.9 µM for GDP (consistent with other reports examining GDP as an inhibitor of FUT8 [36]). Yet, interestingly, the response curve shows a slight decrease in fluorescence intensity with increasing GDP-2F-Fuc (where normally inhibition should result in an increased fluorescent signal). We would interpret from our results that GDP-2F-Fuc is not an inhibitor of FUT8. This contrasts with the results of Okeley et al. [36] who found that GDP-2F-Fuc inhibits the transfer of fucose from radiolabeled GDP-Fuc in FUT8 assays, with an IC50 in the mM range. In other studies, reported by Rillahan et al. [37], it was observed that when cultured human cells were fed with peracetylated 2-deoxy-2-fluorofucose, which is metabolized (slowly) in the cells to generate GDP-2F-Fuc in situ, FUT8-catalyzed modification of glycoproteins is inhibited at 20 µM, but at above 100 µM of peracetylated 2-deoxy-2-fluorofucose this inhibition is reversed. The authors of that study attribute this to a depletion of intracellular GDP-fucose pools via feedback inhibition, but at higher concentrations GDP-2F-Fuc can itself act as a poor substrate (with a high $K_M$) for inhibition.

![Figure 5.](image)
FUT8. Considering this, we rationalize that GDP-2F-Fuc prevents the transfer of fucose from GDP-fucose by acting as a competitive substrate for FUT8 (transferring 2-deoxy-2-fluoro-fucose) rather than strictly as a competitive inhibitor. This is consistent with the results of our assay (with lowered fluorescence indicative of the incorporation of 2-deoxy-2-fluoro-fucose).

It is worth noting that our fluorescence assay does not distinguish modification of MU-G0 by core fucosylation from any other modification that also prevents hydrolysis by chitinase. Many FUT8 inhibitors are structural mimics of fucose and as such become incorporated into the N-glycans of glycoproteins in place of fucose to varying degrees. Our assay strategy may not detect these types of inhibitors as hits but can be of utility if the aim is to screen for inhibitors that prevent core fucosylation without themselves modifying the N-glycan. Such modification may be undesirable, since the inclusion of a foreign moiety on N-glycans can have immunogenic, toxic, or otherwise unforeseen effects.

**FUT8 activity assays in crude tissue lysate**

One major advantage that our strategy presents is its sensitivity and specificity. It results in a change in fluorescence signal as a direct result of the transfer of a sugar unit onto a probe that is specifically recognized by the target glycosyltransferase (FUT8 in this case). This contrasts with other approaches that rely upon the detection (through coupled enzyme reactions) of a nucleoside diphosphate or monophosphate released upon the transfer of a sugar unit from a sugar-nucleotide donor. From various suppliers, commercial assay kits are available enabling sensitive spectrophotometric [38, 39], fluorescence- [40], or luminescence-based [41, 42] detection of released nucleotides—however this signal is not linked to the formation of the glycoside bond that results from the transfer of the sugar unit and it is sensitive to sugar-nucleotide hydrolysis or contaminating nucleotides in the sample. These issues render the nucleotide detection method unsuitable to assay complex samples such as tissue or cell lysates. Using our strategy, we demonstrate that we can detect the activity of FUT8 from a complex sample mixture.

We carried out assays for FUT8 activity using the lysate from homogenized porcine liver tissue. Tissue samples from fresh liver were homogenized using a bead beating homogenizer and the soluble fraction of the lysate was used in varying dilutions for our FUT8 fluorescence assay using MU-G0 as a probe. In reactions containing 10 µM MU-G0 and 100 µM GDP-fucose in buffered solution (50 mM sodium phosphate, pH 7), soluble porcine liver lysate was used in dilutions ranging from 0 to 5 mg/mL of total protein, and the reactions were incubated at 37 °C for varying lengths of time up to 24 hours before being stopped by heat-inactivation (95 °C for 5 minutes). After heat-inactivation, fluorescence was developed with chitinase and measured for each time point (Fig. 6). We observed FUT8 activity from crude lysate that was absent in control experiments wherein lysate was heat-inactivated prior to reactions, indicating that our assay is effective even with a highly impure sample.

**Discussion**

Together our results demonstrate an effective strategy to assay the activity and inhibition of FUT8 with an enzymatically synthesized glycoconjugate, MU-G0. Following a methodology that
we previously devised for glycosidase-dependent glycosyltransferase assays, in this study we have described the development of the tools to apply this approach to study the core fucosylation of complex-type glycans and target this process for inhibition.

We have established a straightforward one-pot enzymatic synthesis to convert SGP, which is readily available from chicken egg yolk or commercially available egg yolk powder, into MU-G0. A simple purification can be accomplished with one chromatographic step and this material can be used in very sensitive assays that require a minimal amount of the probe and enzyme. The synthesis of this probe will allow for high-throughput screening assays with FUT8. Given the sensitivity of our developed assay, 1 mg (0.68 µmol) of MU-G0 probe is sufficient for about 3400 individual assays, and therefore efforts towards screening large libraries of compounds will require only milligram-quantities of the probe. This is easily achievable on a lab-scale given established methods to isolate SGP starting material from consumer-grade chicken eggs or egg yolk powder with reported yields of roughly 8 mg per egg [23] or 0.8 mg per g of egg yolk powder [26] (the other starting material, MU-βGlcNAc, being relatively inexpensive from various suppliers).

The specificity of our strategy in detecting transferase activity makes this a versatile strategy. We show that we can use very impure samples with minimal processing to test for enzymatic activity, demonstrating as an example that liver tissue lysate can be used directly as a source of FUT8 in these enzyme assays. This is something that would not be possible with conventional methods using assay kits that detect glycosyltransferase activity from released nucleotides, and the flexibility in the kinds of samples that can be analyzed may be particularly useful in cases where highly purified mammalian glycosyltransferases are difficult to obtain.

Materials and Methods

SGP extraction and purification

Sialoglycopeptide (SGP) was extracted and purified from chicken egg yolk powder following previously reported procedures, with slight modifications [26]. Prior to extraction, egg yolk powder was first washed with 95% ethanol. An 85 g portion of egg yolk powder (Modernist Pantry) was suspended in 170 mL of 95% ethanol and stirred at room temperature for 30 minutes. The mixture was filtered over a Buchner funnel and dried by suction using a water aspirator. The filtrate, containing ethanol-soluble contaminants, was discarded and the dry material was again resuspended in 170 mL of 95% ethanol for an additional wash, with the mixture once again filtered after 30 minutes of stirring, keeping the solid material and discarding the filtrate. To extract SGP, the solid material was then suspended in 170 mL of 40% ethanol and stirred at room temperature for 30 minutes. The mixture was filtered and dried as before, and the filtrate containing SGP was collected. The solid material was subjected to a second extraction (with an additional 170 mL of 40% ethanol) and the filtrates were pooled and chilled at -20 °C for 30 minutes to precipitate proteins, which were pelleted by centrifugation (3750 rpm, 4 °C, for 10 minutes). The supernatant was collected, concentrated by rotary evaporation to 5 mL, and applied to a 20 g (50 mL) carbon/celite column (1:1 by weight) that had been equilibrated with distilled water + 0.1% TFA. The column was washed with 60 mL of distilled water + 0.1% TFA, then eluted with a gradient between buffer A (water + 0.1% TFA) and buffer B (25% acetonitrile + 0.1% TFA) as follows: 0 to 100% buffer B over 120 mL followed by 100% buffer B for 60 mL. The eluate was collected in 1.5 mL fractions, and those containing the desired material were identified by TLC and confirmed by mass spectrometry. SGP-containing fractions were pooled and concentrated, then applied to a 90 mL P-2 size exclusion column previously equilibrated with 0.1 M ammonium bicarbonate. The
column was eluted with 0.1 M ammonium bicarbonate, and fractions containing pure SGP were lyophilized to dryness, yielding a white, fluffy powder (34 mg, or 0.4 mg of SGP per g of egg yolk powder).

Recombinant proteins

N-terminally His6- or His7-tagged proteins EndoM N175Q [29], BgaA [32], SpHex [30], and FKP [43] were each expressed in Escherichia coli BL21(DE3) cells that were transformed with one of several plasmids (pET29-EndoM-N175Q, pET28-BgaA, p3AHEX-1.8, or pET15-FKP) and cultured in LB medium. Cultures were grown to an OD600 of ~0.6 at 37°C, at which point 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, following by overnight incubation at 18°C. Cells were harvested by centrifugation and lysed by sonication. Each protein was purified from soluble cell extract was using a 1 mL HisPur Ni-NTA column and the proteins were buffer exchanged into 20 mM sodium phosphate (pH 7.5) and 150 mM NaCl for storage (at 4°C for up to two months or at -80°C for longer).

Recombinant exo-α-neuraminidase from Clostridium perfringens (NanI) was purchased from New England Biolabs (P0720S), and chinatase from Streptomyces griseus was purchased from Millipore Sigma (C6137). Recombinant FUT8 was the kind gift of Kenta Yamato (Kaico Ltd.), Jae Man Lee (Kyushu University), and Takahiro Kusakabe (Kyushu University).

MU-G0

A multi-step one-pot synthesis of MU-G0 was carried out in a 2 mL reaction volume. First, 19.5 mg (51.5 µmol) of MU-βGlcNAc was dissolved in 200 µL of DMSO, and then added together with 15.2 mg (10.3 µmol) of purified SGP into a sodium phosphate-buffered solution containing 0.5 mg/mL of EndoM N175Q (with a final composition of 25 mM MU-βGlcNAc, 5 mM SGP, 0.5 mg/mL EndoM N175Q, 50 mM sodium phosphate buffered to pH 7, and 10% DMSO), and the mixture was incubated overnight at 30°C to generate MU-G2S2. Once no further formation of the MU-G2S2 intermediate was observed, SpHex was added to 0.07 mg/mL and the mixture was further incubated at 37°C for an hour prior to heat-inactivation at 75°C for 10 minutes. Addition of exo-α-neuraminidase, NanI (New England Biolabs P0720S), to 0.4 µg/mL (40 U/mL), and exo-β-galactosidase, BgaA, to 0.15mg/ml was followed by incubation at 37°C overnight. After complete digestion, cleaving sialic acid and galactose residues, MU-G0 was purified by a combination of liquid-liquid extraction using a 1:1 volume of ethyl acetate to remove 4-methylumbelliferone, and chromatography using a reverse phase HyperSep C18 cartridge (ThermoFisher, 60108-301) carried out with a stepwise gradient of increasing aqueous methanol (6 mL steps of 0%, 10%, 20%, 30% methanol). Pooled, purified fractions of MU-G0 were lyophilized to dry mass, yielding 2.5mg of MU-G0.

GDP-2F-Fuc

Synthesis of GDP-2F-Fuc was adapted from that reported by Rillahan et al. [37]. In a 1.5 mL reaction, 5 mg (30 µmol) of 2F-Fucose was added along with 21.5 mg (39 µmol) of ATP and 20.4 mg (39 µmol) of GTP to a buffered solution containing 100 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 20 mM MnCl2, and 0.25 mg/ml of FKP. The reaction was left to react at 37°C overnight. The mixture containing GDP-2F-Fuc was then treated with 60 U of alkaline phosphatase (Millipore Sigma, P0114) for an hour at 37°C to hydrolyze free nucleotides. GDP-2F-Fuc was purified on a
Bio-gel P-2 size exclusion column (90 mL resin) eluting with water and fractions containing pure GDP-2F-Fuc were pooled and lyophilized to dry mass, yielding 15 mg (~87%).

**FUT8 activity assay conditions**

In tests varying FUT8 concentration, different dilutions of FUT8 (0, 5, 15, 20, 25, 30, or 35 µg/ml) were prepared in 10 µl of 50 mM sodium phosphate buffer (pH 7). To these solutions were added 10 µl of 20 mM MU-G0, 200 mM GDP-Fucose in 50 mM sodium phosphate buffer (pH 7) (such that the final concentration of MU-G0 was 10 µM, the final concentration of GDP-fucose was 100 µM, and the concentration of GDP-Fucose in 50mM sodium phosphate buffer (pH 7). The reagents were mixed and incubated for 30 minutes at 37 °C then heat-inactivated at 95°C for 5 minutes, and the samples were split into two sets. To one set was added 20 µl of 50 mM sodium phosphate buffer (pH 7) as the blank control group. To the other was added 10 µl of 0.25 mg/mL chitinase in 50mM sodium phosphate buffer (pH 7). Samples were mixed and fluorescence was measured following incubation for 30 minutes at 37°C.

In further tests varying the concentration of donor substrate and incubation time in the assay system, 100 µl solutions containing 10 µM MU-G0, and varying concentrations of GDP-fucose (10, 40, 100, or 200 µM) and 7.5 µg/ml FUT8 in 50 mM sodium phosphate buffer (pH 7) were prepared and incubated at 37 °C for 3 hours. Aliquots of 10 µl were taken at 0-, 30-, 60-, 90-, 120-, and 180-minute time points and heat-inactivated at 95 °C for 5 minutes. To each sample was added 10 µl of 0.25 mg/mL chitinase in 50mM sodium phosphate buffer (pH 7) and incubated for 30 minutes at 37 °C before measuring fluorescence signals.

**FUT8 inhibition assay conditions**

For testing the inhibition of FUT8, 10 µL solutions with varying concentrations of GDP or GDP-2F-Fuc (0 mM, 0.004 mM, 0.012 mM, 0.04 mM, 0.12 mM, 0.4 mM, 1.2 mM, 4 mM) were prepared in 50 mM sodium phosphate buffer (pH 7) then mixed with 10 µl of 15 µg/ml FUT8, also prepared in 50 mM sodium phosphate buffer (pH 7). The enzyme and inhibitor mixtures were incubated at 37 °C for 10 minutes. Then 20 µl solutions of 20 µM MU-G0, 200 µM GDP-fucose in 50 mM sodium phosphate buffer (pH 7) were added to the pre-incubated enzyme-inhibitor samples (such that the final concentration of MU-G0 was 10 µM, the final concentration of GDP-fucose was 100 µM, the final concentration of FUT8 was 7.5 mg/mL and the concentration of inhibitor varied between 0 and 1 mM), then the mixtures were incubated at 37 °C for 30 minutes prior to heat-inactivation at 95 °C for 5 minutes. For fluorescence development 20 µl aliquots were taken from the samples, and 20 µl of 0.25 mg/mL of chitinase in 50 mM sodium phosphate buffer (pH 7) was added to each. Samples were allowed to incubate at 37°C for 30 minutes before measuring fluorescence. The fluorescence measurements were blank corrected against a control where chitinase was excluded, and then normalized to a control in which FUT8 was excluded.

**Conditions for FUT8 activity assays with lysate of homogenized porcine liver**

A 1 g sample of fresh porcine liver was cut into 50 to 300 mg pieces, each placed into a microcentrifuge tube. The tissue was washed three times with PBS buffer (1 mL per piece). To each tube, glass beads (0.5 mm diameter) were added to a mass equal to the mass of tissue. Then, to each tube, for each volume of tissue, two volumes of tissue lysis buffer (50 mM Tris-HCl at pH
7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 2 mM EDTA) were added. The samples were homogenized with a bead beater for a total of three minutes, then the soluble fraction of the lysate was collected by centrifugation (13000 g, 5 minutes, 4 °C) and stored at -80 °C until used for assays.

Samples of soluble liver lysate were diluted to 10, 25, or 50 mg/mL of total protein with tissue lysis buffer. Volumes from each dilution of liver lysate, or from tissue lysis buffer (as a control), were added 1:10 to sodium phosphate-buffered solutions containing MU-G0 and GDP-fucose producing reaction mixtures each with a final composition of 50 mM sodium phosphate (pH 7), 10 µM MU-G0, 100 µM GDP-fucose, and varying amounts of lysate (0, 1, 2.5 or 5 mg/mL of total protein). The reaction mixtures were incubated at 37 °C, and aliquots were stopped by heat-inactivation (95 °C for 5 minutes) at different time points. To 10 µL aliquots of heat-inactivated reaction mixtures were added either 10 µL of 0.25 mg/mL chitinase in 50 mM sodium phosphate buffer (pH 7) or (as a control) 10 µL of 50 mM sodium phosphate buffer (pH 7). Fluorescence readings were taken after development with chitinase and blanked against controls where chitinase was excluded.

HPLC–MS Analysis

Analysis was performed using an Agilent 1290 Infinity II UHPLC instrument in conjunction with an Agilent 6560 Ion Mobility Q-TOF mass spectrometer. Samples obtained from enzymatic assays were diluted 1:1 with distilled water, and 20 µl of each dilution was injected for chromatography using a Phenomenex 150 mm X 2.00 mm Synergi 4µM Hydro-RP column. Samples were eluted from the column with the following gradient (buffer A being water + 0.1% formic acid and buffer B being acetonitrile + 0.1% formic acid): 1 to 10% buffer B at a flow rate of 0.3 mL/min over 4.0 min, 10 to 85% buffer B at a flow rate of 0.3 mL/min over 1.0 min, 85% buffer B at a flow rate of 0.4 mL/min for 1.2 min, 1% buffer B at a flow rate of 0.4 mL/min for 2.1 min, and 1% buffer B at a flow rate of 0.3 mL/min for 2.7 min. Detection of analytes by mass spectrometry was carried out by QTOF in negative mode with a source voltage of 2000 V and a scan range of m/z 50–1750.

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References


