Chapter 10

Activity Detection of GalNAc Transferases by Protein-Based Fluorescence Sensors In Vivo

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Abstract

Mucin-type *O*-glycosylation occurring in the Golgi apparatus is an important protein posttranslational modification initiated by up to 20 GalNAc-transferase isozymes with largely distinct substrate specificities. Regulation of this enzyme family affects a vast array of proteins transiting the secretory pathway and mis-regulation causes human diseases. Here we describe the use of protein-based fluorescence sensors that traffic in the secretory pathway to monitor GalNAc-transferase activity in living cells. The sensors can either be "pan" or isozyme specific.

Key words *O*-glycosylation, GalNAc transferase, Fluorescent biosensor, Fluorescence-activating protein

1 Introduction

Glycosylation enzymes change the surface characteristics of their protein substrates by appending or modifying carbohydrate chains termed glycans. Glycans impact protein solubility, stability, and interactions [1–3]. Mucin-type *O*-glycosylation is a large and important subgroup defined by the initial reaction in which *N*-acetylgalactosamine (GalNAc) is transferred to the hydroxyl group of serine or threonine (and possibly tyrosine) by UDP-*N*-acetyl-α-D-galactosamine polypeptide *N*-acetylgalactosaminyl transferases (GalNAc transferases). This occurs on secretory cargo as it passes through the Golgi apparatus and other Golgi-localized enzymes extend the glycan by further additions of individual monosaccharides. While one or two isoforms exist for each of the enzymes mediating chain extension, there are up to 20 distinct genes encoding GalNAc-transferase isozymes in humans, each with at least partial substrate selectivity [4]. Collectively, the isozymes modify a vast number of substrates and they are already linked to a significant number of medical syndromes [1, 5–8].

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Despite their biological and medical significance, assays have been lacking that monitor GalNAc-transferase activity in living cells and there are no known inhibitors. Therefore, we developed cell-based fluorescence sensors that are particularly sensitive to inhibition of GalNAc-transferase activity [9, 10]. They are transfectable constructs encoding proteins that traffic to the cell surface and become fluorescent if their glycosylation is inhibited. In brief, each sensor has a fluorescence activating protein domain (FAP) followed by a linker to a blocking domain that occludes dimerization necessary for binding of the dye malachite green to the FAP (Fig. 1). The dye itself is nonfluorescent and only becomes fluorescent when bound to the FAP [11]. Within the linker is a glycan acceptor site next to a furin protease site. Because GalNAc-transferases are localized to the cis/medial Golgi and furin is localized to the TGN, glycan addition occurs before, and therefore sterically blocks, processing by furin leaving the sensor inactive. Extending enzymes also act before furin and contribute to glycan-masking (*see* **Note 1**). However, if the GalNAc-transferase activity is inhibited, the entire glycan is absent and furin cleaves the linker releasing the blocking domain and allowing dye activation. The sensors are ratiometric because they also contain a fluorescent protein domain.

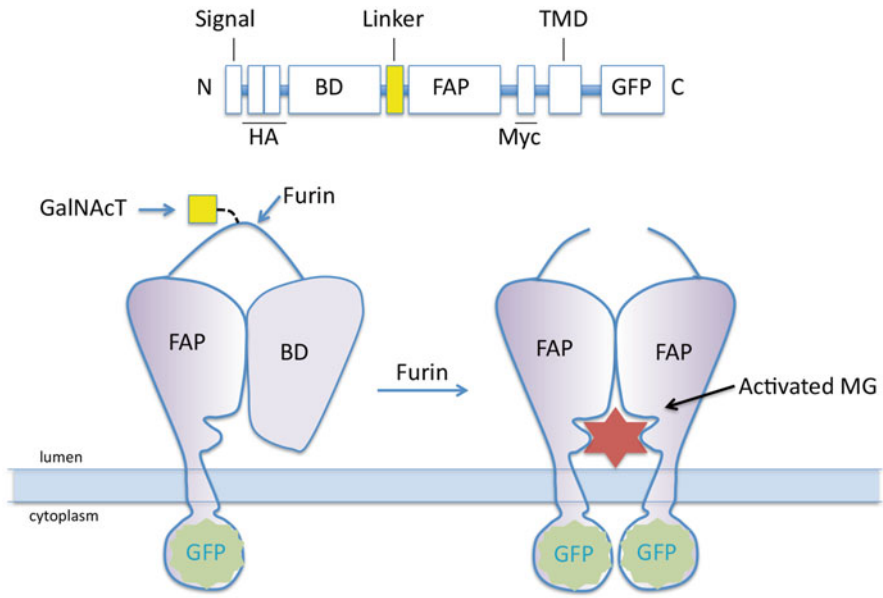

Fig. 1 Sensor schematic. The linear diagram (not to scale) depicts the N-terminal cleaved signal sequence, tandem HA epitopes, blocking domain (BD), linker, fluorescence-activating protein (FAP), myc epitope, transmembrane domain (TMD), and fluorescent protein domain (GFP). The cartoon shows an intact sensor on the *left* with adjacent GalNAc-transferase and furin sites in the linker such that GalNAc addition masks the furin site. Reduced GalNAc-T activity allows furin cleavage releasing the BD. As shown on the *right*, the FAP then dimerizes and binds and activates the dye malachite green (MG). The signal is read as the MG–GFP ratio

There are two critical aspects regarding sensor function. First, the sequence of the glycan acceptor site can either be "pan" or isozyme specific. The pan-specific version contains a minimal sequence recognized by GalNAc-transferases [12]. Although not confirmed for every isozyme, it should be recognized as a substrate by most if not all [13, 14]. For the isozyme-specific versions, the sequences are tailor-made for selective recognition by individual isozymes. At present only T2- and T3-specific versions are available. Their acceptor sequences were derived from known T2 or T3 substrates and then modified to improve selectivity [6, 10, 13, 15, 16]. The second critical aspect is that the starting or ground state signal can be significantly greater than true background (where true background is determined by mutating the furin site). This is advantageous if the desire is to assay enzyme activation. A less than optimal glycosylation site results in incomplete glycosylation and therefore an increased starting signal. Conditions upregulating enzyme activity will then lower the signal. Thus, although the sensors were designed to assay for inhibitors, they can be further modified and used to monitor changes in GalNAc-transferase activity in either direction.

2 Materials

2.1 Cell Culture

1. HEK293 cells.
2. HEK293 growth medium: minimal essential medium containing 10% fetal bovine serum and 100 IU/ml penicillin–streptomycin. Store at 4 °C. Use in 5% humidified CO_2 incubator at 37 °C.
3. Malachite Green-11p-NH2 (MG11P) (Sharp Edge Labs, Pittsburgh, PA) (*see* **Note 2**): Reconstitute at 110 μM (1000× stock) in 95% methanol, 5% acetic acid. Store at 4 °C in the dark.
4. MEM without phenol red: Adjust to 110 nM MG11P just before use.
5. EDTA/PBS: Reconstitute 8 g NaCl, 0.2 g KCl, 0.916 g Na_2HPO_4, and 0.2 g KH_2PO_4 in 1 L water at pH 7.3. Add 2.081 g EDTA (5 mM final concentration) and adjust pH, if necessary. Adjust to 110 nM MG11P just before use.

2.2 Confocal Assay

1. Live cell imaging chamber (or glass bottom dishes) and inverted confocal microscope equipped with a 40× oil immersion objective and 488 nm and 633 nm filter sets (*see* **Note 3**).
2. Image analysis software (e.g., ImageJ, http://imagej.nih.gov/ij/).

2.3 Flow Cytometry Assay

1. 96-well flat-bottom plastic dishes.
2. EDTA/PBS containing 110 nM MG11P.
3. Flow cytometer equipped to read at 488 nm and 640 nm (*see* **Note 3**) and accompanying software.

3 Methods

Below are two protocols for use of the GalNAc transferase biosensors. The first uses live-cell fluorescence microscopy and the second employs flow cytometry. Flow cytometry is preferred because a large number of cells are readily quantified for each condition and multi-well dishes can be used allowing a large number of conditions to be tested (e.g., high-throughput screening). The sensors can also be analyzed using immunoblotting if biochemical verification is desired (*see* **Note 4**). As a starting point, each protocol requires at least 3 cell lines: experimental, negative control and positive control. The experimental cell line(s) stably express the GalNAc-T biosensor(s) of interest. The linker sequence in these will contain either the pan-specific glycan acceptor site or one of the isozyme specific acceptor sites. The signal produced can be compared for differing conditions and normalized to the controls for comparison with other sensor signals. For the negative control, either untransfected cells or, optimally, cells stably expressing a matched version of the sensor with a mutated furin acceptor site (ΔFur) is used. This cell line will establish the true background, i.e., the signal in the absence of any cleavage, which theoretically corresponds to complete glycosylation of all sensor molecules. The positive control is a cell line expressing a matched sensor with a mutated glycan acceptor site (ΔGly). This cell line will establish the maximal possible signal given that it cannot be glycosylated. The protocols are described for use of the preexisting HEK293 cell lines expressing pan-, T2-, and T3-specific sensors and their matched controls. However, the linker sequence can be modified to produce new specificities (*see* **Note 5**). Also, the assay should work in most, if not all, cell lines but each cell type may require minor modifications in cell handling. After processing in the Golgi the sensor accumulates on the cell surface. It takes a minimum of 3 h to develop a strong surface signal, so, after a test condition, a period of at least this duration should be allowed before the cells are analyzed [10].

3.1 Assay GalNAc-T Activity Using Biosensor and Confocal Microscopy

1. Pass the cell lines (ΔFur, biosensor, ΔGly) to achieve 50 % confluence on coverslips for imaging chamber or in glass bottom dishes and incubate for 24 h (*see* **Note 6**).
2. Transfer cells to pre-warmed MEM without phenol red and containing 110 nM MG11P dye.
3. Mount on confocal microscope equipped with a 40× objective (e.g., LSM 510 Meta DuoScan Spectral Confocal Microscope). Use identical confocal settings for all data collection within the experiment (*see* **Note 7**). Focus using the GFP (488 nm) channel to yield sharp cell surface outlines of the near confluent cells (Fig. 2). There should be about 100 cells per field. Acquire a single optical section using both the GFP and MG (633 nm) channels. Repeat for at least eight separate fields per coverslip.

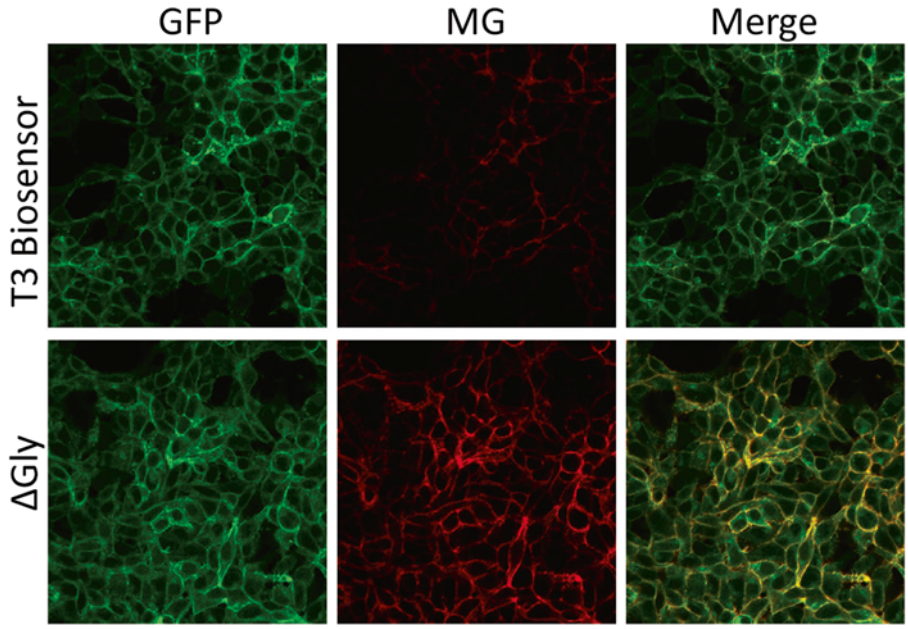

Fig. 2 Fluorescence microscopy data. The figure compares cells expressing the T3 biosensor (*upper row*) to those expressing its activated ΔGly version (*lower row*) in the indicated channels

4. To quantify, for each two-channel image define several regions of interest outside the cells. For each channel in each image, determine the average intensity value in these non-cell areas and use the highest value as the background. For each channel in each image, subtract the background value from all pixels (uniform subtraction). Next, in the GFP channel of each image, use auto threshold to define a threshold and select all above-threshold pixels. Record the average intensity value for these pixels for both the GFP and MG channels. The signal for that image is then expressed as the MG–GFP ratio. Repeat for all eight two-channel images per coverslip. The average of the eight ratios is then the value for a single condition in a single experiment.
5. Comparison of these values between ΔFur, the biosensor, and ΔGly will typically yield a low signal for the biosensor, an even lower signal for ΔFur and a much greater value for ΔGly. To normalize the values for comparison with other sensors subtract the background (ΔFur or untransfected) and then divide by the positive control (ΔGly).

3.2 Assay GalNAc-T Activity Using Biosensor and Multi-well Format Flow-Cytometry

1. Pass the cell lines (ΔFur, biosensor, ΔGly) at 50,000 cells/well into 96-well plates (0.2 ml/well).
2. After 24 h replace the medium with 0.1 ml of the EDTA/PBS solution containing 110 nM MG dye and return to incubator for 5 min (*see* **Note 6**).

3. Suspend cells by pipetting each well up and down 2–3 times with a multichannel pipette with 200 µl tips. Mount plate on flow cytometry instrument (e.g., BD Accuri™ C6 Flow Cytometer). Collect data for 10,000 cells per well using GFP (488 nm) and MG (640 nm) channels (Fig. 3).
4. To quantify, first determine the background. Calculate the geometric mean of values for each channel of each well of the untransfected or ΔFur cells (at least 3 wells). Use the average of these means (one for each channel) as the background values. Next, for each remaining well, determine the geometric mean of the values for each channel. Subtract the channel-specific background values and then calculate the MG–GFP ratio for each well. Averages of these values for multiple trials are then used for comparison.
5. Again, there should be a low signal for the biosensor, an even lower signal for ΔFur (equal to background) and a much greater value for ΔGly. To normalize the values for comparison with other sensors subtract the background (untransfected or ΔFur) and then divide by the positive control (ΔGly).

4 Notes

1. Glycan masking of adjacent protease-processing sites can occur upon addition of a single GalNAc [13, 15] but the current sensors show variable requirements for chain extension. The pan- and T3-specific sensors appear to require extension whereas the T2-specific sensor does not [9, 10].
2. The protocols utilize a membrane impermeant version of the dye and therefore yield surface staining. A membrane permeant version can be used but also yields predominate surface staining

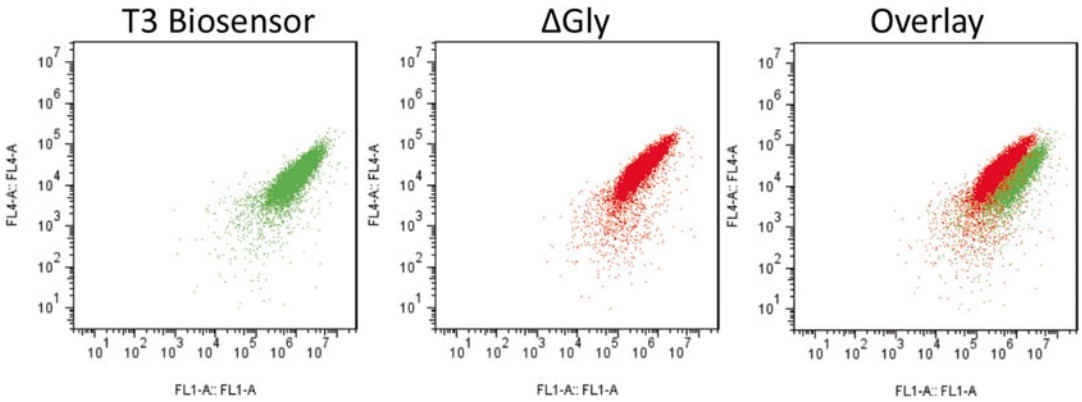

Fig. 3 Flow cytometry data. Shown are scatter plots for the T3 biosensor (*left*), its activated ΔGly version (*middle*), and an overlay. Axes are 488 nm channel (X) and 633 nm channel (Y)

because processing of the biosensor by furin occurs just before its exit from the Golgi. A Golgi signal can be detected if this exit is blocked (e.g., by cell incubation at 20 °C).

3. Excitation and emission spectra for MG11P can be found at http://www.mbic.cmu.edu/images/datasheet/MG-11p-NH2-info_rev21.pdf.
4. Cleavage of the sensor is readily assayed by immunoblot [9]. Briefly, cell lysate proteins are separated by SDS-PAGE, transferred to nitrocellulose and blotted with anti-GFP antibodies to detect uncleaved (≈80 kD) and cleaved (≈60 kD) portions of the sensor. (The missing fragment is secreted into the cell media and detectable with anti-HA antibodies.) Quantify the cleaved fragment as a percent of total. The biosensor should give a value slightly higher than ΔFur and much lower than ΔGly.
5. *Optional linker modification*. The sensor plasmids [9, 10] each contain in-frame fusions encoding a signal sequence, a blocking domain (HL4 heavy chain, [17]), a linker with furin and glycosylation sites, a FAP (L5*, [11]), the myc tag, a transmembrane domain from PDGFR, and either a Venus or GFP domain. The version with the pan-specific glycan acceptor site has a linker with the sequence 5′-c tcg aga aag aag aga tct acc ccc gct cca gct cca tcc ggt ggc ggt ggc agc-3′ encoding NSRKKRSTPAPS where RKKR is the furin site and TPAP comprises the glycan site. To modify selectivity of the sensor, point mutations can be introduced or the entire linker can be substituted with another sequence encoding a glycan acceptor and protease-processing site. The optimal candidates are substrate sequence stretches known to be regulated by glycan masking by a particular GalNAc transferase. In either case, results from published in vitro assays establishing sequence preferences of particular GalNAc transferases [13, 15] can be used to guide further mutagenesis to optimize sensor function [10]. Transient expression can be used for initial tests but ultimately stable cell lines will need to be generated to insure uniform and strong surface expression of the sensors. For any new version, comparison of its signal with and without an intact glycan acceptor site will test its effectiveness. Strong sensors yield maximal activation of 500-fold or more [10]. To confirm isoform specificity of a new sensor, one needs to show that it is strongly activated in cells lacking the particular GalNAc transferase isoform [10]. Genomic editing or RNA interference can be used to suppress expression of the particular isoform.
6. Time after passage can be varied to achieve the desired density. Note that trypsin treatment to passage the cells degrades the surface population of sensor molecules. A minimum of 3 h and a recommended time of at least 6 h post-passage should be used to repopulate the cell surface. Cell release with EDTA/PBS (for passage or analysis) avoids this issue.

7. The intensity of the lasers should be adjusted to ensure maximum sensitivity without saturation. This will depend on expression level, but matched control and experimental sensors should always be analyzed with identical settings.

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