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A Cellular FRET-Based Sensor for β-O-GlcNAc, A Dynamic Carbohydrate Modification Involved in Signaling

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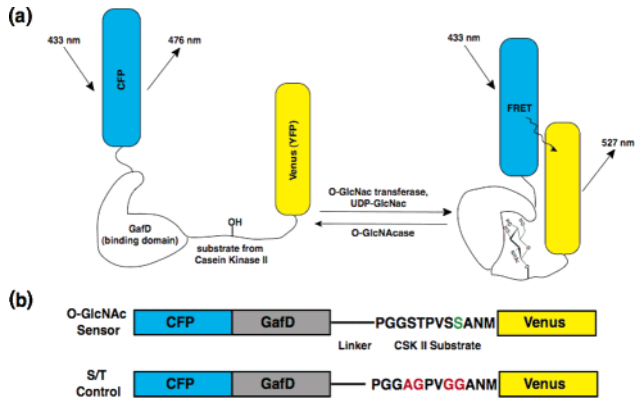
Cellular proteins are regulated by post-translational modifications in response to environmental cues. β-O-*N*-Acetyl-D-glucosamine (O-GlcNAc) is a dynamic carbohydrate modification that is involved in cell signaling and has been implicated in a variety of disease states, including Alzheimer's and type-II diabetes.[1,2] It occurs on serines and threonines in cytosolic and nuclear proteins and is often a reciprocal modification to phosphate. It is controlled by only two enzymes: O-GlcNAc transferase (OGT), which puts on the carbohydrate, and O-GlcNAcase, which removes it. Despite the importance of this modification, little is known about the spatial and temporal localization of O-GlcNAc during signaling. This is due to a complete lack of methods for the study of O-GlcNAc in living cell systems. Herein we report the first genetically encoded FRET-based sensor for the detection of O-GlcNAc dynamics in mammalian cells.

Our sensor design consists of four components, an enhanced cyan fluorescent protein (eCFP), an O-GlcNAc-binding domain, a substrate domain, and the yellow fluorescent protein variant Venus (Scheme 1).[3] This design was based upon similar *in vivo* probes for other post-translational modifications.[4−6] For our binding domain, we chose GafD, a well-defined monomeric bacterial lectin from *Escherichia coli* specific for terminal β-O-GlcNAc.[7,8] Our substrate domain is a known peptide substrate for OGT derived from casein kinase II.[9] We anticipated that, upon O-GlcNAcylation, the GafD domain would bind to the O-GlcNAcylated substrate, bringing the two fluorophores into close proximity and leading to an increase in FRET. As controls, we designed two additional constructs: S/T, in which all of the potential O-GlcNAc sites in the substrate were replaced, and GafD*, which contains only a single point mutation (D88L) in the carbohydrate binding domain that abolishes 80% of binding to GlcNAc.[8] All constructs were cloned into both bacterial and mammalian expression vectors using standard cloning techniques (Scheme 1b; see Supporting Information for methods).

Scheme 1. (a) O-GlcNAc Sensor Design. (b) Sensor Constructs. The Putative O-GlcNAc Site is Shown in Green, and All Serine and Threonine Sites Within the S/T Control Have Been Mutated as Shown in Red



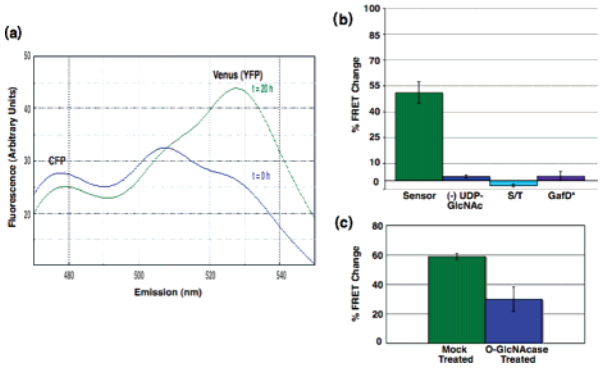


Figure 1. (a) Emission spectra of FRET sensor (ex. = 434 nm) treated with OGT and UDP-GlcNAc at $t = 0$ h (blue line) and $t = 20$ h (green line). The CFP and YFP peaks are indicated. (b) Percent FRET change. O-GlcNAc sensor (Sensor), S/T, and GafD* were treated with OGT and UDP-GlcNAc or O-GlcNAc sensor with OGT alone ((−) UDP-GlcNAc) for $t = 20$ h. The average for a minimum of five replicates is shown. Error bars represent the standard deviation (std. dev.). (c) O-GlcNAcase reverses FRET. O-GlcNAcylated sensor was treated with either buffer (Mock treated) or O-GlcNAcase. The average % FRET for two experiments is shown. Error bars represent the std. dev.

We performed *in vitro* assays using bacterially expressed His_6-tagged sensors. To analyze the FRET response of our sensor, we incubated it with recombinant OGT[10] and the requisite sugar donor UDP-GlcNAc at room temperature. It should be noted that the kinetics of OGT are substrate dependent and can vary widely even for native substrates.[9] Under our assay conditions, the reaction had $t_{1/2} = \sim 6$ h and at 16−20 h was complete as observed by Western blot analysis for O-GlcNAc (see Supporting Information). The slow kinetics of sensor glycosylation mirror the kinetics of self-O-GlcNAcylation by OGT, as seen by our Western blot analysis of the reaction, indicating that our sensor is as good a substrate as a known native substrate (see Supporting Information). However, these kinetics probably do not reflect the situation inside a cell, where the context changes both enzyme activity and local concentrations of substrates. An overlay of the fluorescence emission spectra (ex = 434 nm) for the O-GlcNAc sensor treated with OGT and UDP-GlcNAc at $t = 0$ and 20 h is shown in Figure 1a. As expected, a significant increase in emission at 528 nm is observed (∼51%). This corresponds to a change in ratio from 1.0 to 1.5. In contrast, control experiments in which either the negative control sensors were used or the sugar donor was absent showed minimal FRET changes (<3%, Figure 1b). Control reactions run in the absence of OGT also showed no response (data not shown). Western blot analysis of these reactions confirmed that both the O-GlcNAc sensor and the GafD* construct were substrates for OGT (see Supporting Information). In contrast, the S/T control sensor showed

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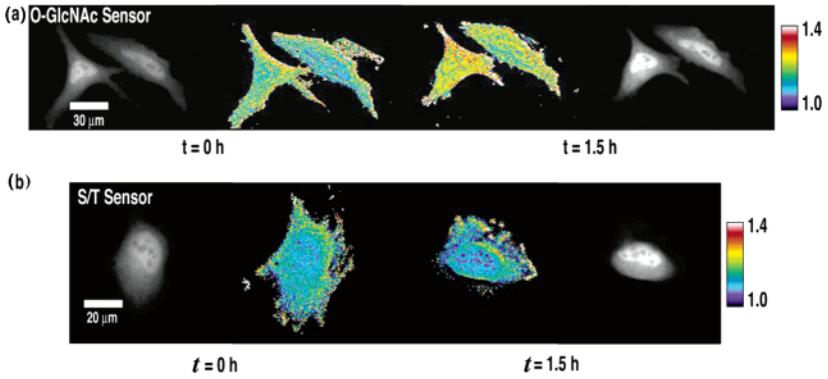

Figure 2. (a) O-GlcNAc response *in vivo*. HeLa cells were transfected with O-GlcNAc sensor. At ~36 h, post-transfection cells were treated with PugNAc (100 μM) and glucosamine (4 mM). YFP and ratiometric FRET images from $t = 0$ and 1.5 h after treatment are shown. See Supporting Information for details. (b) S/T control. HeLa cells transfected with S/T sensor and treated identically to cells in (a) are shown. Images are representative of multiple experiments.

no signs of O-GlcNAcylation, confirming that the O-GlcNAc site on our sensor is within the substrate domain. This work demonstrates the ability of our sensor to observe the addition of O-GlcNAc *in vitro*.

The study of O-GlcNAc dynamics requires a sensor that can examine the removal of this modification, as well as its addition. Therefore, we tested the reversibility of our sensor. To do this, we treated the O-GlcNAcylated sensor with either recombinant O-GlcNAcase[11] or buffer (Mock treatment, Figure 1c). We observed a significant, but incomplete, loss of the FRET signal (~50%) upon treatment with O-GlcNAcase. It should be noted that O-GlcNAc was not completely removed from the sensor as monitored by Western blot analysis, demonstrating that our observations via FRET and Western blot correlate (see Supporting Information). This confirms the reversibility of our sensor. Addition of 10 mM GlcNAc to the sensor also significantly lowered the FRET signal, providing additional confirmation of its reversibility (see Supporting Information).

We studied the ability of our sensor to detect O-GlcNAc dynamics *in vivo* using transiently transfected human cervical carcinoma (HeLa) cells. To promote a dynamic increase in O-GlcNAc, cells were treated with both 100 μM PUGNAc and 4 mM glucosamine. PUGNAc is an O-GlcNAcase inhibitor that causes a slow rise in the total levels of O-GlcNAcylated proteins.[12] Glucosamine enters the hexosamine biosynthetic pathway and results in increased levels of UDP-GlcNAc and thus O-GlcNAc. After 1.5 h of treatment, a significant FRET increase (7−30% dependent on the cell) was observed for HeLa cells transfected with the O-GlcNAc sensor (Figure 2a). This increase coincides with the modest increase in O-GlcNAc observed by Western blot analysis (~30% overall, see Supporting Information). As expected from both literature[12] and Western blot data, the rise in FRET observed is slow but steady. In contrast, little net change in the FRET was seen for cells transfected with either the S/T control (<1%, Figure 2b) or the GafD* control (see Supporting Information). Our data demonstrate that our O-GlcNAc sensor can observe dynamic changes in O-GlcNAc within living cells.

O-GlcNAc plays a role in a wide variety of biological events, including the cell cycle and cellular stress responses.[1] Due to the dynamic nature of signaling, it is crucial to study the regulation of this modification within living cells. The sensor reported herein represents the first cellular sensor for the examination of O-GlcNAc dynamics and will enable us to study response to stimuli. The modular nature of this sensor will allow us to directly study the kinetics of reciprocity between O-GlcNAc and phosphate by altering the substrate domain. This work sets the stage for deconvolution of the complicated intersection of O-GlcNAc with known phosphorylation signaling networks.

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Supporting Information Available: Spectra of O-GlcNAcase experiment, FRET inhibition by GlcNAc, Western blot analysis of *in vitro* and *in vivo* experiments, *in vivo* data for the GafD* control, movies of sensors, and Materials and Methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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