Fluorescent Detection of O-GlcNAc via Tandem Gylcan Labeling

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Abstract

O-GlcNAcylation is a reversible serine/threonine glycosylation on cytosolic and nuclear proteins that are involved in various regulatory pathways. However, the detection and quantification of O-GlcNAcylated substrates have been challenging. Here, we report a highly efficient method for the identification of O-GlcNAc modification via tandem glycan labeling, in which O-GlcNAc is first galactosylated and then sialylated with a fluorophore-conjugated sialic acid residue, therefore enabling highly sensitive fluorescent detection. The method is validated on various proteins that are known to be modified by O-GlcNAcylation including CK2, NOD2, SREBP1c, AKT1, PKM, and PFKFB3, and on the nuclear extract of HEK293 cells. Using this method, we then report the evidence that hypoxia-inducible factor HIF1α is a potential target for O-GlcNAcylation, suggesting a possibly direct connection between the metabolic O-GlcNAc pathway and the hypoxia pathway.

Graphical Abstract
O-GlcNAc refers to a single β-N-acetylglucosamine residue attached to serine/threonine residues on nuclear, cytosolic, and mitochondrial proteins. O-GlcNAc is introduced by O-GlcNAc transferase (OGT) and eOGT from the donor substrate UDP-GlcNAc that is synthesized via the hexosamine biosynthetic pathway. O-GlcNAc is removed by O-β-N-acetylglucosaminidase (OGA). O-GlcNAcylated proteins are primarily found in nuclei and secondary in cytoplasm. Many intracellular proteins that are involved in transcriptional regulation are known to be targets of O-GlcNAcylation. For example, NOD2 and SREBP-1 are regulated by O-GlcNAcylation. NOD2 is a cytoplasmic innate immune receptor that upon activation can activate nuclear factor kappa B (NF-kB). SREBP-1 is a transcription factor that binds to sterol regulatory elements and regulates lipid metabolism. Some intracellular enzymes that play key regulatory roles are also targets of O-GlcNAcylation. Such known enzymes include casein kinase II (CK2) and AKT1 that are involved in regulations of cell metabolism, proliferation, survival, growth, and angiogenesis. One speculative O-GlcNAcylation target is a hypoxia-inducible factor (HIF), a master regulator of cellular and developmental response to hypoxia. HIF consists of a subunit of HIF1α or HIF2α and a subunit of HIF1β. HIF pathway is known to be regulated by O-GlcNAcylation, but it is not clear whether HIF itself is modulated by O-GlcNAcylation.

To study the biological functions of O-GlcNAc, methods for its specific and sensitive detection are critical. In addition to the classical antibody-based methods of detection, many other types of methods have been explored for O-GlcNAc detection, which usually involve an extension or metabolic labeling of O-GlcNAc with radioisotope or chemically modified sugar residues. These methods are rather complicated to perform. Recently, we reported methods of direct fluorescent glycan labeling (DFGL) for detecting the substrate glycans of sialyltransferases and fucosyltransferases. However, these methods cannot be directly applied to detect O-GlcNAc, as O-GlcNAc is not a direct substrate of any of these enzymes. Nevertheless, O-GlcNAc is known as a substrate for the β1,4-galactosyltransferase B4GalT1 with the product of O-linked lactosamine Galβ1,4-GlcNAc-O-S/T. Since lactosamine can be labeled with fluorophore-conjugated sialic acids by the α-2,6-sialyltransferase ST6Gal1, it is surmised that O-GlcNAc may be indirectly detected via a tandem labeling approach with both B4GalT1 and ST6Gal1 (Figure 1).

To test the hypothesis proposed in Figure 1, the recombinant human CK2 (rhCK2) (Figure S1) was first successively treated by OGT, B4GalT1, and ST6Gal1 in the presence of...
their natural donor substrates. When the samples were separated on SDS-PAGE, successive mobility shift was observed (Figure 2A), suggesting that the modifications took place step by step. When the reactions were repeated and analyzed by mass spectrometry, the stepwise additions of O-GlcNAc, Gal, and sialic acid residues to rhCK2 were confirmed (Figure S2). The results of gel analysis and mass spectrometry analysis consistently suggest that the successive modifications on O-GlcNAc by B4GalT1 and ST6Gal1 are highly efficient and O-GlcNAcylation by OGT is rate-limiting. The hypothesis proposed in Figure 1 was further proven on rhCK2 by replacing the natural sialic acid with Alexa Fluor 488, Cy5, and Alexa Fluor 555-conjugated sialic acids. Figure 2B shows the successive mobility shift caused by OGT, B4GalT1, and ST6Gal1 on rhCK2 and the incorporation of these fluorophore-conjugated sialic acids.

The method of fluorescent detection of O-GlcNAc with tandem labeling was first tested on the nuclear extract of HEK293 cells (Figure 3). Labeling of the extract resulted in numerous fluorescent bands across the SDS-gel. Furthermore, these bands were obliterated by OGA pretreatment and augmented by OGT pretreatment, confirming that the signals were due to O-GlcNAcylation. The results not only confirm the abundance of O-GlcNAcylation on nuclear proteins but also suggest that the labeling method is applicable to detect O-GlcNAc on unknown proteins. The method was also validated on various purified proteins (Figure S3). As expected, AKT1, PFKFB3, PFKFB4, and PKM that are known to be targeted for O-GlcNAcylation were modified by OGT and subsequently labeled by Cy5.

We then applied this method to analyze E. coli expressed truncated version of HIF1α (Figure S4) along with some other known targets of O-GlcNAcylation including SREBP1c and NOD2 (Figure 4). To our excitement, it was found that recombinant HIF1α was labeled after OGT treatment, suggesting that HIF1α is a target for O-GlcNAcylation.

To find out whether HIF1α can be modified by O-GlcNAc in vivo, full-length HIF1α expressed in HEK293T cells (Figure S4) was purified and probed for O-GlcNAc with tandem glycan labeling (Figure 5). Direct labeling resulted in multiple fluorescent bands above and beneath the position of the unmodified HIF1α but did not when B4GalT1 was absent from the labeling mix or the sample was pretreated with OGA (right panel in Figure 5), suggesting the presence of O-GlcNAc on the sample. Consistent with the positive labeling on the truncated version of HIF1α (Figure 4 and lane 7 in Figure 5), OGT pretreatment resulted in significantly increased labeling on a band corresponding to the unmodified HIF1α. Western blotting of the gel with anti-FLAG antibody revealed protein species with a molecular weight higher than that of the unmodified full-length HIF1α (middle panel in Figure 5), suggesting the presence of ubiquitinated HIF1α. Ubiquitination is known to lead the mobility shift of HIF1α and its further degradation in SDS-PAGE, which may explain the appearance of the labeled bands above and beneath the unmodified HIF1α.

HIF1α is the master regulator in the hypoxia pathway and is activated when the oxygen level is low. An increased level of HIF1α during hypoxia leads to the upregulation of the glycolysis and hexosamine pathways, therefore, an increased level of O-GlcNAcylation, which leads to feedback regulation of the HIF pathway. However, it is not known
whether HIF1α is directly regulated by O-GlcNAcylation. Here, we provide evidence that HIF1α can be O-GlcNAcylated by OGT in vitro and might be O-GlcNAcylated in vivo. HIF1α is best known to be modified by proline hydroxylation\textsuperscript{32,33} and subsequent poly-ubiquitination for proteasomal degradation.\textsuperscript{30,34} The exact biological roles of O-GlcNAcylation on HIF1α remains to be investigated. O-GlcNAcylation on HIF1α likely counteracts the effect of poly-ubiquitination; therefore, positive feedback regulates the HIF pathway, which explains several observations reported previously including that a high level of HIF1α correlates to elevated OGT in human breast cancers.\textsuperscript{16}

Compared to the previous methods of O-GlcNAc detection via click chemistry reaction,\textsuperscript{23,35} the current method has the following features. First, the method is more convenient as it eliminates click chemistry reaction and allows direct imaging of samples separated on an SDS-PAGE gel without the time-consuming membrane transfer and subsequent chemiluminescent detection. Second, the method has eliminated all side effects caused by click chemistry reagents, such as oxidative cleavage of target proteins by copper ions and nonspecific labeling observed with alkyne-fluorophores (Figure S5 and S6). Third, by eliminating the side reactions, the method has also improved the labeling specificity. Besides, specificity under questioning can always be confirmed with additional OGA and OGT pretreatment. Fourth, the method is expected to have high sensitivity. Since a femtomole level of Cy5-labeled glycans can be measured,\textsuperscript{27} it is reasonable to expect that the same level of O-GlcNAc can be detected. As ST6Gal1 can add fluorophore-conjugated sialic acid to other types of glycans with terminal β-4 linked Gal residues, particularly N-glycans,\textsuperscript{26,36} it is not recommended that the current method be applied to samples containing N-glycans, such as samples of the membrane and extracellular proteins.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fluorescent detection of O-GlcNAc via tandem glycan labeling. Fluorophore-conjugated activated sialic acids were prepared by copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) of azido-sialic acid and alkyne-fluorophores. O-GlcNAc is first galactosylated by B4GalT1 to become an O-linked lactosamine structure and then labeled with fluorophore-conjugated sialic acids by sialyltransferases, such as ST6Gal1.
Figure 2.
Method validation with recombinant human CK2 (rhCK2). (A) O-GlcNAc on rhCK2 can be sequentially galactosylated and sialylated. Stepwise modification rhCK2 (labeled as a) by OGT, B4GalT1, and ST6Gal1 (their products are labeled as b, c, and d, respectively) resulted in successive mobility shift on SDS-PAGE that was visualized by trichloroethanal (TCE) imaging. (B) Labeling O-GlcNAc on rhCK2 with different fluorophore-conjugated sialic acids. O-GlcNAc was first introduced to rhCK2 and then labeled by different fluorophore-conjugated sialic acids. Samples were separated on SDS-PAGE and visualized with TCE imaging (upper panel) and fluorescent imaging (lower panel). From lane 4 to 6, O-GlcNAcylated rhCK2 was labeled with AlexaFluor 555 (555), Cy5, and AlexaFluor 488 (488), respectively. Free AlexaFluor 555 dye was not removed in lane 4 (marked by a green arrow). M, molecular marker (range in kDa).
Figure 3.
Detection of \(\alpha\)-GlcNAc on nuclear extract (NE) of HEK293 cells. \(\alpha\)-GlcNAc was detected without pretreatment (lane 1 and 2), after OGA treatment (lane 3 and 4), after OGT treatment (lane 5 and 6), in the absence of B4GalT1 (lane 7 and 8). No NE was applied in lane 9 and 10. All samples were separated on an SDS-PAGE and visualized with TCE imaging (left panel) and fluorescent imaging (right panel).
Figure 4.
Detection of $O$-GlcNAc on truncated HIF1α ($\Delta$HIF1α). Recombinant SREBP1c, NOD2, and CK2 were probed for $O$-GlcNAc as positive controls with Cy5 before and after OGT treatment and separated on SDS-PAGE and visualized with TCE imaging (left panel) and fluorescent imaging (right panel). FUT8 served as a negative control. OGT and ST6Gal1 exhibited self-modification.
Figure 5.
Detecting O-GlcNAc on HEK293T cell-expressed HIF1α. Both HEK293T cell and E. coli expressed HIF1α samples were detected for O-GlcNAc with AlexaFluor 555 under indicated conditions. Lane 3 was pretreated with OGA. Lanes 4 and 7 were pretreated with OGT. In lanes 1 and 5, labeling was performed in the absence of B4GalT1. The gel was visualized by trichloroethanal (TCE) imaging (left panel) and fluorescent imaging (right panel) and then subject to Western blotting with anti-FLAG antibody (middle panel). While OGA pretreatment abolished the labeling, OGT pretreatment increased the labeling on unmodified HIF1α (red arrow). M, molecular marker.