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MS-based proteomics for comprehensive investigation of protein O-GlcNAcylation

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Abstract

Protein O-GlcNAcylation refers to the covalent binding of a single N-acetylglucosamine (GlcNAc) to the serine or threonine residue. This modification primarily occurs on proteins in the nucleus and the cytosol, and plays critical roles in many cellular events, including regulation of gene expression and signal transduction. Aberrant protein O-GlcNAcylation is directly related to human diseases such as cancers, diabetes and neurodegenerative diseases. In the past decades, considerable progress has been made for global and site-specific analysis of O-GlcNAcylation in complex biological samples using mass spectrometry (MS)-based proteomics. In this review, we summarized previous efforts on comprehensive investigation of protein O-GlcNAcylation by MS. Specifically, the review is focused on methods for enriching and site-specifically mapping O-GlcNAcylated peptides, and applications for quantifying protein O-GlcNAcylation in different biological systems. As O-GlcNAcylation is an important protein modification for cell survival, effective methods are essential for advancing our understanding of glycoprotein functions and cellular events.

Introduction

The hexosamine biosynthetic pathway (HBP) is a branch of glycolysis that consumes 2-5% of glucose in cells and generates uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).[1, 2] An enzyme named O-GlcNAc transferase (OGT) uses UDP-GlcNAc to modify the serine and threonine residues of proteins, and this modification can be reversibly removed by O-GlcNAcase (OGA).[3, 4] Since its discovery, O-GlcNAcylation has been gradually recognized as an important nutrient sensor because the synthesis of UDP-GlcNAc requires critical metabolites including glucose, glucosamine, acetyl-CoA, and nucleotides.[5, 6] The flux of these metabolites may affect the level of UDP-GlcNAc, and the expression of OGT/OGA, which determines the O-GlcNAcylation of many proteins involved in gene transcription, protein translation, and signal transduction to regulate cell metabolism. Indeed, O-GlcNAcylation is pivotal for cellular homeostasis, and its dysregulation is related to multiple diseases, including diabetes, cancers, and neurodegenerative diseases.[7, 8] Furthermore, O-GlcNAcylation is involved in extensive crosstalks with other important modifications, such as ubiquitination and phosphorylation,[9, 10] and these interplays are critical regulators of many cellular events.

Despite its importance, O-GlcNAcylation is very challenging to be studied using conventional techniques. Specifically, O-GlcNAcylation is a neutral modification with the molecular weight of 203 Da, and thus it is difficult to be differentiated from the unmodified form of the corresponding proteins with conventional gel separation. Moreover, many O-GlcNAcylated proteins are of low abundance in cells because O-GlcNAcylation normally happens at the sub-stoichiometric level and the parent proteins may be low abundant, making them more challenging to be detected without effective enrichment.[11, 12] As O-GlcNAcylation regulates protein functions

site-specifically, identification of O-GlcNAcylation with site information adds another dimension of difficulty. Over last decades, considerable progress has been made, and to date, O-GlcNAcylation has been identified on many proteins. Studies further unveiled its biological importance and roles in disease pathologies. MS-based proteomics has become one of the most powerful methods for studying O-GlcNAcylation because it enables us to globally and site-specifically characterize protein O-GlcNAcylation.[13-15] With some excellent reviews[8, 16, 17] that already discussed the detection of O-GlcNAcylation, here, we focus on using MS-based proteomics for comprehensive analysis of protein O-GlcNAcylation, ranging from enrichment, fragmentation, to quantification.

Enrichment methods for O-GlcNAcylated proteins/peptides

(a) Lectin-based enrichment methods

Lectins are glycan binding proteins that exist ubiquitously in nature, and they can recognize specific sugar moieties. Among them, some can bind to terminal β-linked GlcNAc, such as Wheat Germ Agglutinin (WGA), Aleuria aurantia lectin (AAL), Psathyrella velutina lectin (PVL), Griffonia simplicifolia lectin-II (GSL-II), and Boletopsis leucomelas lectin (BLL).[18-20] WGA has been extensively applied for O-GlcNAcylation studies. WGA binds to both terminal GlcNAc and sialic acid, and it has relatively low binding affinity towards GlcNAc (~10 mM).[21] Succinylated WGA can increase its selectivity towards β-GlcNAc over sialic acid, but its affinity also decreased.[22, 23] Therefore, WGA is not effective for specific recognition of O-GlcNAcylated proteins/peptides. Nevertheless, when WGA is used to construct an affinity column, in which

unmodified peptides elute earlier, but peptides with terminal GlcNAc can be retained and then eluted later. This method, termed lectin weak affinity chromatography (LWAC), was extensively employed to enrich glycopeptides for MS analysis.[24, 25] Specifically, it was applied to globally analyze O-GlcNAcylated proteins from synapse cells in mice and human,[26, 27] Schwann cells,[28] and osteoblast cells,[29] and in plant *Arabidopsis*.[30] In murine synapses and *Arabidopsis*, 1750 and 971 unique O-GlcNAcylated peptides were identified, respectively, demonstrating that this method was relatively effective in separating O-GlcNAcylated peptides.[27, 30]

(b) Antibody-based enrichment methods

Several antibodies were developed to target O-GlcNAc.[31, 32] When O-GlcNAc antibodies are conjugated to beads, they can be further used to immunoprecipitate O-GlcNAcylated proteins/peptides. For example, using CTD 110.6 coupled resins, 51 O-GlcNAcylated proteins were enriched and identified from Cos-7 cells.[33] Combining four types of O-GlcNAc antibodies (CTD110.6, RL2, HGAC39, and HGAC85) with WGA, 60 glycopeptides were identified from MEF cells.[34] Teo et al. reported the generation of a large panel of O-GlcNAc-specific antibodies by combining three-component immunogen methodology with hybridoma technology, which resulted in the identification of more than 200 mammalian O-GlcNAc modified proteins. Nevertheless, some limitations hinder the wider applications of O-GlcNAc antibodies for proteomics research. First, besides that RL2 and CTD 110.6 are pan-specific for O-GlcNAcylated proteins, other antibodies recognize peptide sequences in addition to GlcNAc, so they are biased towards only a subset of glycopeptides. Second, some of these antibodies have relatively low binding affinities towards O-GlcNAc.[35] Finally, the specificity could be an issue because other

sugar moieties may interfere the enrichment.[36, 37] Therefore, the performance of antibody-based methods may be improved by increasing the binding affinity and the specificity towards O-GlcNAc.

(c) Inactive OGA mutant for enriching O-GlcNAcylated peptides

OGA binds to O-GlcNAcylated proteins and then hydrolyses the sugar moiety. As OGA is the only enzyme that catalyzes the removal of O-GlcNAc, it can interact with various glycoprotein structures to catalyze the hydrolysis reaction. This makes OGA an attractive tool for the enrichment of O-GlcNAcylated proteins, as the affinity of OGA to O-GlcNAc can be as low as in the nanomolar range. The Van Aalten group developed an OGA mutant (*Cp*OGA^{D298N}) that disabled its catalytic activity, but retained their binding affinity to O-GlcNAc, to enrich O-GlcNAcylated proteins and peptides.[38] The mutated enzyme was first coupled to beads and tested against O-GlcNAcylated transforming growth factor β-activated kinase 1 binding protein 1 (TAB1). Only the mutated enzyme successfully pulled down the glycoprotein compared to the negative control. *Cp*OGA^{D298N} also bound to an O-GlcNAcylated peptide with an affinity of 36 μM. The results demonstrated that the affinity of *Cp*OGA^{D298N} was sufficient to enrich O-GlcNAcylated proteins. Next, it was used to enrich O-GlcNAcylated proteins from a HeLa cell lysate, and 61 *O*-GlcNAcylated peptides was identified with high confidence. To profile O-GlcNAcylation events in the developing *Drosophila* embryo, a total of 52 O-GlcNAcylated peptides were identified on 43 proteins.[39] In general, the OGA mutant is attractive for the enrichment of O-GlcNAcylated proteins, but the affinity of the protein to O-GlcNAc and the overall workflow could be further improved.

(d) Chemical methods for glycopeptide enrichment

The unique chemical properties of the sugar moiety and the glycosidic bond were exploited to enrich the modified peptides. One popular method for N-glycoprotein enrichment is hydrazide chemistry, and it is also applied for O-GlcNAc studies (Fig. 1A). First, glycopeptides were treated with peroxide, which generated the aldehyde groups from GlcNAc. Then they were enriched with hydrazide resins. The method was applied to profile the O-GlcNAcylated proteins in *Drosophila* proteasome complex, and 7 O-GlcNAcylated peptides were detected.[40] One potential drawback of this method is that the oxidation of GlcNAc requires much higher concentrated peroxide and a longer treatment time compared with the oxidation of other monosaccharides with *cis*-diols, for instance, sialic acid, because the vicinal diols are in the *trans* but not *cis* conformation. Therefore, side reactions can happen during the treatment, which could negatively impact the downstream identification of glycopeptides using MS.

Another chemical method used β-elimination followed by the Michael addition of dithiothreitol (BEMAD) to tag O-GlcNAc sites. Under alkaline conditions, O-GlcNAc was converted to α, β-unsaturated ketone, which can be further functionalized by dithiothreitol (DTT). The modified peptides were then enriched using a thiol column (Fig. 1B).[41, 42] Using this method, 5 O-GlcNAcylation sites were identified from mouse contractile protein homogenates.[43] Overath et al. further modified the method using a novel biotin-cystamine tag, and in the murine 20S proteasome core complex, 6 O-GlcNAcylation sites were identified.[44] BEMAD was also employed in tandem with other enrichment methods to replace GlcNAc with DTT. For example, HEK293 cells were metabolically labeled with azide-modified GlcNAc, and the labeled O-GlcNAcylated

proteins were enriched by the alkyne resins through click chemistry. β-elimination was then carried out to selectively elute O-GlcNAcylated peptides from the resins, which resulted in the identification of 185 O-GlcNAc modified sites on 80 proteins.[45] This indirect method for the identification of protein O-GlcNAcylation has some unique advantages. First, β-elimination generates α, β-unsaturated ketone, which is a good chemical handle for the following enrichment. Second, the glycosidic bond is labile during fragmentation in MS, which is more challenging for accurate localization of the modification site. After BEMAD, O-GlcNAc of glycopeptides replaced by DTT makes the peptides more stable during MS analysis, facilitating the site localization. However, as β-elimination can not only remove O-GlcNAc, but also cleave the phosphate group from the serine or threonine residues, false positive identifications could occur. Therefore, phosphatase treatment should be performed before BEMAD to remove the phosphate groups.[43] Moreover, because β-elimination is performed under very basic conditions (pH ~12), undesired side reactions can happen, including the hydrolysis of the peptide backbone.[46]

Boronic acid can form reversible covalent bonds with *cis*-diols for glycoprotein enrichment.[47-50] It was also applied to enrich O-GlcNAcylated proteins. From the HeLa cell nuclei, 105 O-GlcNAcylated peptides were identified from 42 proteins.[51] Using a dendrimer-conjugated boronic acid derivative (DBA), which significantly enhanced the interaction between boronic acid and glycan, Xiao et al. identified 304 unique O-GlcNAcylated peptides on 131 proteins in HEK 293T cells.[52]

(e) Chemoenzymatic enrichment methods

One beautiful method for the detection of O-GlcNAcylation was using β1,4-galactosyltransferase (GalT) that transfers radioactive galactose to glucose or GlcNAc.[5, 53] Wild-type GalT has very stringent donor specificity which only catalyzes the transfer of UDP-galactose. However, with a point mutation in the active site, the donor specificity of the enzyme was expanded to substrates with bulkier groups on C2.[54] UDP-GalNAc analogs carrying a chemical handle could be transferred to terminal GlcNAc by the engineered GalT, which enabled the enrichment of glycoproteins. Using the engineered enzyme, the Hsieh-Wilson group developed a beautiful chemoenzymatic method for the enrichment and detection of protein O-GlcNAcylation.[55, 56] It has become one of the most popular methods to identify O-GlcNAcylated proteins and has been applied to study various samples (Fig. 1C). For example, in *Trypanosoma cruzi*, 6 O-GlcNAcylated peptides were found.[57] In *Caenorhabditis elegans*, 108 O-GlcNAcylated proteins and 64 modification site were identified.[15] More than 600 peptides with one or more O-GlcNAcylation sites were detected on 342 proteins from skeletal muscle cells.[58] The O-GlcNAc level was found to be closely related to the progression of Alzheimer's disease (AD).[59] For a study of O-GlcNAcylation in AD, 8 O-GlcNAcylation sites were mapped in the tau-enriched sample from rat brain.[60] In another study, 458 O-GlcNAcylation sites on 195 proteins were identified in mouse brain tissues. Among the sites identified, some were found on EGF-repeats on the extracellular domains of 5 membrane proteins, providing evidence for O-GlcNAcylation on the cell surface.[13] Histone modifications are critical regulators for gene expression. Using the chemoenzymatic method, O-GlcNAcylation sites on H2A, H2B, and H4 were identified, and this histone modification was dynamic during the mitosis and heat shock.[61]

Regarding the chemoenzymatic method, the engineered GalT is required, and the production of the enzyme mutant is not trivial for many labs. Furthermore, the sugar donor with a

chemical handle (azide/alkyne) is not easy to synthesize. To solve these problems, a novel chemoenzymatic method was recently reported for site-specific analysis of O-GlcNAcylated proteins, which used a wild type GalT and UDP-galactose as the sugar donor. The labeled glycopeptides can be further oxidized with galactose oxidase (GAO) and then be enriched using the hydrazide resins (Fig. 1D). In the biological triplicate experiments, 251 O-GlcNAcylated proteins were detected in MCF7 cells with site information.[14] Additionally, 18 O-glucose modified peptides were also identified using this method. Without the need of the engineered enzyme and the click chemistry reagents, this chemoenzymatic method is more accessible and cost-effective. Furthermore, without any sample restrictions, this method can be applied to study protein O-GlcNAcylation in various samples, from whole cell lysates to clinical samples.

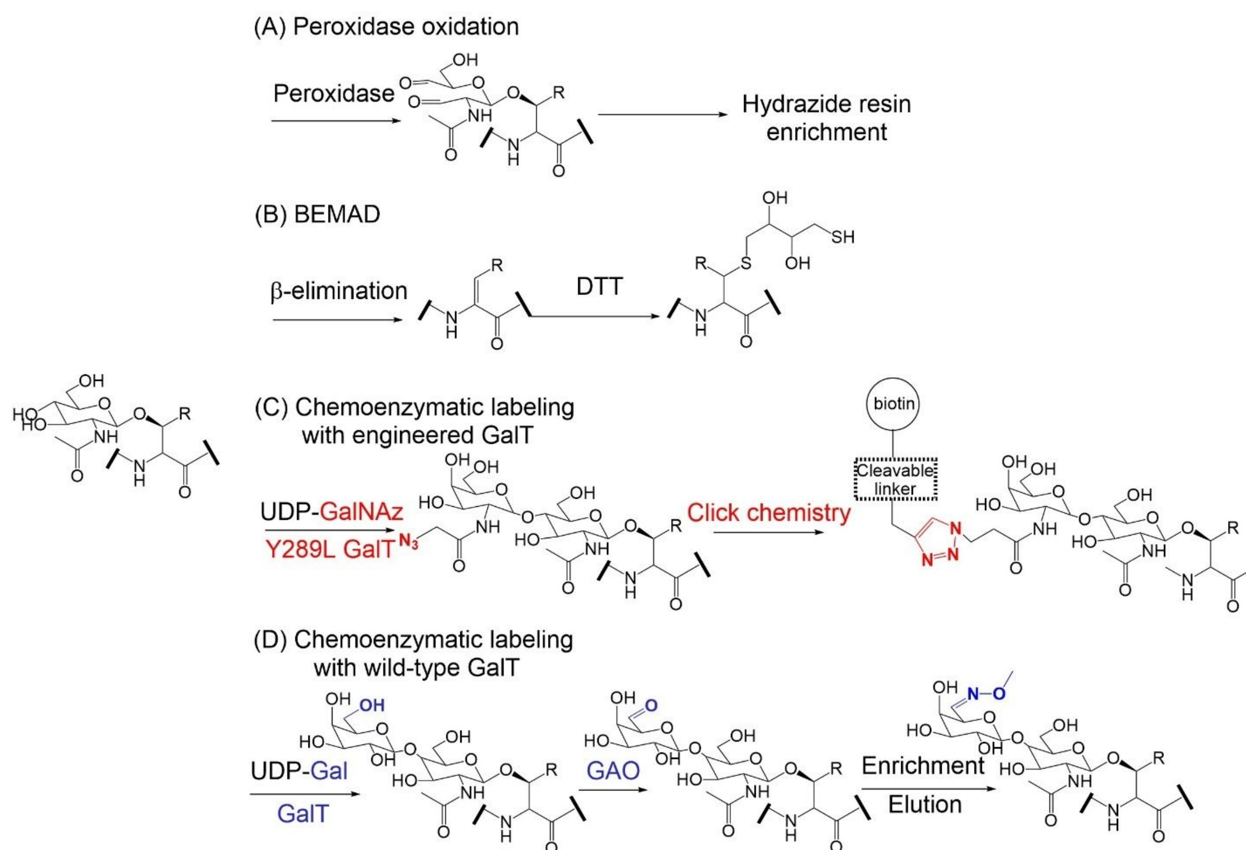

Fig. 1. Schemes of the chemical and chemoenzymatic enrichment methods.

(f) Metabolic labeling-based methods

Metabolic labeling with sugar analogs has been proven to be powerful to study protein glycosylation.[62-65] Peracetylated *N*-azidoacetylglucosamine (Ac_4GlcNAz) was used to label O-GlcNAcylated proteins,[63] and the hydrophobicity of the acetyl residues facilitated cells to uptake the sugar analog, which can be hydrolyzed by esterase in cells. Then the sugar analog was further converted to UDP-GlcNAz by the corresponding enzymes, which was a substrate of OGT and was able to be used to modify many intracellular proteins. The azido group in the labeled glycoproteins served as a chemical handle for glycoprotein enrichment and detection through bioorthogonal

chemistry, such as copper(I) catalyzed azide alkyne cycloaddition (CuAAC), strain-promoted azide–alkyne cycloaddition (SPAAC), or Staudinger ligation.[66-68] It was effective to integrate metabolic labeling with bioorthogonal chemistry to study protein glycosylation.[58, 62, 69-75] Different sugar analogs were examined for global analysis of protein O-GlcNAcylation (Fig. 2). Boyce et al. found that the peracetylated *N*-azidoacetylgalactosamine ($Ac_4GalNAz$) treatment resulted in a more robust labeling of O-GlcNAc than $Ac_4GlcNAz$.[69] As UDP-GlcNAz and UDP-GalNAz were interconvertible by UDP-galactose 4'-epimerase (GALE), a more specific chemical reporter for GlcNAc was desired.[76] The UDP sugar originated from peracetylated N-(4-pentynoyl)-glucosamine ($Ac_4GlcNAlk$) cannot be converted to GalNAlk in cells, and thus this chemical reporter could be more specific for protein O-GlcNAcylation.[77] However, $Ac_4GlcNAlk$ could still label N-glycoproteins. To solve this problem, peracetylated 6-azido-6-deoxy-N-acetyl-glucosamine ($Ac_36AzGlcNAc$) and 6-Alkynyl-6-deoxy-GlcNAc ($Ac_36AlkGlcNAc$) were developed to increase the specificity for labeling intracellular O-GlcNAcylated proteins (Fig. 2).[78, 79]

Isotope-targeted glycoproteomics (IsoTaG) was coupled with metabolic labeling to study protein O-GlcNAcylation.[80] In human T cells, over 2000 O-GlcNAc modified peptides were identified through metabolic labeling with $Ac_4GalNAz$ and the IsoTaG platform.[81] O-GlcNAc can occur co-translationally and it prevents the degradation of nascent polypeptide chains. To study co-translationally O-GlcNAc modified nascent proteins, a tandem metabolic engineering strategy was developed to label glycosylated nascent polypeptides by O-propargyl-puromycin (OPP) and $Ac_4GalNAz$. A set of co-translationally modified O-GlcNAcylated proteins were identified, and three of them were further validated to demonstrate the effectiveness of the method.[82]

Studies of chemical probes for O-GlcNAcylation has surprisingly resulted in the identification of protein O-glucosylation. Chemical reporters that mimic the structure of glucose, peracetylated 2-Azido-2-deoxy-glucose ($Ac_4$2AzGlc) and 6-azido-6-deoxy-glucose ($Ac_4$6AzGlc), can also be used by OGT to modify proteins, due to its promiscuity for accepting substrates.[83, 84] Inspection of the data from previous mass spectrometric studies have confirmed the presence of endogenous O-glucose. To study O-GlcNAcylated proteins and their binding partners, a probe carrying diazirine photo-crosslinker, Ac_3GlcNDAz-1-P(Ac-SATE)$_2$, was synthesized (the structure is in Fig. 2). The results revealed the interactions between O-GlcNAcylated nucleoporins and nuclear transport factors.[85] Furthermore, a direct one-step fluorescent labeling of O-GlcNAcylated proteins was developed by using a fluorophore linked to N-acyl group of the sugar analog (Fig. 2).[86]

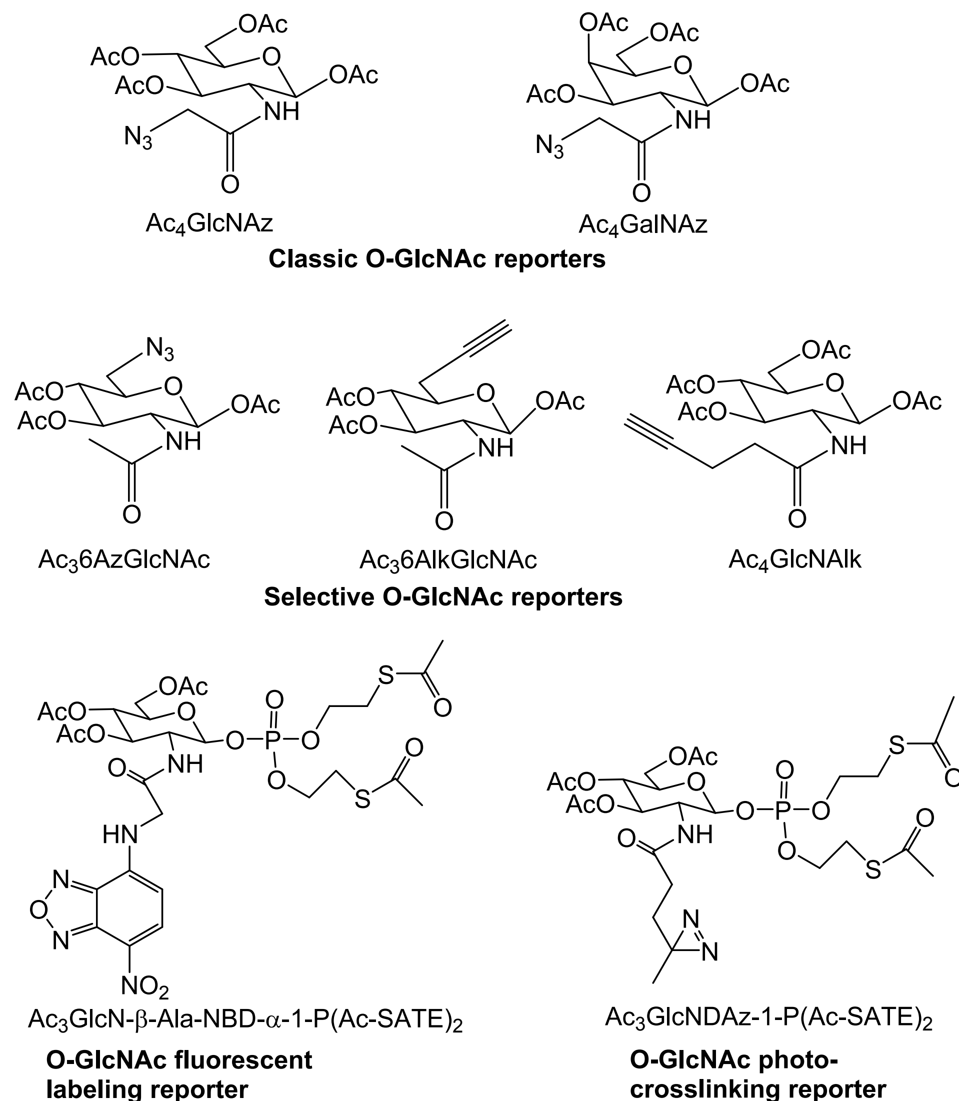

Fig. 2. Sugar analogs used for O-GlcNAcylation studies.

Fragmentation methods for localizing O-GlcNAcylation sites

When enriched O-GlcNAcylated peptides are analyzed by MS, it is important to determine the exact site of O-GlcNAcylation. It can be determined by the detection of peptide fragments containing O-GlcNAc. One of the most popular fragmentation methods, collision-induced dissociation (CID), for peptide sequencing is not well-suitable to site-specifically analyze O-

GlcNAcylated peptides because the glycosidic linkage is very labile under CID.[87] Therefore, the neutral loss of the glycan frequently happens, resulting in the loss of the glycosylation site information. Alternatively, higher-energy collisional dissociation (HCD) generates a series of oxonium ions that can serve as evidence for the presence of GlcNAc. Hahne et al. took advantage of this phenomenon, and developed the software called Oscore that can automatically analyze tandem mass spectra with the presence of the O-GlcNAc diagnostic ions. By re-analyzing previous published large-scale proteomics data using Oscore, 158 O-GlcNAcylated peptides were identified.[88] However, glycans can still be lost prior to peptide fragmentation under HCD, making the localization of the exact glycosylation site very challenging (Fig. 3A).

To confidently localize O-GlcNAcylation sites, election-transfer dissociation (ETD) was employed, which does not break the linkage between O-GlcNAc and peptide.[89] Therefore, ETD becomes a popular fragmentation method for confident O-GlcNAcylation site assignment. However, limitations of ETD fragmentation are that peptides with lower positive charges are not normally fragmented well. Additionally, ETD generally does not fragment peptides from the N-terminus to proline due to the cyclic secondary amine structure of this amino acid.[90] Therefore, ETD is usually used in tandem with HCD, namely electron-transfer/higher-energy collision dissociation (EThcD) for better fragmentation of the peptide backbone.[91] Recently, ultraviolet photodissociation (UVPD) was also applied to fragment O-GlcNAcylated peptides, which offered extensive fragmentations of the peptide backbone, while leaving the glycosidic bond intact (Fig. 3B).[92] When being used in tandem with HCD, UVPD is triggered by the advent of the oxonium ions of O-GlcNAc by HCD, and the same precursor ion is fragmented by UVPD in the subsequent scan. To evaluate the effectiveness of UVPD, the same peptide was fragmented by UVPD or

EThcD (Figs. 3C and D). The comparison of the tandem spectra showed that UVPD provided a better coverage of the middle region of the glycopeptide.

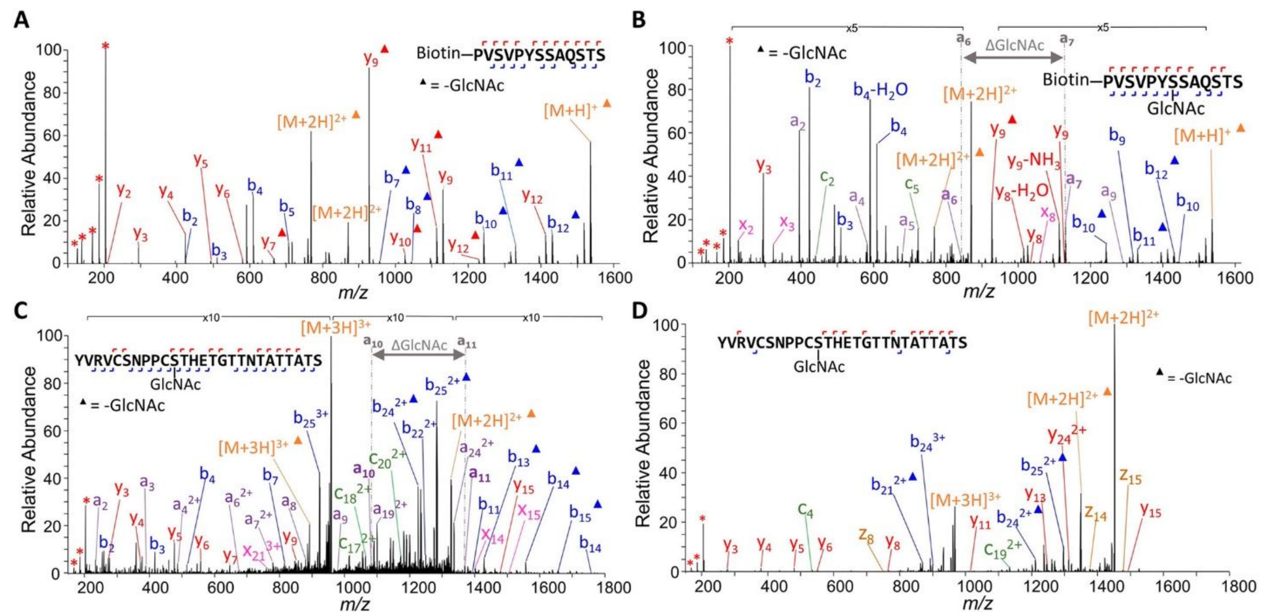

Fig. 3. Fragmentation methods for identification and confident site localization of O-GlcNAcylated peptides. (A) HCD mass spectrum of an O-GlcNAcylated peptide from TAB1. (B) UVPD mass spectrum of an O-GlcNAcylated peptide from TAB1. (C) 193 nm UVPD mass spectrum of an O-GlcNAcylated peptide from HCF. (D) EThcD mass spectrum of an O-GlcNAcylated peptide from HCF. (Adapted from reference[92], with the permission from American Chemical Society).

Quantitative proteomics for comprehensive analysis of protein O-GlcNAcylation

Quantitative proteomics is very powerful for biological studies as it enables us to systematically and quantitatively analyze proteins in different samples.[93] There are three main methods to quantify proteins by MS: label-free quantification (LFQ),[94] stable isotope labeling with amino

acids in cell culture (SILAC),[95] and isobaric tagging.[96-98] These methods can also be applied for quantification of protein modifications, including O-GlcNAcylation.

Combining metabolic labeling and SILAC, Cox et al. developed a workflow to quantify stimulus-dependent changes in O-GlcNAcylated proteins.[99] The workflow was applied to study the role of O-GlcNAcylation in protein trafficking from cells treated with BFA (Brefeldin A). Eleven O-GlcNAcylation sites were identified on coatomer subunit gamma-1 (COPγ1), and these modifications on COPγ1 were reduced in cells with the BFA treatment, which halted COPγ1 trafficking. Therefore, the results revealed the potential role of O-GlcNAcylation in regulating protein trafficking. Furthermore, Qin et al. developed a quantitative time-resolved proteomic strategy that used sugar analog labeling and pulse-chase SILAC to quantify the turnover of O-GlcNAcylated proteins.[100] In total, the turnover rates of 533 O-GlcNAcylated proteins in NIH 3T3 cells were quantified, and about 14% of the proteins had slow turnover rates. Among them, three core proteins of the box C/D small nucleolar ribonucleoprotein complexes (snoRNPs) were studied, and it was shown that O-GlcNAcylation stabilized these proteins and was essential for the snoRNP assembly.

As O-GlcNAcylation plays important roles in human diseases, quantitative proteomics can be very useful to unveil the disease mechanisms. To study O-GlcNAcylation in the pathogenesis of sporadic Alzheimer’s disease (AD), Wang et al. employed the tandem mass tag (TMT) reagents to label human brain tissues with or without AD, and enriched O-GlcNAcylated peptides by chemoenzymatic labeling using the engineered GalT. A total of 1094 O-GlcNAcylation sites were quantified from 530 proteins in the human brain tissue samples.[101] Among them, 131 O-GlcNAc peptides from 81 proteins were altered in the AD brain samples compared with the control ones.

Some proteins known as regulators in AD were found with altered O-GlcNAcylation level, which could pave the way for future studies of the pathologies of sporadic AD.

For some methods, the enrichment of O-GlcNAcylated peptides requires a designed tag that reacts with O-GlcNAc specifically, it provides an opportunity to use an isobarically encoded tag for quantification by MS. For instance, β-elimination of O-GlcNAc generated an α, β-unsaturated ketone, and later derivatization using the isotopically encoded DTT or biotin-cystamine tag allowed for quantification of glycopeptides.[102, 103] Similar principle was adopted for the design of isotope-tagged cleavable linkers, which were applied to quantitatively study the level of O-GlcNAcylated proteins across the samples under different conditions (Fig. 4).[104, 105] Qin et al. designed a cleavable probe containing an isotope-encoded linker to quantify protein O-GlcNAcylation. The method was applied to site-specifically quantify O-GlcNAcylation levels under different concentrations of the metabolic labeling probe. Furthermore, O-GlcNAcylation stoichiometry levels were quantified in the placenta samples from male and female mice. The results confirmed that the male placenta had a lower O-GlcNAcylation level compared with the female ones.

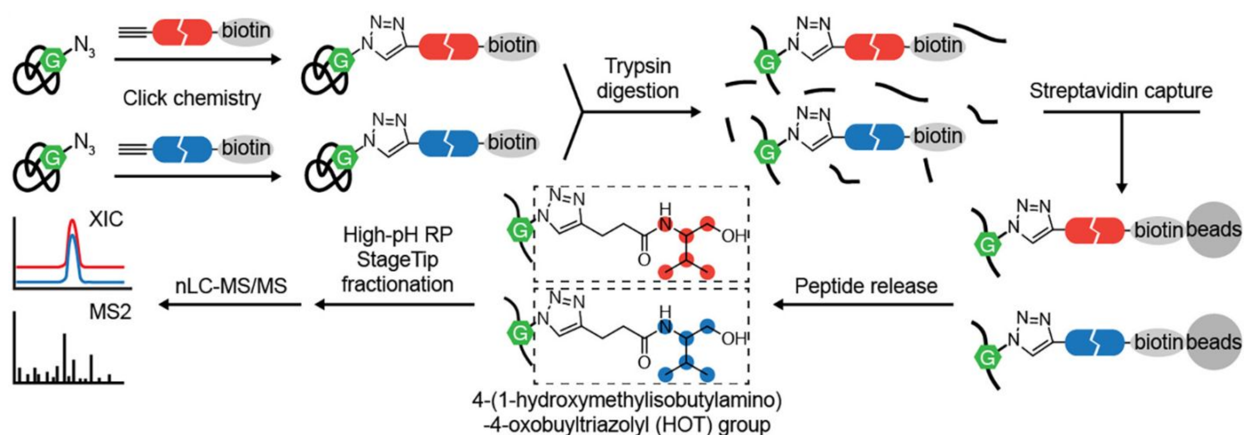

Fig. 4. Quantitative analysis of protein O-GlcNAcylation using an isotope-encoded cleavable probe. (Adapted from reference[105], with the permission from American Chemical Society)

Conclusion and outlook

Protein O-GlcNAcylation has attracted great attention due to its close link to cell metabolism and critical roles in regulating cell signaling and gene expression. Aberrant O-GlcNAcylation is directly related to human diseases, including diabetes, cancers, and neurodegenerative disorders. In recent years, several methods were developed to effectively capture O-GlcNAcylated proteins/peptides. In combination with MS-based proteomics, many O-GlcNAcylation sites on proteins from samples of various origins were confidently identified. Recently, O-GlcNAcylation sites identified so far were included in two databases, providing a comprehensive resource that will greatly benefit future research in protein O-GlcNAcylation.[106, 107] Moreover, coupled with quantitative proteomics, functions of protein O-GlcNAcylation in biological processes, and their changes in diseases can be studied. However, some limitations of the existing methods still impede their wide applications to study protein O-GlcNAcylation. Novel methods are urgently needed for more effectively and specifically capturing GlcNAcylated peptides/proteins in complex biological

samples. Furthermore, as the glycosidic bond for O-GlcNAcylation is fragile under the commonly used CID/HCD, ETD would be preferred for accurate site localization. However, few labs are equipped with ETD instrumentation. Finally, quantitative analyses of protein O-GlcNAcylation are still very limited. As O-GlcNAcylation is critical for cellular homeostasis, comparison of O-GlcNAcylated proteins in different cellular compartments or under various conditions could further facilitate our understanding of the properties and functions of protein O-GlcNAcylation. Systematic and quantitative analysis of protein O-GlcNAcylation in many diseased samples will help unveil the disease mechanisms and explore potential drug targets. Compared with protein phosphorylation, which is extensively studied over the past decades, O-GlcNAcylation has received much less investigation. With novel and effective methods and advanced MS instrumentation, more in-depth studies will lead to unveil the roles of O-GlcNAcylation in biological systems.

Conflicts of interest

There are no conflicts to declare.

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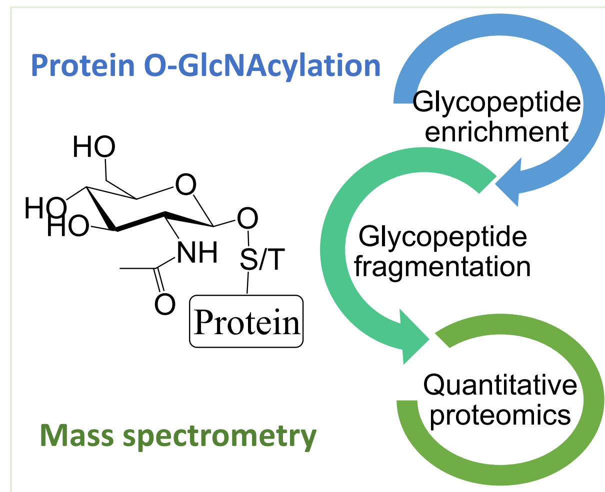

Protein O-GlcNAcylation plays critical roles in mammalian cells, and here we review MS-based proteomics methods for comprehensive and site-specific analysis of protein O-GlcNAcylation, ranging from enrichment, fragmentation, to quantification.