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Review

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Review

Demystifying *O*-GlcNAcylation: hints from peptide substrates

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Abstract

O-GlcNAcylation, analogous to phosphorylation, is an essential post-translational modification of proteins at Ser/Thr residues with a single β-*N*-acetylglucosamine moiety. This dynamic protein modification regulates many fundamental cellular processes and its deregulation has been linked to chronic diseases such as cancer, diabetes and neurodegenerative disorders. Reversible attachment and removal of *O*-GlcNAc is governed only by *O*-GlcNAc transferase and *O*-GlcNAcase, respectively. Peptide substrates, derived from natural *O*-GlcNAcylation targets, function in the catalytic cores of these two enzymes by maintaining interactions between enzyme and substrate, which makes them ideal models for the study of *O*-GlcNAcylation and deglycosylation. These peptides provide valuable tools for a deeper understanding of *O*-GlcNAc processing enzymes. By taking advantage of peptide chemistry, recent progress in the study of activity and regulatory mechanisms of these two enzymes has advanced our understanding of their fundamental specificities as well as their potential as therapeutic targets. Hence, this review summarizes the recent achievements on this modification studied at the peptide level, focusing on enzyme activity, enzyme specificity, direct function, site-specific antibodies and peptide substrate-inspired inhibitors.

Key words: *O*-GlcNAc transferase, *O*-GlcNAcase, O-linked *N*-acetylglucosamine modification, peptide substrates, post-translational modification

Introduction

O-GlcNAcylation is a ubiquitous post-translational modification (PTM) of serine or threonine residues with a single O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) on numerous proteins. In contrast to complex glycans that typically function on the cell surface by mediating numerous ligand–receptor interactions (Gambaryan et al. 1995; Gallegos et al. 2012; Torreno-Pina et al. 2014), O-GlcNAc modifications of proteins ubiquitously occur inside the cells where they regulate many basic cellular processes. Proteomics studies estimated that ~19% of the O-GlcNAcylated proteins, including NF-κB, p53 and STAT, are involved in regulation of gene expression (Hahne et al. 2013). O-GlcNAcylation regulates protein activity and integrates cellular signaling transduction by itself or in combination with intensive cross-talk with other PTMs (Guinez et al. 2008), especially phosphorylation (Wang et al. 2008; Ande et al. 2009; Zhong et al. 2015). Histone proteins are also targets of O-GlcNAcylation, which is suggested to control the cell cycle and cell survival (Zhang et al. 2011; Fong et al. 2012). In addition, O-GlcNAc is also involved in the regulation of proteasome function by modifying the Rpt2 subunit (Zhang et al. 2003). To date, ca. 1492 O-GlcNAcylation sites have been recorded in the PhosphoSitePlus® database (www.phosphosite.org) (Hornbeck et al. 2015) and aberrant regulation of O-GlcNAcylation has been linked to chronic diseases such as type 2 diabetes, cancer and Alzheimer's disease (AD; Liu et al. 2004; Banerjee et al. 2015). In the case of cancer, modulation of O-GlcNAcylation by inhibitors showed an anticancer effect by inhibiting breast tumor

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growth and invasion, suggesting that *O*-GlcNAcylation is a potential therapeutic target for cancer treatment (Caldwell et al. 2010).

Like protein phosphorylation, the *O*-GlcNAc modification of proteins is also reversible, but it is under the control of only a pair of antagonistic enzymes. *O*-GlcNAc transferase (OGT) catalyzes the addition of GlcNAc onto the protein using uridine 5′-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) as the sugar donor, while *O*-GlcNAcase (OGA) removes the *O*-GlcNAc from the modified protein. Remarkably, OGT activity is largely influenced by the intracellular concentration of UDP-GlcNAc which is the metabolic end product of the hexosamine biosynthetic pathway (HBP; Kreppel and Hart 1999; Swamy et al. 2016). Since the HBP integrates metabolisms of glucose, acids (acetate), amino acids (glutamine) and nucleotides (UTP) to generate UDP-GlcNAc, *O*-GlcNAcylation is considered as a nutrient sensitive PTM (Hawkins et al. 1997; Liu et al. 2007; Singleton and Wischmeyer 2008; Onodera et al. 2014; Durning et al. 2016).

The gene encoding OGT is localized on the X-chromosome in human and mouse and is considered to be essential in the mammalian development (Shafi et al. 2000). Although only one gene is responsible for OGT, it can generate three separate transcripts that encode three different isoforms of the enzyme. All OGT isoforms consist of a tetratricopeptide repeats (TPR) domain, a linker region and a C-terminal catalytic domain. In humans, all three OGT isoforms have an identical catalytic domain but differ in the number of TPR repeats, each of which includes ca. 34 amino acids (Lubas et al. 1997; Hanover et al. 2003). The longest one contains 13.5 TPR repeats (Jinek et al. 2004), is localized in both the nucleus and the cytoplasm and has been termed as nc-OGT. The second longest one has 9.5 TPR repeats, contains a mitochondrial targeting sequence and has been termed m-OGT. The shortest one has only 2.5 TPR repeats, is expressed in both the nucleus and the cytoplasm and is termed s-OGT, referring to the short nature of the TPR tail. Crystal structures of the OGT catalytic pocket with bound substrates have revealed that an ordered bi-bi mechanism was involved in the glycosylation reaction where the sugar donor UDP-GlcNAc binds first and facilitates the binding of the acceptor peptide (Lazarus et al. 2011). UDP-GlcNAc is bound in the OGT catalytic site by evolutionarily conserved residues important for catalytic activity. The peptide is bound in the OGT catalytic site by its amide backbone interaction with OGT side chains and the UDP moiety with an extended conformation. In the transfer of GlcNAc, the α-phosphate of UDP-GlcNAc is possibly used as a catalytic base, together with catalytical residues of OGT such as Lys842 (Schimpl, Zheng et al. 2012), although the identity of the base has not been firmly established (Withers and Davies 2012). Surprisingly, OGT was also shown to have a proteolytic activity in the maturation process of the cell cycle host cell factor 1 (HCF1) and the cleavage of HCF1 occurs within the OGT catalytic domain in a UDP-GlcNAc dependent manner (Capotosti et al. 2011; Lazarus et al. 2013). The underlying mechanism for this unexpected activity of OGT seems to involve glycosylation of the HCF1 glutamate side chain and conversion of this glycosylation product to an internal pyroglutamate which undergoes backbone cleavage (Janetzko et al. 2016). Studies of TPR repeat structures and interactors have demonstrated that TPR repeats form important protein–protein interactions which might contribute to the substrate specificity of OGT (Iyer and Hart 2003; Iyer et al. 2003; Jinek et al. 2004; Lazarus et al. 2013). Recent studies further suggested that OGT recognizes the protein substrate TAB1 through extensive interactions with the TPR domain, suggesting an essential role of TPR in OGT recognition of physiological substrates (Rafie et al. 2017). In addition, it was proposed that transient interactions of OGT and its partner interactors may form holoenzymes which might be substrate-specific (Deng et al. 2014). While aspects of TPR recognition and holoenzymes formation may play significant roles, all OGT substrates will additionally have to obey the specificity constraints imposed upon them especially from the −3 to +3 positions around the modification sites by the characteristics of the OGT active site, which can be efficiently studied with short peptide OGT substrates (Lazarus et al. 2012, 2013; Schimpl, Zheng et al. 2012).

The human OGA gene is localized on chromosome 10q24 and it has been implicated in AD (Kim et al. 2006). It has been reported that humans have two OGA splicing transcripts which produce the long OGA (OGA-L or OGA-FL) and the small nuclear variant of OGA (OGA-S or OGA-NV), respectively (Li et al. 2010). OGA-L is a 103 kDa protein that typically has an N-terminal catalytic domain, a stalk domain and a C-terminal histone acetyltransferase (HAT) like domain. OGA-S (76 kDa) does not have the HAT like domain and it shows a 400-fold lower hydrolase efficiency than OGA-L, although they have similar catalytic properties (Kim et al. 2006; Macauley and Vocadlo 2009; Li et al. 2010). Despite ample progress in the investigation of human OGA, its mechanisms and inhibitors have been mostly derived from studying the bacterial homologs of OGA. In the hydrolysis reaction by bacterial CpOGA, Asp401 (Asp175 in human OGA) forms major hydrogen bonds with the sugar portion of the substrate, while Asp298 (Asp285 in human OGA) protonates the glycosidic bond (Rao et al. 2006; Selvan et al. 2017). As a boost for research on the human OGA, it should be noted that crystal structures of human OGA became available recently (Elsen et al. 2017; Li et al. 2017; Roth et al. 2017). In agreement with bacterial OGA studies, it was found that human OGA mainly uses Asp285 to anchor the GlcNAc moiety of the substrate through hydrogen bonds and uses Asp174 as a catalytic base and Asp175 as a catalytic acid (Cetinbas et al. 2006; Li et al. 2017).

Recent research work on demystifying *O*-GlcNAc processing enzymes, using short peptide substrates, has seen progress and further advanced our understanding of this essential PTM. This motivated us to summarize the usefulness of peptide substrates to gain insights into the properties of OGT and OGA. This review covers five aspects of the use of peptides: (1) it describes peptide substrate-based methods for the study of *O*-GlcNAcylation; (2) it summarizes the OGT and OGA substrate preferences; (3) it discusses the incorporation of *O*-GlcNAc into peptides as well as proteins by chemical synthesis; (4) it summarizes the generation of site-specific *O*-GlcNAc antibodies using glycopeptides and (5) it discusses the early development of OGT inhibitors utilizing the peptide substrate information.

Identifying activities of *O*-GlcNAc processing enzymes using peptide-based approaches

OGT activity profiling using peptide substrates

The relevance of the use of short peptides, as substrates, to profile activity and acquire substrate specificity for kinases and phosphatases has been shown on numerous occasions for kinases and phosphatases, which has lead to great successes in the field of phosphorylation (Jarboe et al. 2012; Deng Y et al. 2014; Stanford et al. 2014; Hovestad-Bijl et al. 2016; Miller and Turk 2016). By taking advantage of their flexibility and availability, peptide substrates of OGT have become efficient tools to predict OGT activity (Lubas and Hanover 2000; Shen et al. 2017) as well as a means to study aspects

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of binding and catalysis in crystal structures of complexes (Lazarus et al. 2011; Schimpl, Borodkin et al. 2012). One commonly used peptide substrate of OGT is the CKII_340_352 peptide derived from the α-subunit of casein kinase II which is *O*-GlcNAcylated at residue Ser347 *in vitro* and *in vivo* (Kreppel and Hart 1999; Zachara et al. 2011; Tarrant et al. 2012). Several excellent OGT peptide substrates have also been discovered through different means including RBL2_410_422 (Pathak et al. 2015; Shi et al. 2016), TAB1_389_401 (Pathak et al. 2012; Schimpl, Borodkin et al. 2012) and ZO-3_357_371 (Table I) (Shi et al. 2017). All these peptide substrates have individual protein origins, and therefore provide entries into the study of the biology of the parent proteins.

In recent years, protein and peptides arrays of different formats have been applied to identify *O*-GlcNAcylation and the study of OGT activities (Deng RP et al. 2014; Ortiz-Meoz et al. 2014; Pathak et al. 2015; Shi et al. 2016, 2017). A human protein microarray containing 8000 unique proteins has been successfully used to study OGT activity and hundreds of novel proteins have been identified as OGT substrates with diverse functions, including kinases, transcription factors and apoptosis-related proteins (Ortiz-Meoz et al. 2014). However, the protein array cannot be used to generate straightforward sequence information. While it has its own valuable uses, the use of small peptides focuses more readily on the specificity of the enzyme. The use of a peptide microarray to study OGT offers advantages in generating inherent sequence-specificity information. As such, the method is complementary to the protein array where additional protein–protein interactions may additionally determine the specificity. The first example of a peptide microarray study described a library of 720 synthesized biotinylated peptides with sequences derived from the human proteome, using a scintillation proximity assay (Pathak et al. 2015). In this assay (Figure 1A), the OGT reaction was carried out in 384-well polypropylene plates, with one peptide in each well, in the presence of radio labeled UDP-[^{3}H]GlcNAc. Then, the *O*-GlcNAcylated peptides were immobilized on a streptavidin coated scintillant flashplate, which allowed the direct quantification of radio labeled peptide by using scintillation counting. This approach revealed comprehensive sequence information which is discussed in Section 3. Disadvantages include the fact that this approach involved radio labeling which requires time for signal development and is therefore less convenient for real time kinetic analysis.

A different peptide microarray with kinetics monitoring was described for OGT activity by our group (Figure 1B; Shi et al. 2016). This dynamic peptide microarray technology was initially developed for kinase activity profiling (Lemeer et al. 2007; Anderson et al. 2015). In the kinase field, the use of immobilized peptide sequences derived from knowledge of protein phosphorylation sites has proven to be highly valuable for the study of kinase activity (Hilhorst et al. 2009). In this array, each peptide was prepared with additional Cys-Gly residues at the N-terminus, which allows immobilization of a maleimide functionalized porous membrane surface. However, the nature of this immobilization chemistry also makes the array results less quantifiable when studying peptides with multiple cysteines, due to additional non-terminal attachment modes. The OGT reaction was performed in the presence of a fluorescently labeled detection antibody. The reaction mixture can be pumped up and down periodically through the membrane during the entire reaction time and the fluorescent signal of only the bound fluorescent antibody is acquired at regular intervals. This is possible by temporarily placing the solution with unbound fluorescent antibody below the chip, outside the detector range (Figure 1B). As a result, this enables a kinetic reading of the enzymatic modification of each peptide on the array without removing the unbound antibody by extra washing. *O*-GlcNAcylation of peptides with this array can be achieved not only by using purified OGT enzyme but also by using a cell lysate. In addition, this array-based identification of OGT substrates from peptides known to contain phosphorylation sites, also made it possible to study cross-talk between these two modifications (Shi et al. 2017), which will be discussed in Section 4. Kinase activity profiling with such arrays has been demonstrated to be of relevance for cancer diagnosis and help in the prediction of efficacy of cancer drugs for a specific patient (Folkvord et al. 2010), so ultimately *O*-GlcNAcylation on peptide arrays may exhibit a similar medicinal relevance. Although these peptide-based techniques provide a tool for OGT substrate identification, these hits were not validated at the protein level.

Detection of OGA activities with peptide substrates

In vitro OGA activity is normally detected by using the color producing compound *p*-nitrophenyl 2-acetamido-2-deoxy-*β*-D-glucopyranoside (*p*NPGlcNAc) and the fluorogenic compounds 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MuGlcNAc) and fluorescein di(*N*-acetyl-beta-D-glucosamine) (FDGlcNAc) as substrates (Dong and Hart 1994; Macauley et al. 2005; Kim et al. 2006). However, these compounds are not natural substrates of OGA and cannot fully reflect OGA activity with a natural glycopeptidic substrate. Similarly to the OGT array assay, a fluorescent method for the detection of OGT activity was used to study the removal of GlcNAc from a more natural substrate (Figure 1C). This peptide microarray-based approach was recently described to measure OGA activity from both purified OGA as well as OGA present in cell lysates derived from several cancer cell lines (Sharif et al. 2017). A series of glycopeptides as OGA substrates was prepared based on peptides which are known OGT targets and these were immobilized on the array. The array was subjected to purified OGA or cell lysates, followed by detection of the remaining *O*-GlcNAc on each peptide with a fluorescent antibody. In addition, a more comprehensive version of this glycopeptide microarray with more diversity has to be used to further validate its potential to profile OGA catalytic properties.

Table I. A list of peptides mentioned in this review

Primary sequence	Protein	Start and end	Modification site
PGGSTPVSSANMM	CKII	340–352	Ser347
PVSVPYSSAQSTS	TAB1	389–401	Ser395
KENSPCVTPVSTA	RBL2	410–422	Ser420
RESSYDIYRVPSSQS	ZO-3	357–371	Ser369
QVAHSGAKAS	hOGA	401–410	Ser405
VYKSPVVSGDTSPRH	Tau	393–407	Ser400
KTAPVQLWVDSTPPPGTRVRA	p53	139–159	Ser149

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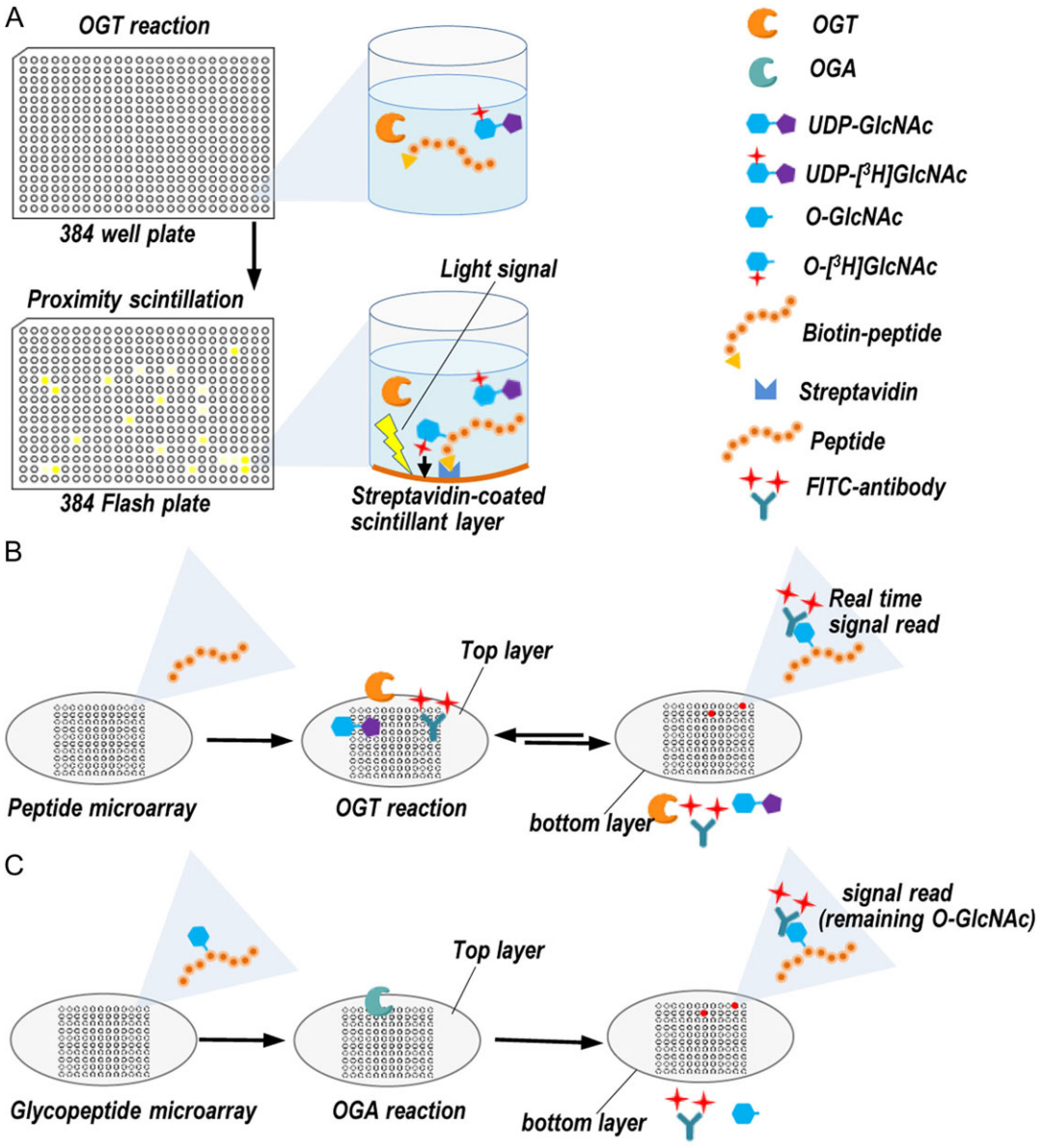

Fig. 1. Schematic depiction of high-throughput assays for activity of OGT and OGA against peptide substrates. (**A**) Scintillation proximity-based OGT assay. (**B**) Peptide microarray-based real time OGT assay. (**C**) Glycopeptide microarray-based OGA assay.

Essential role of amino acids around the *O*-GlcNAc site in substrate recognition

OGT substrate recognition

It was recently reported that OGT has a peptide sequence preference around its modification site on short peptide substrates (Jochmann et al. 2014; Liu et al. 2014; Pathak et al. 2015). A high density peptide microarray is an ideal platform to study such enzymatic specificities (Lai et al. 2016). In an array analysis of 720 Ser/Thr peptides for OGT activity, ~10% of these peptides were identified as OGT substrates (Pathak et al. 2015), suggesting that OGT has the ability to specifically recognize a substrate by its primary sequence among hundreds of peptides (Shi et al. 2016, 2017). With the sequence information of these identified peptides, an unbiased OGT substrate motif was obtained as [TS][PT][VT]S/T[RLV][ASY] (from −3 to +2 with respect to *O*-GlcNAcylation site) which was also verified in select cases by crystal structures (Pathak et al. 2015). This motif is generally in line with the motif PPV[S/T]SATT (Liu et al. 2014) and PPV[S/T]TA (Wu et al. 2014) obtained by frequency analysis of known OGT substrates. The clear preference of Pro at the −2 site by OGT enables the peptide substrate to display an extended conformation, which facilitates its binding to the OGT active site (Lazarus et al. 2011; Pathak et al. 2015). Interestingly, it was also reported that OGT has a strictly negative preference for Pro at the +1 subsite, which could prevent *O*-GlcNAcylation at a Ser/Thr/Pro rich region even if other sites match with the substrate motif (Leney et al. 2017).

Despite this progress, the current motifs are not able to accurately predict OGT peptide substrates. We have found that only a few of our experimentally identified novel substrates matched with these reported motifs and some matched peptides in our experiments were not experimentally identified as a substrate (Shi et al. 2016, 2017). Nevertheless,

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for the best discovered hit peptide from our experiments the motif successfully identified the modification site. This site was confirmed through mutation experiments by making a series of peptides (Shi et al. 2017). Regardless of predictable accuracy, all these findings strongly suggest that the primary sequence is a very important factor in determining the substrate specificity of OGT.

It should be noted, also, that no obvious consensus motif has been found by proteomic studies of *O*-GlcNAc proteins from murine synaptosome although moderate preferences for Pro (−2 and −3 positions) and Val (−1 or −3 positions) by OGT have been observed (Trinidad et al. 2012, 2013; Vosseller et al. 2006). There is a possibility that *in vivo* OGT might achieve substrate specificity following a less stringent motif and that other determinants such as the TPR repeats and holoenzymes are involved as discussed in Section 1. Regardless of this, it should still be possible to generate a more complete and comprehensive OGT substrate motif, since short peptides only interact with OGT at the active site, which would be a very valuable tool for OGT protein substrate prediction and identification.

OGA substrate recognition

The investigation of human OGA specificity was primarily limited by the lack of available OGA crystal structures with bound substrate. Hence, a bacterial OGA orthologue such as *Cp*OGA was widely used as a model to explore human OGA catalytic properties (Rao et al. 2006; Schimpl, Borodkin et al. 2012). The crystal structure of *Cp*OGA in complex with synthetic glycopeptides derived from p53 (Yang et al. 2006), TAB1 and OGA (Table I) was used to gain insights into the human OGA specificity (Schimpl, Borodkin et al. 2012). In this structure, extensive hydrogen bonds were observed between the GlcNAc moiety and *Cp*OGA (Schimpl, Borodkin et al. 2012), suggesting an important role of GlcNAc for OGA substrate binding and recognition. Interestingly, although no peptide side chain was found to be involved in direct interaction with the enzyme, a combination of intramolecular hydrogen bonds and hydrophobic interactions between the peptide backbone and *Cp*OGA forced the glycopeptide into a conserved "V-shape" conformation that makes it possible to access the *Cp*OGA active site (Schimpl, Borodkin et al. 2012). In addition, kinetic studies showed that human OGA has similar catalytic parameters for five *O*-GlcNAcylated proteins and a glycopeptide derived from a protein substrate is a better substrate than the protein itself. These data further suggest that GlcNAc is the primary determinant in OGA catalysis but amino acid residues may hinder (Shen et al. 2012). Now that crystal structures of human OGA have recently become available (Elsen et al. 2017; Li et al. 2017; Roth et al. 2017) with, e.g., the complexed *O*-GlcNAcylated p53_144-154 peptide, again an essential role of GlcNAc for OGA substrates recognition is indicated (Li et al. 2017). However, in this case also the peptide side chains were involved in hydrophobic interactions with OGA. Substitution of substrate peptide residues and OGA residues involved in the interaction, both lead to a reduction of substrate binding (Li et al. 2017). Although *O*-GlcNAc is the dominant structural feature for OGA substrate recognition, additional specific interactions involving substrate amino acids might exist, which could lead to differential preferences of substrates by OGA. Whether OGA requires a distinct substrate motif could be further studied, e.g., by using an array of synthetic glycopeptides. Based on the above fact that the sugar moiety is the major determinant for OGA activity, a hydrolytically stable sulfur-linked glycopeptide (S-GlcNAc) was synthesized and incorporated into the protein α-synuclein (De Leon et al. 2017) where indeed stability against OGA was observed.

Global regulation of *O*-GlcNAc cycling

While several comprehensive reviews have discussed the regulatory mechanisms of O-GlcNAc cycling (Love et al. 2010; Bond and Hanover 2013), recent findings add to this picture. *O*-GlcNAcylation cycling is critically controlled by many factors (Figure 2). OGT activity is greatly dependent on the intracellular concentration of UDP-GlcNAc, which is a metabolic product of the HBP together with fatty acid metabolism, amino acid metabolism and nucleotide metabolism (Hawkins et al. 1997; Liu et al. 2007; Singleton and Wischmeyer 2008; Onodera et al. 2014). The altered levels of OGT and OGA in cancer cells suggests the involvement of regulation in the enzyme expression levels (Krzeslak et al. 2012; Lynch et al. 2012; Sharif et al. 2017). UDP, a product of the O-GlcNAcylation reaction, might act as a feedback inhibitory regulator of OGT, considering its strong binding to the enzyme of ca. 2 μM (Dorfmueller et al. 2011). The extensive cross-talk of phosphorylation and *O*-GlcNAcylation represent novel regulatory mechanisms from the substrate side, which is slowly becoming more clear (Dias et al. 2009; Wang et al. 2010; Leney et al. 2017; Shi et al. 2017). Furthermore, the inherent specificity of especially OGT (Shi et al. 2016) and now seemingly also of OGA, enables their ability to selectively act on substrate proteins based on their amino acid sequence (Li et al. 2017). Finally, the role of OGT's TPR repeats have been implicated in the substrate preference based on protein–protein interactions with the substrate protein (Rafie et al. 2017). Globally, it seems that to maintain a normal *O*-GlcNAcylation status, the addition of *O*-GlcNAc involves stringent and multiple levels of regulation, while for the removal of *O*-GlcNAc this seems to be less so. Nevertheless, additional OGT/OGA sequence specificities and cross-talk mechanisms will likely be uncovered in the future.

Direct role of *O*-GlcNAcylation from synthetic glycopeptide to glycoprotein

Elucidating the physiological function of a specific O-GlcNAcylation event on a specific protein is a challenging task because of the complex natural context of proteins within the cell. Removing of a putative modification site by gene mutation has been widely performed to indirectly observe the effect of a specific PTM in both *in vitro* and *in vivo* situations, resulting often in unconfirmed hypotheses. Using synthetic peptides is an efficient way to unambiguously introduce specific PTMs in a peptide which can subsequently be incorporated into a protein using advanced chemical ligation protocols. This then enables the direct study of the role of that modification.

The advantage of peptide chemistry in studying a specific PTM has been well-demonstrated in the study of the Tau protein, which is linked to assembly and stabilization of microtubules for the proper function of neurons (Himmler et al. 1989; Smet-Nocca et al. 2011). Misfolding and aggregation of Tau has been implicated in AD and other neurodegenerative disorders, which is mainly caused by abnormal PTMs including phosphorylation and O-GlcNAcylation (Yuzwa et al. 2012; Morris et al. 2015). Using a synthetic phosphorylated Tau peptide as an OGT substrate or an *O*-GlcNAcylated Tau peptide as a kinase substrate, mass spectrometry experiments revealed a reciprocal relationship between phosphorylation (on Serines 396 and 404) and *O*-GlcNAcylation (on serine 400) in peptide Tau-392_411 (Smet-Nocca et al. 2011). The phosphate group at Ser400 completely prevents *O*-GlcNAcylation of this peptide by direct occupation of the *O*-GlcNAcylation site, and the phosphate groups at Ser396 and Ser404 led to a decreased *O*-GlcNAcylation

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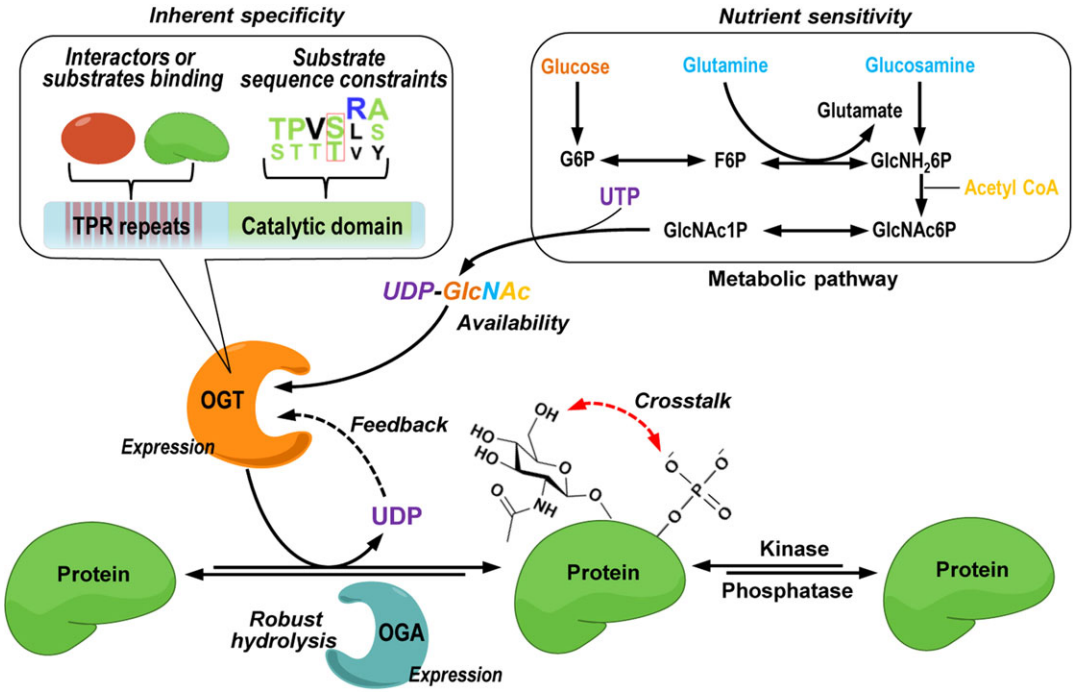

Fig. 2. The global regulation of *O*-GlcNAc cycling. The cellular *O*-GlcNAcylation of proteins can be controlled by factors including OGT expression, inherent specificity (TPR and consensus sequence), nutrient availability, UDP-GlcNAc availability, UDP feedback inhibition, cross-talk with phosphorylation, OGA expression and OGA's robust hydrolysis ability.

level at Ser400. In the other direction, *O*-GlcNAcylation of Ser400 reduced the phosphorylation of Ser404 mediated by the Cdk2 kinase complex and completely suppressed the phosphorylation of Ser396/400/404 mediated by GSK3β (Figure 3A).

Recently, a dynamic peptide microarray approach has been described to study the interplay between *O*-GlcNAc and tyrosine phosphorylation in a direct and real time manner (Shi et al. 2017). In this work, dual substrates of OGT and the Jak2 tyrosine kinase were identified from an array of peptides derived from known tyrosine kinase protein substrates. With these dual substrates, the effect of prior *O*-GlcNAcylation by OGT on the subsequent tyrosine phosphorylation by Jak2 was determined, and vice versa. This approach revealed that tyrosine phosphorylation of six peptides prevented their subsequent *O*-GlcNAcylation, whereas no correlation was found in the other direction. Peptides derived from the ZO-3 protein were synthesized with a phosphate group on Tyr364 and a GlcNAc group on Ser369, respectively. They were immobilized on the microarray to further validate the identified cross-talk kinetically (Figure 3B). Besides using purified enzymes, this cross-talk was also observed using a HeLa cell lysate as the source of both OGT and the kinase. Although useful kinetics information was achieved in this case, future studies should also confirm these observations with *in vivo* experiments. This type of cross-talk, might prove to be more general with additional studies (Leney et al. 2017).

A modified peptide can be employed to synthetically access a modified full-length OGT protein substrate through a semi-synthetic strategy using native chemical ligation (Dawson et al. 1994; Smet-Nocca et al. 2011). This enables validation of its physiological functions at the protein level. Recently, a synthesis of *O*-GlcNAcylated α-synuclein by ligating synthesized peptide and expressed protein portions, has indicated that *O*-GlcNAcylation of this protein has an inhibitory effect on its aggregation, suggesting a crucial role of *O*-GlcNAcylation in the progression of AD and Parkinson's disease (Marotta et al. 2015). In this study, the *O*-GlcNAc modified peptide was prepared by an Fmoc-based solid phase peptide synthesis strategy, resulting in a C-terminal thioester and an N-terminal thioproline-protected cysteine. Through an expressed protein ligation strategy (Figure 3C), the N-terminal cysteine of a recombinant protein segment was transesterified with the C-terminus of the above peptide and then rapidly rearranged to form a stable peptide bond. Similarly, the ligation of the newly formed construct and a recombinant protein segment with a C-terminal thioesters was carried out. After radical-based desulfurization of the introduced extra cysteine, a full-length α-synuclein containing an *O*-GlcNAc modification was obtained. The synthetic protein showed secondary structure characteristics that were the same as those derived from the unmodified protein. With this synthetic *O*-GlcNAc modified α-synuclein, it was demonstrated that a single *O*-GlcNAcylation at Thr72 has a notable and substoichiometric inhibitory effect on α-synuclein aggregation, suggesting that modulating a specific *O*-GlcNAcylation is perhaps a viable therapeutic strategy for PD. In addition, *O*-GlcNAcylation of this protein at Thr72 was shown to be able to slightly increase its phosphorylation at Ser87 by the CK1 kinase, but inhibit its phosphorylation at S129 by the GRK5, CK1 and PLK3 kinases (Figure 3C). As a result, such cross-talk may also affect α-synuclein aggregation.

Another well-demonstrated example is the synthesis of *O*-GlcNAcylated CK2 which is involved in cell growth and proliferation through its kinase activity (Lebrin et al. 2001). In this case (Figure 3C), an *O*-GlcNAcylated protein was prepared using the same expressed protein ligation strategy, except that the synthetic peptide was prepared with metabolically stable thio-linked GlcNAc (S-GlcNAc) (Tarrant et al. 2012). Using this protein, it was found that *O*-GlcNAcylation of Ser347 inhibits its phosphorylation at Thr344 and modulates CK2 kinase substrate selectivity. This methodology is very powerful

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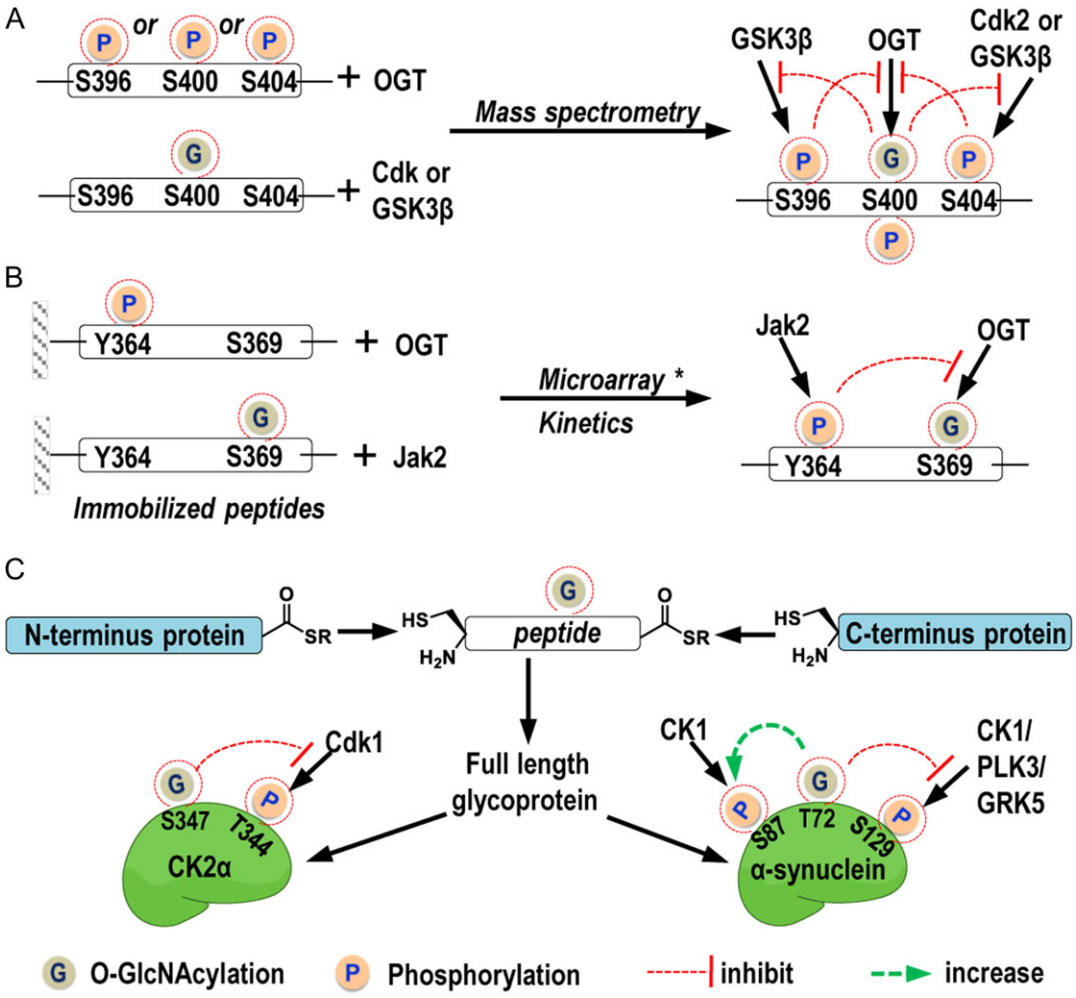

Fig. 3. Study of *O*-GlcNAcylation and its cross-talk in a direct manner using synthetic peptide and synthetic proteins. Mass spectrometry-based study of cross-talk on Tau_392-411 (**A**) and peptide microarray-based study of cross-talk on ZO-3_357-371 (**B**) with synthetic glycopeptide and phosphopeptide. *This kinetic peptide microarray assay is described in Figure 1B. (**C**) Functional study of *O*-GlcNAc modification on CK2α and α-synuclein using synthetic glycoprotein.

although the synthesis is challenging and in certain cases protein folding issues may occur.

Generation of specific *O*-GlcNAc antibodies with small OGT substrate peptides

A well-studied substrate peptide provides important sequence-specific information which can be used as an antigen to generate modification-site-specific antibodies. Such specific antibodies would be powerful tools for the further validation of *O*-GlcNAcylation on a specific protein at the more complex endogenous levels. Take the Tau protein for example, as an early attempt with a synthetic glycopeptide (CSPVV-(*O*-GlcNAc)-SGDTS) as an antigen. Its use led to the generation of a polyclonal antibody (termed 3925) that recognizes Tau with the *O*-GlcNAc modification at Ser400 *in vitro*, and both the peptide sequence and the GlcNAc moiety were found to contribute to the antibody specificity (Yuzwa et al. 2011). However, this antibody was also found to recognize a range of other *O*-GlcNAc proteins, which makes it unsuitable for use in *in vivo*. Since then, a more specific monoclonal antibody against the Tau protein *O*-GlcNAcylation at Ser400 (termed OTau (S400)) was generated using the *O*-GlcNAc peptide (VYKSPVV-(*O*-GlcNAc))-SGDTSPRH based on a comprehensive study of *O*-GlcNAcylation by peptide scanning (Smet-Nocca et al. 2011; Cameron et al. 2013). This antibody was successfully applied to study the *O*-GlcNAcylation of the full tau protein at Ser400 *in vitro* and *in vivo*, which may further facilitate the elucidation of the function of Tau in AD (Cameron et al. 2013). There are other examples of site-specific *O*-GlcNAc antibodies generated by using glycopeptides, such as the monoclonal antibody against Thr58 *O*-GlcNAc in c-Myc (Kamemura et al. 2002), a polyclonal antibody against Ser1011 *O*-GlcNAc in IRS-1 (Klein et al. 2009) and a polyclonal antibody against Ser395 *O*-GlcNAc in TAB1 (Pathak et al. 2012). These works indicate that the sequence information generated in the peptide-based studies of *O*-GlcNAcylation facilitates the detection of protein *O*-GlcNAcylation with site-specific antibodies.

Specific OGT inhibitors/bisubstrate inhibitors from OGT substrates

Specific and potent inhibitors are valuable tools for deeper investigation of the biological functions of OGT. The development of OGT

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inhibitors is still in its early stages and the number of selective OGT inhibitors with suitable pharmaceutical properties is still quite limited. Current OGT inhibitors through rational designs are mostly small analogs of UDP-GlcNAc and UDP targeting the sugar donor binding site of OGT, such as C-UDP (IC_{50} = 9 μM) (Dorfmueller et al. 2011), Alloxan (IC_{50} = 18 μM) (Konrad et al. 2002; Dorfmueller et al. 2011), UDP-GlcNAc glycosyl C-phosphate (UDP-C-GlcNAc, IC_{50} = 41 μM) (Chang et al. 2006; Hajduch et al. 2008) and a thioglycosyl analog of UDP-GlcNAc (UDP-S-GlcNAc, IC_{50} = 93 μM) (Dorfmueller et al. 2011). Remarkably, UDP, the byproduct from *O*-GlcNAcylation, showed the strongest inhibitory effect of them all with an IC_{50} value of 1.8 μM (Dorfmueller et al. 2011). Although low micromolar inhibitors have been generated by mimicking the sugar donor, they are not cell permeable or show unfavorable side effect by affecting other enzymes that utilizing UDP or UDP-GlcNAc (Dorfmueller et al. 2011). A noteworthy rational approach described is to produce sugar donor mimic UDP-5SGlcNAc in cells by using the metabolic nature of GlcNAc salvage and HBP pathways with a precursor Ac-5SGlcNAc (Gloster et al. 2011). UDP-5SGlcNAc (K_i = 8 μM) produced by this approach has been shown to be able to disrupt OGT activity in cases (Andres et al. 2017), but it might have side effects on cell surface glycosylation and other metabolic pathways. A high throughput screening assay was also used for the discovery of OGT inhibitors, such as a substrate analog displacement assay in which a fluorescent UDP-GlcNAc analog was used as a probe to determine the binding of small molecules by fluorescence polarization (Gross et al. 2005). In this work, a cell permeable OGT inhibitor (OSMI-1, IC_{50} = 2.7 μM) was identified with a good but not perfect selectivity (Ortiz-Meoz et al. 2015). Recently, a natural product OGT inhibitor L01 was discovered with an IC_{50} value of 2.7 μM through a structure-based virtual screening and this compound showed lower toxicity and a higher selectivity *in vitro* and *in vivo* (Liu et al. 2017).

One study reported on developing OGT inhibitors by mimicking the sugar acceptor peptide (Borodkin et al. 2014). The use of the enzyme–substrate interacting motif as a template to develop specific peptide inhibitor has been well-demonstrated in the field of kinases (Nishikawa et al. 2000; van Wandelen et al. 2013), although high affinity may not be easily achieved with a relatively flexible peptide backbone only. In our recent work, we have shown that an OGT substrate peptide in which the serine (Ser420) at the *O*-GlcNAcylation site was replaced with an alanine, which cannot be *O*-GlcNAcylated, exhibited ~50% OGT activity inhibition at 500 μM (Shi et al. 2016). A closely related peptide substrate was shown to bind the OGT catalytic site with several hydrogen bonds and Van der Waals interactions (Pathak et al. 2015), so it was hypothesized that the alanine mutant peptide still binds the active site of OGT. These findings provide the possibility that peptide acceptors can be starting points of OGT inhibitor development by taking advantage of the specific contacts with OGT.

Conclusion and perspectives

In the last decade, the use of peptide substrates has remarkably facilitated the study of *O*-GlcNAcylation and its processing enzymes at the active sites. OGT was shown to be selective with respect to the amino acids around the modification site, but the current OGT substrate motif or sequon derived from a limited number of peptide substrates is not complete and not yet able to accurately predict substrates. It has to be noted that there might be other synergistical modes of selectivity for OGT with respect to protein substrates, involving TRP repeat binding and holoenzymes, but this is not the case for short peptide substrates. With further profiling of OGT peptide substrates, it should be possible to generate a more comprehensive motif that could be an important predictive tool with applications, e.g., in cancer. Further research by profiling cancer cell lines and patient tissues would lead to the identification of biomarkers with diagnostic and predictive value. Although a significant number of peptides have already been identified as OGT substrates, whether its parental proteins are indeed physiological targets of OGT in cells is mostly unknown. Site-specific *O*-GlcNAc antibodies would be useful to discover the biological function of a specific *O*-GlcNAcylation event and its relevance for disease, especially for cancer, diabetes and neurodegenerative disease. Specific and cell permeable OGT inhibitors are still urgently required, not only for research tools to study OGT but also for pharmacological applications.

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Conflict of interest statement

None declared.

Abbreviations

AD, Alzheimer's disease; GlcNAc, *N*-acetyl-D-glucosamine; HAT, histone acetyltransferase; HBP, hexosamine biosynthetic pathway; OGA, *O*-GlcNAcase; OGT, *O*-GlcNAc transferase; PTM, post-translational modification; TPR, tetratricopeptide repeats; UDP-GlcNAc, Uridine 5′-diphospho-*N*-acetylglucosamine; HAT, histone acetyltransferase.

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