Proteomic Approaches to Analyze the Dynamic Relationships Between Nucleocytoplasmic Protein Glycosylation and Phosphorylation

Stephen A. Whelan, Gerald W. Hart

Abstract—O-linked β-N-acetylglucosamine (O-GlcNAc) is both an abundant and dynamic posttranslational modification similar to phosphorylation that occurs on serine and threonine residues of cytosolic and nuclear proteins in all metazoans and cell types examined, including cardiovascular tissue. Since the discovery of O-GlcNAc more than 20 years ago, the elucidation of O-GlcNAc as a posttranslational modification has been slow, albeit similar to the rate of acceptance of phosphorylation, because of the lack of tools available for its study. Identifying O-GlcNAc posttranslational modifications on proteins is a major challenge to proteomics. The recent development of mild β-elimination followed by Michael addition with dithiothreitol has significantly improved the site mapping of both O-GlcNAc and O-phosphate in functional proteomics. β-Elimination followed by Michael addition with dithiothreitol facilitates the study of the labile O-GlcNAc modification in the etiology of disease states. We discuss how recent technological innovations will expand our present understanding of O-GlcNAc and what the implications are for diabetes and cardiovascular complications. (Circ Res. 2003;93:1047-1058.)

Key Words: O-linked β-N-acetylglucosamine • proteomics • BEMAD • OGT • O-GlcNAcase

O-linked β-N-acetylglucosamine (O-GlcNAc) was described in 19841 in studies designed to specifically label terminal GlcNAc with tritiated galactose [3H]Gal on proteins of lymphocytes. Subsequent product analysis showed that the incorporation was predominantly on nucleocytoplasmic proteins and that, unlike prototypical glycosylation, the modification consists of a single O-linked β-N-acetylglucosamine residue.2 O-GlcNAc is one of many posttranslational modifications, such as phosphorylation, acetylation, methylation, and ubiquitination, that contribute to the complexity of the cell’s proteome, attributable to multiple, competing, and various combinations of site occupancy.3–7 Although O-GlcNAc seems to be as abundant and dynamic and in some cases acts reciprocal to phosphorylation, it is not as well characterized because of its comparatively recent discovery 20 years ago compared with the discovery of phosphorylation 70 years ago and because of the technical difficulty associated with the study of this saccharide modification.8 How-
### Proteins Modified by O-GlcNAc

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ever, much progress has been made in the development of several tools for the identification and characterization of the O-GlcNAc modification.

Serine/threonine-O-GlcNAc is as abundant and dynamic as O-phosphate on nucleocytosolic proteins.\(^9\)\(^{-11}\) O-GlcNAc has been detected on a myriad of proteins, including RNA polymerase II and many of its associated transcription factors, kinases, phosphatases, cytoskeletal proteins, nuclear hormone receptors, nuclear pore proteins, signal transduction molecules, and actin regulatory proteins (Table).\(^9\)\(^{-11}\) Like phosphate, O-GlcNAc is dynamic; the half-life of O-GlcNAc is much shorter than the half-life of the proteins it modifies. Thus far, all O-GlcNAc–containing proteins are also phosphoproteins.\(^9\)\(^{-11}\) A global reciprocal relationship between O-GlcNAc and O-phosphorylation has been observed,\(^12\)\(^{-13}\) as well as a dynamic relation at specific sites.\(^12\)\(^{-14}\) Specific examples of the reciprocal relationship in which O-GlcNAc and O-phosphorylation have been mapped to the same residue include the Ser16 on the estrogen receptor/\(H9252\),\(^17\) the SV 40 T antigen,\(^18\) the Thr58 of c-Myc,\(^14\) and the RNA pol II,\(^15\) among others. O-GlcNAc and O-phosphorylation may also compete for adjacent sites, such as in casein kinase II (CKII; unpublished results) and RNA pol II,\(^15\) where the respective sites lie within a few residues of each other. The O-GlcNAc modification also plays an important role in protein-protein interactions.\(^19\) For example, O-GlcNAc–modified Sp1 seems
been shown to be inducible in response to glucose metabo-

cification of several key proteins in the insulin-signaling

pathway (insulin receptor substrate-1 \([\text{IRS-1}]\) and


directly causes insulin resistance, the hallmark of type II

diabetes.\(^{27}\) In addition, Federici et al.\(^{28}\) demonstrated a corre-


ticulate 75-kDa form and a splice variant that migrates at an apparent

molecular weight of 75 kDa.\(^{45}\) The 130-kDa form is

and the O-GlcNAcase activity,\(^{25}\) and the major O-GlcNAc

attachment site on the proto-oncogene c-Myc (Thr58) is a

phosphorylation site, at the same site as a phosphorylation site,

residues. O-GlcNAc modification may occur alone, adjacent to a

phosphorylation of tau is associated with the etiology of Alzhei-

mer disease, and given the current data, O-GlcNAc may be

Figure 1. Multiple states of O-GlcNac posttranslational modification. O-GlcNac modification occurs on serine and threonine residues. O-GlcNac modification may occur alone, adjacent to a phosphorylation site, at the same site as a phosphorylation site, or at multiple sites in any number of combinations. O-GlcNac and phosphorylation in some cases have a reciprocal relationship where they compete for the same site or adjacent sites. Specific examples of adjacent-site O-GlcNac modification are c-Fos kinase II \((\text{CKII}); \text{unpublished results}) and SRF; same site, c-Myc and estrogen receptor \((\text{ER}-\beta); \text{and multiple sites, RNA Pol II and SRF.}\)

to inhibit its binding to TATA-binding protein–associated factor II \(110\) and decreases Sp1 transcriptional activity on certain promoters.\(^{19}\) The O-GlcNac modification has also been shown to be inducible in response to glucose metabo-

tinulin signaling, and cell-cycle progression.\(^{20,21}\) Therefore, serine and threonine residues may exist in at least three states: glycosylated, phosphorylated, and unmodified (Figure 1).

O-GlcNAc has been implicated in several disease states. Many proteins that are O-GlcNAc modified are associated with Alzheimer disease, including tau,\(^{16}\) clathrin assembly proteins,\(^{22}\) and \(\beta\)-amyloid precursor protein.\(^{23}\) Hyperphosphorylation of tau is associated with the etiology of Alzheimer disease, and given the current data, O-GlcNAc may be protective to this hyperphosphorylation.\(^{24}\) O-GlcNAc also plays a role in cancer. Primary breast carcinomas have decreased levels of O-GlcNAc–modified proteins and increased O-GlcNAcase activity,\(^{25}\) and the major O-GlcNAc attachment site on the proto-oncogene c-Myc (Thr58) is a mutational hot spot for Burkitt’s lymphoma.\(^{14}\) Juang et al.\(^{26}\) have shown that in most patients with systemic lupus erythema-

sotus, there is reduced O-GlcNAc modification of Elf-1 transcription factor, which results in decreased T cell receptor \((\text{TCR})/\text{CD3}\) gene transcription. Elevation of O-GlcNAc levels in \(3T3-L1\) adipocytes, including the O-GlcNAc modification of several key proteins in the insulin-signal-

ming pathway \((\text{insulin receptor substrate-1} \ ([\text{IRS-1}]\) and \(\beta\)-catenin),

directly causes insulin resistance, the hallmark of type II diabetes.\(^{27}\) In addition, Federici et al.\(^{28}\) demonstrated a correlation between the elevation of O-GlcNAc on IRS-1, IRS-2, phosphatidylinositol 3-kinase \((\text{PI3K})\) regulatory subunit \((\text{p85}),\) and endothelial NO synthase \((\text{eNOS})\) and insulin resistance when human coronary artery endothelial cells were treated with excess glucose or glucosamine. Determining the sites of O-GlcNAc modification and their functional interplay with phosphorylation on the proteins within signaling pathways is required to begin to understand the molecular role of O-GlcNAc in the etiology of these diseases, including diabetes and cardiovascular disease.

O-Linked \(\beta-N\)-Acetylglucosamine Transferase

and \(\beta-d-N\)-Acetylglucosaminidase

O-linked \(\beta-N\)-acetylglucosamine transferase \((\text{OGT})\) and \(\beta-d-N\)-acetylglucosaminidase \((\text{O-GlcNAcase})\) enzymes are re-

sponsible for the dynamic glycosylation and deglycosylation of nucleocytoplasmic proteins, respectively, similar to the phosphorylation and dephosphorylation of proteins by kinases and phosphatases.\(^{2,29,30}\) OGT was cloned from rat, humans, and \(\text{Caenorhabditis elegans}.\(^{29,33}\) Recently, multiple splice vari-

ants of OGT were identified,\(^{32}\) with one variant localized to the mitochondria.\(^{33,34}\) The major OGT contains 11.5 tetraetri-

copeptide repeats that serve as protein-protein interactions, allowing OGT to interact with a large variety of proteins,\(^{35,36}\) mSin3A and OIP106/GRIF-1 protein are two of a potentially long list of OGT-interacting proteins.\(^{37,38}\) There are also more than 30 unique cyclic DNA clones of potential OGT-interacting proteins produced in an initial yeast two-hybrid screen.\(^{38}\) OGT is necessary for survival at the single-cell level, and the OGT gene maps to \(\text{Xq13, a locus commonly associated with neurodegenerative diseases}.\(^{39}\) OGT is modi-

fied by O-GlcNAc and by tyrosine phosphorylation, suggesting that it may be regulated by tyrosine kinase–mediated signal transduction cascades.\(^{29,35}\) High glucose causes hyper-

O-GlcNAc modification of OGT.\(^{40,41}\) In addition, OGT is highly responsive to intracellular uridine \(5’\)-diphosphate \((\text{UDP})\)-GlcNAc concentrations.\(^{38}\) Presently, no specific in-

hibitors of OGT have been isolated. However, UDP is a potent inhibitor,\(^{35}\) and alloxan \((\text{a uracil analog})\) is also a nonspecific inhibitor.\(^{42}\)

Like OGT, O-GlcNAcase is found in all tissues examined,

with the highest expression in brain, placenta, and pancreas.\(^{30}\) O-GlcNAcase shows little homology to acidic lysosomal hexosaminidase, is insensitive to \(N\)-acyt
glactosamine, and is specific for \(\beta-O-GlcNAc\).\(^{30,43}\) It was initially designated a hexosaminidase \(C\) based on its characteristics of having a neutral \(\text{pH}\) optimum, nucleocytoplasmic distribution, and selectivity in removing GlcNAc and not GalNAc.\(^{30,43}\) O-GlcNAcase has recently been placed in the family of \(\text{GCN5-related family of acetyltransferases based on sequence homology comparisons}.\(^{44}\) There is a 130-kDa O-GlcNAcase form and a splice variant that migrates at an apparent molecular weight of 75 kDa.\(^{45,46}\) The 130-kDa form is primarily found in the cytosolic fraction, whereas the 75-kDa splice variant is a nuclear protein.\(^{45,47,48}\) The 75-kDa splice variant, which does not have a portion of the carboxyl termi

nus, is not as active as an O-GlcNAcase.\(^{49}\) The function of the 75-kDa O-GlcNAcase is presently unknown, but it has been proposed that it may compete with O-GlcNAcase or O-GlcNAc for interacting proteins. O-GlcNAcase is cleaved by caspase 3, generating a 65-kDa carboxyl terminal frag-

ment.\(^{48}\) This cleavage has no effect on O-GlcNAcase activity in vitro. Other caspase 3 substrates also retain complete activity on cleavage, such as protein kinase \(C\) \(\delta\) and the
Hexosamine Biosynthetic Pathway, Diabetes, and Cardiovascular Complications

Diabetes-associated complications, such as cardiovascular disease, arise as a result of excess flux of glucose through at least one of the nutrient-sensing pathways, the hexosamine biosynthetic pathway (HBP) (Figure 2). Approximately 2% to 5% glucose that is used by the cell is converted to products of the HBP. Several groups have suggested that OGT and O-GlcNAc may be a nutritional sensor in response to the HBP. The HBP was first implicated in glucose-induced insulin resistance through the rate-limiting enzyme glutamine, fructose-6-phosphate amidotransferase (GFAT) in the multistep conversion of glucose to glucosamine. Marshall et al\(^{58}\) showed that an inhibitor of GFAT, azaserine, reversed hyperglycemia-induced insulin resistance, but glucosamine, downstream of GFAT, not only reversed the affect but also potentiated induced insulin resistance compared with glucose. Glucosamine potently induces insulin resistance in several models in vitro and in vivo, eg, murine adipocytes and skeletal muscle.\(^{2,58,61}\) Transgenic mice both overexpressing GFAT in skeletal muscle and adipose tissue have decreased glucose disposal rates.\(^{62}\) The primary end product of the HBP is UDP-GlcNAc, the donor sugar nucleotide used by OGT. OGT responds to UDP-GlcNAc concentrations well beyond the ranges occurring in cells from nanomolar to several millimolar, and its activity toward different peptide substrates is altered at different UDP-GlcNAc concentrations.\(^{35}\) Increased levels of UDP-GlcNAc induced by insulin and glucosamine infusion render mice insulin resistant, and skeletal muscle proteins contain elevated O-GlcNAc levels. In another case, a transgenic mouse overexpressing OGT in muscle and adipose tissue displayed lowered glucose disposal rates, hyperglycemia, and hyperleptinemia, which are hallmarks of type 2 diabetes.\(^{64}\) Hyperglycemia also increases OGT expression and activity in rat aortic smooth muscle cells, and OGT itself seems to be hyperglycosylated in response to elevated glucose.\(^{65}\) However, the most convincing evidence that O-GlcNAc has a direct role in attenuating insulin signaling is the concomitant increase in O-GlcNAc and induction of insulin resistance on PUGNAc inhibition of O-GlcNAcase in the absence of hyperglycemia.\(^{67,68}\) Hence, elevations in O-GlcNAc dynamically upset the balance between O-phosphate and O-GlcNAc modification, leading to defective insulin signaling.

Patti et al\(^{67}\) first demonstrated that IRS-1 and IRS-2 were O-GlcNAc modified by elevated glucosamine treatment and that this may result in a reduction in IRS/PI3K p85 interactions in rat skeletal muscle. IRS proteins bind to the regulatory subunit (p85) of PI3K via its SH2 domains.\(^{69}\) Federici et al\(^{70}\) also demonstrated that elevated glucose and glucosamine treatment increased the O-GlcNAc modification of IRS-1 and IRS-2, as well as the PI3K regulatory subunit p85 in human coronary artery endothelial cells. In addition, the IRS-1 and IRS-2 interaction with p85 and activity of AKT was shown to be attenuated.\(^{28}\) Interestingly, Vosseller et al\(^{77}\) specifically demonstrated that treatment of 3T3-L1 adipocytes with PUGNAc both increased O-GlcNAc on IRS-1 and attenuated the activity of downstream effector proteins AKT and GSK-3β, decreasing insulin-stimulated glucose uptake. The O-GlcNAc modification of IRS-1 and IRS-2 may be interfering at or near the site of interaction with p85. These data corroborate early findings that insulin resistance is attributed to postreceptor defects.\(^{69}\)

Parker et al\(^{70}\) have demonstrated in NIH3T3-L1 adipocytes that high-glucose and high-glucosamine concentrations induced insulin resistance and increased the O-GlcNAc modification of glycogen synthase. This elevation of O-GlcNAc on glycogen synthase caused it to become resistant to insulin-stimulated protein phosphatase 1–mediated activation.\(^{70,71}\) The O-GlcNAc modification is probably disrupting the interaction of protein phosphatase 1 or other interacting proteins necessary for glycogen synthesis. GSK3β is also
O-GlcNAc modified, contributing to the number of possible check points for glycogen synthesis. Therefore, O-GlcNAc modification of GSK3β might act as a nutrient sensor sensitive to the influx of glucose through the HBP. A complete understanding of the complex regulation of protein activity and protein-protein interactions attributable to the interplay of O-GlcNAc and O-phosphate will require site mapping of these sites under normal and insulin-resistant conditions.

Increased flux through the HBP has also been proposed to cause vascular complications commonly associated with diabetes-related glucose toxicity. Du et al. demonstrated that the elevation of the O-GlcNAc modification on eNOS is associated with reduced activity of eNOS in diabetic rats as well as in hyperglycemia-induced bovine aortic endothelial cells. They demonstrated a 2-fold increase in O-GlcNAc modification of the protein and a reciprocal decrease in phosphorylation at Ser1177 in eNOS. The site of O-GlcNAc modification seems to be at a different location than Ser1177, because a mutation at Ser1177 did not affect the level of O-GlcNAc modification on eNOS. In addition, a similar model of eNOS activity and posttranslational modification was detected in aortae of diabetic animals. As mentioned above, the increase in O-GlcNAc modification of upstream insulin-signaling proteins such as IRS-1, IRS-2, and p85 is associated with a defect in AKT activation. This defect in AKT activity affects production of NO, which is important in prevention of vascular disease. The reduction in eNOS activity and decrease in NO production results in an increase in matrix metalloproteinase (MMP) expression and activity and a decrease in tissue inhibitor of metalloproteinase 3 activity and expression. MMP is responsible for the degradation of extracellular matrix scaffold of the vessel wall in vascular remodeling. The MMP/tissue inhibitor of metalloproteinase imbalance has been suggested to be a contributing cause to matrix degradation and the development of macrovascular disease.

Hyperglycemia-induced mitochondrial superoxide production activates the hexosamine pathway, leading to eNOS inactivity and increased O-GlcNAc. Inhibition of the rate-limiting step in the HBP, GFAT, and blocking mitochondrial superoxide overproduction with uncoupling protein-1 or manganese superoxide dismutase reversed the inhibition of eNOS and its O-GlcNAc modification. Uncoupling protein-1 is a specific protein uncoupler of oxidative phosphorylation and disrupts the electrochemical gradient that drives superoxide production, whereas the overexpression of manganese superoxide dismutase catalyzes the dismutase of superoxide to hydrogen peroxide, preventing the effects of hyperglycemia-induced hexosamine O-GlcNAc modification of eNOS. The overproduction of mitochondrial superoxide results in inhibition of GAPDH activity and activates the hexosamine pathway through GFAT, resulting in elevated levels of O-GlcNAc modification. Hyperglycemia also increases the O-GlcNAc modification of Sp1 by ~70% and results in a reciprocal decrease in its phosphoserine and phosphothreonine by approximately the same amount. Haltiwanger et al. demonstrated the reciprocal relationship between O-GlcNAc and O-phosphate on the Sp1 transcription factor with the O-GlcNAcase inhibitor PUGNAc. The hyperglycemia-induced O-GlcNAc modification of Sp1 results in Sp1 transactivation and expression of transforming growth factor-α (TGF-α), TGF-β, and plasminogen activator inhibitor-1, which contains two Sp1 sites. TGF-β production leads to increased matrix production, leading to nodular diabetic glomerulosclerosis in mesangial cells. Elevated plasminogen activator inhibitor-1 production is not only found in diabetic patients but also plays a role in atherothrombotic disorders and development of vascular disease in patients with diabetes. The effects of hyperglycemia are reversed by inhibitors that disrupt the hexosamine pathway and mitochondrial superoxide. The O-GlcNAc-modified Sp1 is less transcriptionally active on some promoters and seems to have a reciprocal relation with serine/threonine phosphorylation. O-GlcNAc modification of these signaling kinases and transcription factors seems to be acting as a nutritional sensor through the hexosamine biosynthetic pathway by modulating signaling cascades and transcriptional machinery in response to the cell’s nutritional state.

**Studying O-GlcNAc**

In vitro enzymatic labeling of O-GlcNAc with UDP[3H]Gal using galactosyltransferase has been the primary method for the detection and characterization of O-GlcNAc until the recent development of O-GlcNAc–specific antibodies. Although time consuming, in combination with product analysis, the galactotransferase method remains the gold standard for the detection and analysis of O-GlcNAc, because it shows unequivocally that proteins were modified by a single O-linked N-acetylglucosamine residue. PNGase F treatment is used to specifically cleave any N-linked oligosaccharides that were nonspecifically tagged with UDP[3H]Gal. The O-linkage of GlcNAc to protein is resistant to PNGase F. However, O-GlcNAc may be rapidly removed by endogenous O-GlcNAcase or hecosaminidase before it can be detected; therefore, it is necessary to use PUGNAc or GlcNAc as inhibitors of these enzymes in cellular homogenates.

The development of RL-2 and CTD 110.6 antibodies has significantly increased the efficiency of identifying O-GlcNAc–modified proteins. The CTD 110.6 antibody was raised against an O-GlcNAc–modified peptide from the large subunit RNA polymerase II CTD. This antibody demonstrates the broadest immunoreactivity to the O-GlcNAc modification. The development of O-GlcNAc site-specific antibodies has proven to be a useful tool, as illustrated in the studies with c-Myc. Antibodies specific for O-GlcNAc-Thr58 and unmodified Thr58 were created, in conjunction with a commercially available O-phosphate-Thr58 antibody, to study the reciprocal functions of the posttranslational modifications at this key residue. These tools helped determine that the alternative glycosylation and phosphorylation of Thr58 regulate the myriad of functions of c-Myc in cells. O-GlcNAc may also be detected using a lectin, succinylated wheat germ agglutinin (sWGA), that recognizes terminal N-acetylglucosamine. Because sWGA binds any terminal O-GlcNAc residue, several controls to confirm O-GlcNAc modification should be used. PNGase F is used to remove the
N-linked modifications. Hexosaminidase treatment may also be used as a negative control to remove the O-linked modifications. A sWGA-conjugated Sepharose column may also be used to isolate and enrich O-GlcNAc–modified proteins from cell extracts. Alternatively, low abundant and short half-life proteins may be expressed using the rabbit reticulocyte lysate transcription/translation system to generate [35S]Met-labeled protein. The rabbit reticulocyte lysate contains sufficient amounts of OTG and UDP-GlcNAc to O-GlcNAc modify in vitro synthesized peptides. Metabolic labeling may also be conducted with [3H]glucosamine.

Original efforts at site-mapping O-GlcNAc sites involved time-consuming multiple-step procedures. The peptides were separated by high-performance liquid chromatography (HPLC), and fractions were analyzed by fast atom bombardment mass spectrometry to detect fractions containing peptides that differed in mass by 203 daltons, which could correspond to unmodified and GlcNAc-modified versions of the same peptide. Fractions containing the modified version are subjected to in vitro galactosyltransferase labeling using UDP-[3H]Gal to radioactively tag the GlcNAc residues. Glycopeptides are purified by several rounds of HPLC. The sites of modification are then determined by Edman degradation analysis by monitoring the release of radioactive modified amino acids. However, this method of O-GlcNAc site mapping is time consuming and is complicated by the number of HPLC peptide purifications and manual Edman degradation reactions compounded by low stoichiometry of the O-GlcNAc modification. Therefore, it was necessary to start with 5 to 10 nmol of purified protein. In one case, the serum response factor (SRF) was overexpressed in baculovirus and was site mapped by sequential enzymatic digest with a combination of enzymes, including trypsin and proline-specific enzymes. Greis et al used alkaline β-elimination and collision-induced dissociation (CID) in combination with electrospray ionization mass spectrometry to identify O-GlcNAc–modified peptides with <5 pmol of peptide. Alkaline β-elimination results in the conversion of O-GlcNAc-serine to 2-amino propenoic acid and O-GlcNAc-threonine to 2-amino-2-butenolic acid and decreased both the mass of the glycopeptide by 222 daltons and the CID fragment ion from 87 daltons (Ser) to 69 daltons (2-amino propenoic acid). Without the alkaline β-elimination step, the CID energy needed to ionize and fragment the peptide for sequencing removes the O-GlcNAc modification from the peptide. Hence, the alkaline β-elimination chemically enables the site mapping of O-GlcNAc modifications. This method has also been successfully used for mapping O-GalNAc and O-LacNAc. However, under the alkaline β-elimination conditions, significant peptide degradation occurs. To unambiguously distinguish O-GlcNAc and O-GalNAc, the O-GlcNAc peptides may be isolated and enriched by affinity chromatography (ie, CTD 110.6 or sWGA).

Haynes and Aebersold developed a method using synthetic O-GlcNAc–peptides and CID that was subsequently capable of identifying a low abundant O-GlcNAc–modified bovine α-crystallin in complex mixture. Chaulky and Burlingame also used a similar method by lowering the CID energy in subsequent MS/MS scans on precursor ions that only required 5 to 10 pmol of gel-purified SRF using a quadrupole time-of-flight mass spectrometer. They were able to identify some of the previous O-GlcNAc sites and two additional O-GlcNAc sites on SRF. In addition, they detected a novel phosphorylation site. Most recently, the transcription factor cAMP-responsive element-binding protein has been shown to be O-GlcNAc modified by similar methods. The O-GlcNAc modification was shown to inhibit TAFII130 association and thereby repress transcriptional activity. These recent methods provide an alternative means to manually map O-GlcNAc sites on purified proteins. However, these methods do not provide a basis for conducting comparative quantitation and do not allow for enrichment of low-abundance PTM-modified peptides. Therefore, mild β-elimination followed by Michael addition with dithiothreitol (BEMAD) was developed to address the former problems and limitations of O-GlcNAc functional proteomics.

Wells et al and others developed BEMAD based on the previous methods using β-elimination followed by Michael addition of an affinity tag to map phosphorylation sites. Posttranslational modifications are substoichiometric and often labile, making their identification difficult. β-Elimination from serine or threonine followed by attack of the resulting α,β-unsaturated carbonyl with a nucleophilic tag stabilizes the O-linkage during collision-induced dissociation, allowing for site identification by LC-MS/MS. This tag confers a unique molecular weight to the modified amino acid that is easily identifiable via database searches and also allows for the enrichment via affinity chromatography of the peptide containing the modification. However, any modification of serines and threonines, including O-GlcNAc, O-GalNAc, O-LacNAc, and O-phosphate, that is susceptible to β-elimination may be targeted by this method as well as alkylated cysteines or methionines. Dithiothreitol (DTT) was chosen as the tag because it is easily identified against a nonredundant database, inexpensive compared with other tags such as ICAT, and easily adaptable for performing quantitative mass spectrometry using commercially available deuterated (d10) DTT.

The BEMAD method is successful in identifying O-GlcNAc sites not only on purified proteins but also in semicomplex mixtures. The BEMAD protocol may be modified to identify O-GlcNAc peptides, O-phosphate peptides, and stoichiometric changes in control versus induced cellular experiments or normal tissue states versus disease states. Because less-abundant proteins may be masked by abundant proteins in complex mixtures, it may be necessary to enrich target proteins by affinity chromatography or fractionation. Purified proteins are oxidized with performic acid and digested with trypsin before mild BEMAD to oxidize cysteine (Δ48.0 daltons), tryptophan (Δ48.0 daltons), and methionine (Δ32.0 daltons) so that the cysteine does not bind the thiol column on purification of the DTT-modified peptides. As internal controls for both O-GlcNAc and O-phosphate site mapping, the peptides are spiked with synthetic phospho- AKT peptides and O-GlcNAc peptides and treated with alkaline phosphatase to remove all phosphorylation modifications or with O-GlcNAc case to remove all of the O-GlcNAc
The versatility of the BEMAD method allows the flexibility to cation has led to several rapid and useful methods. The development of mass O-GlcNAc as a dynamic posttranslational modification with many functional roles in the cell. The development of tools and techniques for the study of the O-GlcNAc modification has established the acceptance of O-GlcNAc peptides and cysteines, digested with trypsin, and then spiked with O-GlcNAc peptides and O-phosphate peptides. Then both the control protein sample and the experimental sample are each split into two fractions and treated with either alkaline phosphatase or O-GlcNAcase. BEMAD is conducted on the two fractions from the control sample with deuterated DTT (heavy) and the two fractions from the experimental sample with DTT (light). The alkaline phosphatase–treated fractions are combined, and the O-GlcNAcase fractions are combined before thiol column enrichment and LC-MS/MS. A mass increase of 136.2 and 142.2 daltons is allowed for the light and heavy DTT-modified cysteines, respectively. The deuterated d10 has four deuterated hydrogens that are exchangeable in solution and therefore loses four deuterated hydrogens before LC-MS/MS analysis. The quantity of heavy DTT-labeled O-GlcNAc–modified peptides in the control sample is then compared with the light DTT-labeled O-GlcNAc–modified peptides in the experimental sample. The same quantity comparison is made in the O-phosphate sample. The spiked O-GlcNAc peptides and O-phosphate peptides are again used as internal controls and should be present in each sample at equal ratios. In addition, it is possible to quantitate protein expression levels between control and experimental cells by alkylating the cysteines with iodoacetamide instead of performic acid treatment and then using light DTT and heavy DTT, respectively, to label the cysteines. The combination of these quantitative BEMAD methods may be used to distinguish the reciprocal role of O-GlcNAc and O-phosphate as well as the level of protein expression, protein activity, and protein–protein interactions in disease states compared with normal states.

Concluding Remarks

The development of tools and techniques for the study of the O-GlcNAc modification has established the acceptance of O-GlcNAc as a dynamic posttranslational modification with many functional roles in the cell. The development of mass spectrometry techniques to site-map the O-GlcNAc modification has led to several rapid and useful methods. The versatility of the BEMAD method allows the flexibility to simultaneously study O-GlcNAc and O-phosphate quantitatively and for the enrichment of either posttranslational modification in the study of normal versus induced cellular states. Presently we are applying BEMAD in the study of O-GlcNAc in several disease states, including diabetes and cardiovascular complications.

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Proteomic Approaches to Analyze the Dynamic Relationships Between Nucleocytoplasmic Protein Glycosylation and Phosphorylation

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