The intersections between *O*-GlcNAcylation and phosphorylation: implications for multiple signaling pathways

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Journal of Cell Science 123, 13-22 Published by The Company of Biologists 2010
doi:10.1242/jcs.053678

Summary

A paradigm-changing discovery in biology came about when it was found that nuclear and cytosolic proteins could be dynamically glycosylated with a single *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) moiety. *O*-GlcNAcylation is akin to phosphorylation: it occurs on serine and/or threonine side chains of proteins, and cycles rapidly upon cellular activation. *O*-GlcNAc and phosphate show a complex interplay: they can either competitively occupy a single site or proximal sites, or noncompetitively occupy different sites on a substrate. Phosphorylation regulates *O*-GlcNAc-cycling enzymes and, conversely, *O*-GlcNAcylation controls phosphate-cycling enzymes. Such crosstalk is evident in all compartments of the cell, a finding that is congruent with the fundamental role of *O*-GlcNAc in regulating nutrient- and stress-induced signal transduction. *O*-GlcNAc transferase is recruited to the plasma membrane in response to insulin and is targeted to substrates by forming transient holoenzyme complexes that have different specificities. Cytosolic *O*-GlcNAcylation is important for the proper transduction of signaling cascades such as the NFκB pathway, whereas nuclear *O*-GlcNAc is crucial for regulating the activity of numerous transcription factors. This Commentary focuses on recent findings supporting an emerging concept that continuous crosstalk between phosphorylation and *O*-GlcNAcylation is essential for the control of vital cellular processes and for understanding the mechanisms that underlie certain neuropathologies.

Key words: *O*-GlcNAcylation, Phosphorylation, Crosstalk, Signal transduction, Transcription, Insulin, Neurodegeneration

Introduction

Protein glycosylation traditionally refers to the covalent attachment of complex oligosaccharides to proteins in intralumenal compartments or cellular membranes, or that are destined for secretion. A novel form of glycosylation emerged when proteins in the nucleus and cytosol were found to be modified with a single β-*N*-acetylglucosamine monosaccharide moiety through an *O*-β-glycosidic attachment (*O*-linked β-*N*-acetylglucosamine; *O*-GlcNAc) (Torres and Hart, 1984) to serine and/or threonine side chains of the polypeptide backbone (Holt and Hart, 1986; Holt et al., 1987) (Fig. 1). *O*-GlcNAcylation differs from classical complex glycosylation in that the sugar moiety is not further modified or elongated into complex structures. Furthermore, the addition and removal of *O*-GlcNAc moieties cycles, sometimes very rapidly, in a dynamic and inducible manner in response to cellular changes (Kearse and Hart, 1991) (for reviews, see Copeland et al., 2008; Hanover, 2001; Hart et al., 2007).

The addition of *O*-GlcNAc to proteins is catalyzed by uridine diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase (*O*-GlcNAc transferase; OGT), which uses uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) as the direct sugar donor (Haltiwanger et al., 1992) (Fig. 1). OGT is enriched in the nucleus, but is also present in the cytosol of all cells as a multimer. Its structure comprises a tetratricopeptide repeat (TPR)-rich N-terminal domain, which mediates multimerization and protein-protein interactions, and a C-terminal catalytic domain. The overall catalytic activity of OGT is regulated linearly by a wide range of intracellular concentrations of UDP-GlcNAc (Kreppel and Hart, 1999) that fluctuate proportionally in response to the flow of nutrients (glucose, glutamine, energy) through the hexosamine biosynthetic pathway (Marshall et al., 2004; McClain and Crook, 1996). Thus, the *O*-GlcNAcylation of substrates represents a 'metabolic sensor' that adjusts protein function according to the nutritional status of the cell.

The attachment of *O*-GlcNAc is also aided by targeting proteins that transiently associate with OGT and deliver it to specific substrates (Cheung and Hart, 2008; Cheung et al., 2008; Housley et al., 2009; Iyer et al., 2003; Iyer and Hart, 2003). For example, p38 kinase associates with the C-terminal portion of OGT during glucose deprivation in neuronal cells and targets it directly to Neurofilament H (neurofilament heavy polypeptide; NF-H), which, upon *O*-GlcNAcylation, becomes soluble and disassembles from cytoskeletal filaments. This increased activity of OGT towards Neurofilament H occurs even when the pool of intracellular UDP-GlcNAc is significantly reduced, indicating that modulation of the substrate specificity of OGT by accessory proteins is a major mechanism for regulating the addition of *O*-GlcNAc to target substrates (Cheung and Hart, 2008). One of the first OGT-targeting proteins described (then termed OIP106; now known as *Drosophila* Milton) has since been shown to be a key protein that regulates the axonal transport of mitochondria in neurons (Goldstein et al., 2008).

The removal of *O*-GlcNAc from proteins is catalyzed by a neutral β-*N*-acetylglucosaminidase (*O*-GlcNAcase) (Fig. 1), a 130-kDa enzyme that is highly concentrated in the cytosol but is also present in the nucleus of all cell types (Dong and Hart, 1994; Gao et al., 2001). The structure of *O*-GlcNAcase consists of an N-terminal glycosidase domain and a C-terminal region with homology to histone acetyltransferases (HATs) (Schultz and Pils, 2002; Toleman et al., 2004). Caspase-3 is known to cleave at a site between these two domains in vitro and upon apoptosis in vivo; however, the two

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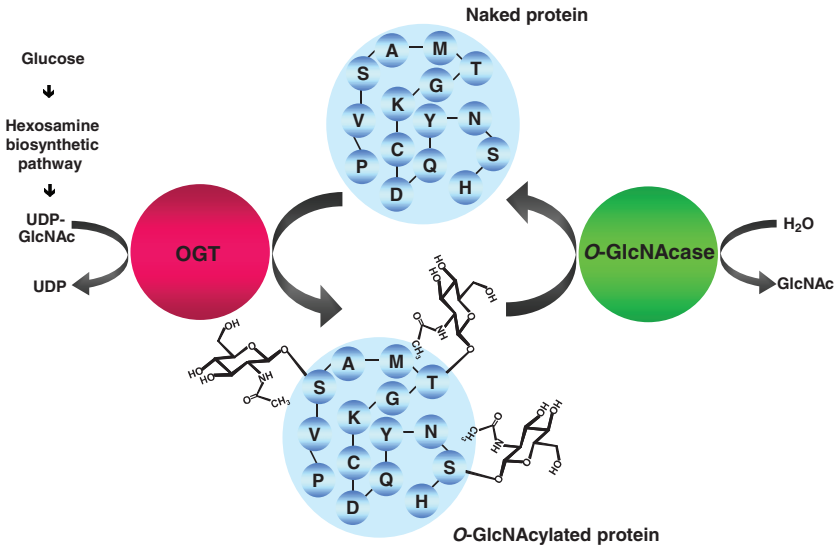

Fig. 1. The *O*-GlcNAcylation cycle. Glucose is metabolized through the hexosamine biosynthetic pathway to form the high-energy intermediate UDP-GlcNAc, which serves as the sugar donor for OGT. The *O*-GlcNAc cycle starts when OGT catalyzes the transfer of GlcNAc from UDP-GlcNAc to serine and/or threonine residues of a protein substrate through β-glycosidic attachment. Although no consensus sequence has been identified for OGT, proline and valine are the most common amino acids found prior to the serine or threonine target site. The *O*-GlcNAcylated protein can be a substrate for *O*-GlcNAcase, which hydrolyses the glycosidic linkage to generate free GlcNAc and naked protein.

halves of cleaved *O*-GlcNAcase remain physically associated and the glycosidase retains full activity (Butkinaree et al., 2008; Wells et al., 2002a). Interestingly, an alternatively spliced variant of *O*-GlcNAcase that lacks the HAT domain and localizes predominantly in the nucleus has also been identified (Comtesse et al., 2001). Although much remains to be investigated regarding how this enzyme is regulated, it is known that *O*-GlcNAcase is a substrate for *O*-GlcNAcylation (Khidekel et al., 2007) and that it can exist in a functional complex with OGT (Whisenhunt et al., 2006). These findings illustrate that a unique mechanism regulates the activities of two enzymes with opposing activity (*O*-GlcNAcase and OGT) during *O*-GlcNAc cycling (removal-addition) of target proteins.

For almost every type of major post-translational modification that involves the attachment of functional biochemical groups to polypeptides, the human genome encodes a vast array of modifying enzymes that is almost comparable in number to that of their specific substrates (Venter et al., 2001). So, given that it is only catalyzed by the action of two enzymes, how extensive and important is *O*-GlcNAcylation? Studies throughout the past 25 years have established that *O*-GlcNAc might be absent in many but not all protozoans (Previato et al., 1994) and yeast, but ubiquitous in viruses and all metazoans including plants, worms, insects and mammals. *O*-GlcNAc is essential for life in mammalian cells; for example, the *ogt* gene is required for embryonic-stem-cell viability in mice (Shafi et al., 2000). Using Cre-*lox* methodology, one group has demonstrated that tissue-specific deletions of *ogt* result in apoptosis and a reduction in cell number in T cells and neurons (O'Donnell et al., 2004). By contrast, knocking out either the *ogt-1* or *oga-1* (the latter encodes *O*-GlcNAcase) allele in *Caenorhabditis elegans* results in viable and fertile animals that have abnormal regulation of macronutrient storage. Furthermore, when either *ogt-1* or *oga-1* is deleted in temperature-sensitive insulin-like receptor (*daf-2*) *C. elegans* mutants, alterations in dauer larvae formation are observed, suggesting a role for *O*-GlcNAc-cycling enzymes in controlling the insulin signaling pathway in response to nutrient flux (Forsythe et al., 2006; Hanover et al., 2005). *O*-GlcNAcylation of proteins represents a signaling mechanism by which cells sense and respond to a variety of stress stimuli. The rapid and dynamic increase in the *O*-GlcNAcylation of numerous proteins renders cells more tolerant to stress, in part by regulating the expression of heat-shock proteins (Zachara et al., 2004). In addition to promoting cell survival, *O*-GlcNAcylation of specific substrates has been shown to modulate diverse protein functions, including protein-protein interactions, protein turnover, subcellular localization and changes in activity.

One of the main mechanisms that underlies the biological actions of *O*-GlcNAc involves its interplay with another post-translational modification: protein phosphorylation (for reviews, see Copeland et al., 2008; Hanover, 2001; Hart et al., 2007). In this Commentary, we explore past and current data on the extensive crosstalk between *O*-GlcNAc and phosphate, and discuss how the regulation of protein function by this interplay affects numerous signaling pathways in virtually every compartment of the cell.

Evidence for *O*-GlcNAc–phosphate crosstalk at the global level

Recent proteomic advances that involve the selective enrichment of low-abundance *O*-GlcNAcylated species from complex mixtures, followed by high-technology mass spectrometric (MS) methods, have allowed the mapping and quantification of multiple *O*-GlcNAcylation sites (Khidekel et al., 2007; Wang et al., 2009; Wells et al., 2002b). These analyses have led to the conclusion that *O*-GlcNAc parallels phosphorylation in terms of dynamics, abundance and impact on protein function. Several early site-mapping studies showed that *O*-GlcNAc and phosphate often compete for the same sites (for a review, see Hart, 1997). Global hyperphosphorylation induced by okadaic-acid treatment in a neuroblastoma cell line decreased *O*-GlcNAcylation levels of both nuclear and cytoplasmic proteins (Lefebvre et al., 1999). Alterations in the activities of protein kinase A or C, and of tyrosine kinases, resulted in a reciprocal effect on the *O*-GlcNAcylation of cytoskeletal proteins in mouse neurons (Griffith and Schmitz, 1999). These data suggest that the two modifications regulate each other globally in a reciprocal manner. A more direct indication of the interplay between *O*-GlcNAcylation and phosphorylation was shown in a large-scale proteomic study in which inhibition of glycogen synthase kinase-3 (GSK3) by lithium resulted in drastic changes in the *O*-GlcNAcylation of different protein substrates (Wang et al., 2007). In this study, 45 *O*-GlcNAcylated proteins of different functional classes were identified and the fold change

of *O*-GlcNAcylation of 29 of these proteins in response to GSK3 inhibition was quantified using a SILAC (stable isotope labeling with amino acids in cell culture)-MS approach. In line with the idea of competitive regulation by *O*-GlcNAc and phosphate, a portion of the proteins showed a significant increase in their *O*-GlcNAcylation levels upon inhibition of GSK3. However, in a large percentage of proteins, the levels of *O*-GlcNAcylation decreased as a consequence of inactive GSK3, indicating that the relationship between the two modifications is not simply reciprocal. A follow-up study quantified the phosphorylation dynamics at the single-site level upon global elevations in *O*-GlcNAcylation by selective inhibition of *O*-GlcNAcase (Wang et al., 2007). By using a phospho-enrichment strategy followed by a proteomic approach known as iTRAQ (isobaric tag for relative and absolute quantification) labeling and MS analysis, 711 phosphorylation sites were detected and quantified, of which approximately 48% were not actively cycling in the non-stimulated cells. However, an increase in global *O*-GlcNAcylation affected nearly every actively cycling phosphorylated site, resulting in decreased stoichiometry of phosphate at 280 sites, and increased phosphate content at 148 sites. The proteins on which phosphorylation rapidly cycles upon elevated *O*-GlcNAcylation belonged to a wide array of functional types, including cytoskeletal proteins, metabolic enzymes, kinases, transcription factors and RNA-processing factors. A recent study of murine postsynaptic density preparations purified through lectin weak-affinity chromatography and analyzed by electron transfer dissociation (ETD) assigned as many *O*-GlcNAc sites as known phosphorylated residues on the murine protein Bassoon (28 each), strongly supporting the notion that both modifications are highly abundant, at least in the brain (Chalkley et al., 2009).

The results obtained from these high-throughput analyses indicate that there is an extremely complex crosstalk between *O*-GlcNAcylation and phosphorylation, and that the two modifications not only regulate each other in a reciprocal manner, but also coexist at different sites (adjacent, proximal or distant) that can regulate one another to generate a vast array of chemical diversity amongst substrates. This dynamic molecular diversity facilitates the adjustment to the constant changes in the cellular environment.

Signaling at the plasma membrane and in the cytosol

The activities of the molecules involved in the insulin pathway are regulated by on-and-off phosphorylation events. However, recent

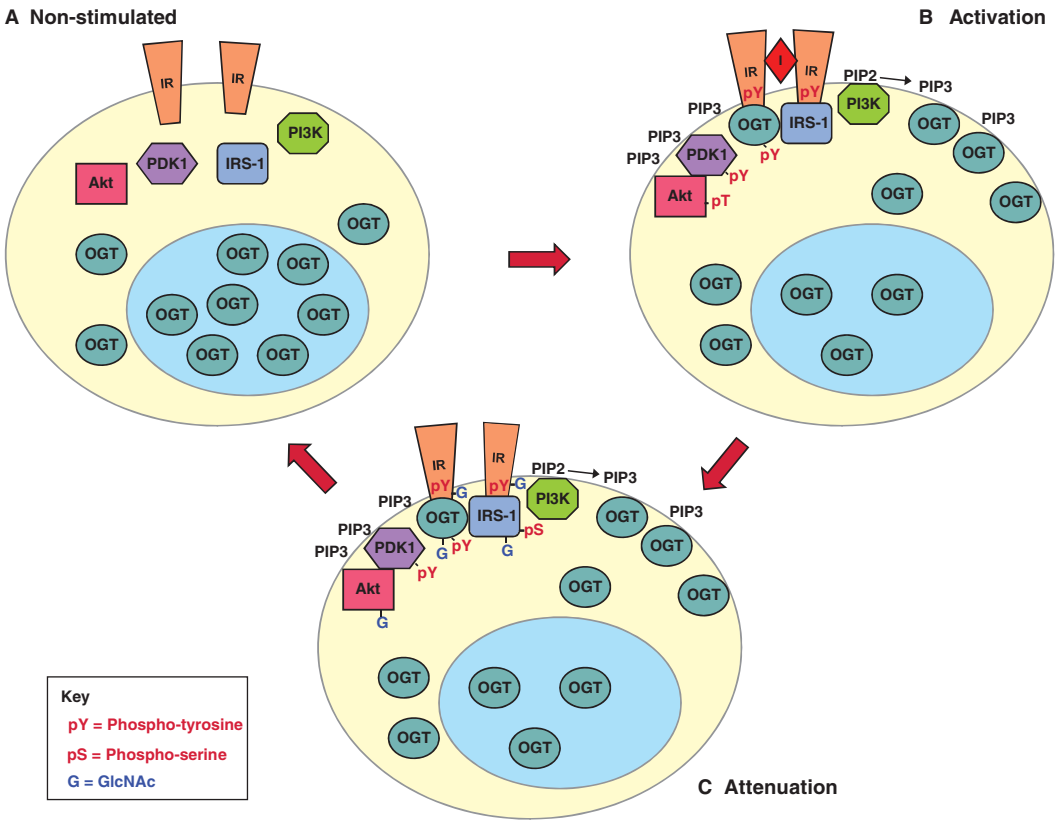

Fig. 2. *O*-GlcNAc and phosphate regulate the insulin signaling pathway at the plasma membrane. (**A**) In the basal state, OGT is concentrated in the nucleus of the insulin-responsive cell. (**B**) Upon insulin (I) binding to its receptor (IR), active PI3K catalyzes the phosphorylation of the plasma-membrane phospholipid phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2; PIP2] to PtdIns(3,4,5)P_3 (PIP3), driving the translocation of OGT from the nucleus to the plasma membrane by its binding to PtdIns(3,4,5)P_3. OGT is then tyrosine phosphorylated by active IR. This occurs simultaneously with the activation of other molecules of the pathway such as phosphoinositide-dependent kinase-1 (PDK1), IRS-1 and Akt, which is mediated in part by phosphorylation events. (**C**) Active OGT can now *O*-GlcNAcylate substrates of the pathway, including IR, IRS-1, Akt and OGT itself. *O*-GlcNAcylation of IRS-1 occurs in parallel with its phosphorylation at several serine residues that are known to inactivate IRS-1. Comparatively, *O*-GlcNAcylation of Akt inhibits its (activating) phosphorylation at a threonine residue, thereby inhibiting its kinase activity. Thus, the overall effect of *O*-GlcNAcylation of insulin signaling molecules leads to attenuation of the pathway.

studies have established that the cycling of *O*-GlcNAc is also necessary for the correct transduction and amplification of signals initiated at the plasma membrane by insulin (Fig. 2). Upon stimulation, activation of phosphoinositide 3-kinase (PI3K) drives the localization of OGT from the nucleus to the plasma membrane, where it is recruited by binding to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3]. The interaction between OGT and PtdIns(3,4,5)P_3 does not increase the basal activity of OGT (Yang et al., 2008); however, upon translocation to the plasma membrane, OGT associates with the insulin receptor (IR) and becomes tyrosine phosphorylated, which in turn increases OGT activity (Whelan et al., 2008). This redistribution and activation of OGT allows for the dynamic modification by *O*-GlcNAcylation of downstream targets in the pathway, including IR-β, insulin receptor substrate 1 (IRS-1) (Ball et al., 2006; Klein et al., 2009), Akt and OGT itself (Vosseller et al., 2002; Whelan et al., 2008; Yang et al., 2008).

The sites of OGT modification in the rat IRS-1 protein are serine 914, serine 1009, serine 1036 and serine 1041, whereas human IRS-1 is modified at serine 984/985, serine 1011 and within residues 1025-1045. In both species, these residues are all located in the C-terminus, which is rich in docking sites for Src-homology 2 (SH2)-domain-containing proteins (Ball et al., 2006; Klein et al., 2009). In livers of mice that are overexpressing OGT, the addition of *O*-GlcNAc to IRS-1 directly correlates with an increase in IRS-1 phosphorylation at serine 307 and serines 632/635, which are sites known to attenuate the insulin signaling cascade (Yang et al., 2008). Insulin-stimulated *O*-GlcNAcylation of Akt inhibits its phosphorylation at threonine 308, which shuts down its kinase activity and downregulates signaling (Vosseller et al., 2002; Yang et al., 2008). Improper inactivation of the insulin pathway by interfering with this site-specific crosstalk between *O*-GlcNAc and phosphate underlies the insulin-resistance phenomenon that is associated with increased activity of the hexosamine pathway (McClain and Crook, 1996). Blocking *O*-GlcNAc cycling with the *O*-GlcNAcase inhibitor *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) in adipocytes significantly decreases insulin-stimulated glucose uptake (Vosseller et al., 2002) and inhibits translocation of the glucose transporter GLUT4 to the plasma membrane (Park et al., 2005). Hepatic overexpression of OGT in mice via adenoviral delivery results in significantly increased levels of plasma insulin and impaired insulin-tolerance tests, in addition to disturbing glucose and lipid homeostasis (Yang et al., 2008). Similarly, in *C. elegans*, altered *O*-GlcNAcylation dysregulates carbohydrate and lipid metabolism (Forsythe et al., 2006; Hanover et al., 2005).

Despite these observations, perturbing *O*-GlcNAc–phosphate interplay cannot solely explain insulin desensitization. Upon acute insulin stimulation, decreasing global *O*-GlcNAcylation by adenoviral overexpression of *O*-GlcNAcase or knocking down OGT expression through siRNA fails to restore the impaired activation of Akt or the inhibition of glucose uptake in 3T3-L1 adipocytes pre-incubated in high-glucose medium with low-dose insulin (Robinson et al., 2007). Furthermore, increasing global *O*-GlcNAc levels with 1,2-dideoxy-2′-propyl-α-D-glucopyranoso-[2,1-D]-Δ2′-thiazoline (NButGT), a more selective inhibitor of *O*-GlcNAcase than is PUGNAc, does not produce insulin-desensitizing effects as measured by glucose uptake and Akt phosphorylation in 3T3-L1 adipocytes (Macauley et al., 2008). These latter reports indicate that the abnormal increase in the levels of *O*-GlcNAc is neither required nor sufficient for the induction of insulin resistance. The conflicting results of previous studies that used PUGNAc might be the result of non-selective inhibition of lysosomal hexosaminidases. In addition, hepatic overexpression of OGT in mouse liver results in aberrant increased expression of gluconeogenic and lipogenic genes (Yang et al., 2008), suggesting that it is the effect of elevated OGT and/or the resulting increased *O*-GlcNAcylation on transcription, translation and/or protein turnover that is the cause of the insulin-resistance phenotype observed in these mice.

The crosstalk between *O*-GlcNAcylation and phosphorylation that is initiated upon insulin signaling has also been implicated in modulating the cytosolic enzymatic activity of endothelial nitric oxide synthase (eNOS) during vasodilation. In cultured bovine aortic endothelial cells (BAECs) and human coronary-artery endothelial cells (HCAECs), hyperglycemia-induced inhibition of eNOS activity correlated with a significant increase in its *O*-GlcNAcylation and a reciprocal decrease of phosphorylation at the Akt site (serine 1177) (Du et al., 2001; Federici et al., 2002). These effects were reversed by overexpressing uncoupling protein 1 (UCP-1) or manganese superoxide dismutase (MnSOD), suggesting that activation of the hexosamine pathway and increased *O*-GlcNAc levels might arise owing to hyperglycemia-induced overproduction of superoxide in mitochondria. Some of these findings were also observed in aortae from diabetic rats, suggesting that dysregulation of the dynamics between *O*-GlcNAcylation and phosphorylation of eNOS is one of the physiological causes of early endothelial dysfunction in diabetes-induced atherosclerosis (Du et al., 2001). Moreover, hyper-*O*-GlcNAcylation of eNOS seems to contribute to diabetes-associated erectile dysfunction in rats (Musicki et al., 2005).

In addition to regulating the insulin cascade, increased *O*-GlcNAcylation of cytosolic proteins in response to hyperglycemia also regulates the signaling pathway that leads to activation of the transcription factor nuclear factor-κB (NFκB). Cultured cells that have lost the tumor suppressor gene *p53* switch to using a highly glycolytic metabolism resulting in increased glucose consumption and elevated global protein *O*-GlcNAcylation. One of the molecules whose activity is tightly regulated by such increased stoichiometry of *O*-GlcNAc is inhibitor of NFκB (IκB) kinase-β (IKKβ). In response to pro-inflammatory stimuli, IKKβ phosphorylates IκB, promoting its degradation by the ubiquitin-proteasome machinery. Because IκB sequesters NFκB in the cytosol, IKKβ activity results in NFκB translocation to the nucleus and subsequent transcriptional activity. In HepG2 cells exposed to high glucose or treated with streptozotocin (STZ), and in $p53^{-/-}$ MEFs, *O*-GlcNAcylation of IKKβ is elevated concomitantly with its activation by phosphorylation. These phenomena are not only dependent on the p65 subunit of NFκB, but they also correlate with a sustained increase in the DNA-binding and transcriptional activities of NFκB in response to tumor necrosis factor-α (TNFα) stimulation, suggesting that *O*-GlcNAcylated IKKβ is a constitutively active form of the kinase. By using site-directed mutagenesis, the exact residue of *O*-GlcNAc modification on IKKβ was mapped to serine 733 at the C-terminus, which was previously characterized as an inactivating phosphorylation site, thus highlighting the competitive occupancy of the two modifications on the same amino acid. These results were reproduced in transformed human cell lines (Kawauchi et al., 2009), demonstrating that *O*-GlcNAcylation of IKKβ is responsible, at least in part, for the constitutive activation of NFκB that is a hallmark of many cancers.

Nuclear transcription

Initial studies on the subcellular distribution of *O*-GlcNAc in rat liver revealed that the modification was present in nearly every

compartment of the cell, and highly enriched in the nucleus, including in chromatin (Holt and Hart, 1986; Kelly and Hart, 1989). This observation sparked an interest in the study of proteins involved in the transcriptional machinery as candidate substrates for *O*-GlcNAcylation. Subsequent work showed that the C-terminal domain (CTD) of RNA polymerase II (RNA pol II), which is known to be phosphorylated, is also extensively modified by *O*-GlcNAcylation (Kelly et al., 1993). Strikingly, *O*-GlcNAc and phosphate on RNA pol II are mutually exclusive at the level of their modifying enzymes: the phosphorylated protein is not a substrate for OGT, whereas the presence of *O*-GlcNAc inhibits RNA pol II phosphorylation by CTD kinase. The presence of two chemically distinct subpopulations of RNA pol II in the nucleus suggests that *O*-GlcNAc and phosphate regulate, in a concerted manner, the initiation and elongation cycles of class II gene transcription (Comer and Hart, 2000). It has been proposed that the *O*-GlcNAcylated form of the RNA pol II holoenzyme localizes to promoters in a transcriptionally inactive poised state and is ready for transcriptional elongation only after *O*-GlcNAc is removed and the CTD becomes hyperphosphorylated upon gene activation (Comer and Hart, 2001).

In addition to RNA pol II, early studies also established that many general and promoter-specific transcription factors that are responsible for the expression of class II genes are also *O*-GlcNAcylated as well as phosphorylated (Jackson and Tjian, 1988). The transcription factor Myc, a crucial regulator of cell growth and division, is modified by *O*-GlcNAc at threonine 58, a residue that is mutated in lymphomas (Chou et al., 1995a; Chou et al., 1995b). Interestingly, threonine 58 resides in the protein's transactivation domain (TAD) and is also a target site of phosphorylation by GSK3. The modification of this amino acid by *O*-GlcNAc and phosphate is competitive and reciprocal; growth-inhibited cells that have been serum starved predominantly contain the threonine-58 *O*-GlcNAcylated form of Myc, whereas serum stimulation re-establishes the phosphate at this site. Interestingly, a protein containing a serine-to-alanine mutation at position 62, at which phosphorylation is required before the threonine-58 residue can be phosphorylated, shows a marked increase in *O*-GlcNAcylation at threonine 58. These results suggest that the inverse occupancy of threonine 58 by *O*-GlcNAc and phosphate, and their influence on phosphorylation at serine 62, might determine the biochemical properties of Myc's TAD, which is important for controlling the interaction between Myc and its functional binding partners during mitogenic growth stimulation (Kamemura et al., 2002).

Competitive amino acid occupancy of *O*-GlcNAc and phosphate has also been shown for the mouse estrogen receptor-β (mER-β) transcription factor. Site-mapping studies using galactosyltransferase-labeling fingerprinting of tryptic peptides followed by MS analysis demonstrated that serine 16 of mER-β is a site for either *O*-GlcNAcylation or phosphorylation, which occur in a mutually exclusive manner. This residue is located near the N-terminus of the protein, and is present within a motif with a high PEST [proline (P), glutamic acid (E), serine (S) and threonine (T)] score, a sequence that regulates protein degradation. It is plausible that the interplay between *O*-GlcNAc and phosphate at serine 16 controls the turnover of mER-β, with phosphate acting as the signal for rapid degradation and *O*-GlcNAc serving to stabilize the protein (Cheng et al., 2000).

The biological activities of the transcription factors p53 and CCAAT-enhancer-binding protein-β (C/EBPβ) are regulated by another type of *O*-GlcNAc–phosphate interplay: competitive occupancy at different sites. For example, treatment of MCF-7 cells with STZ results in an accumulation of p53 concomitantly with its increased *O*-GlcNAcylation at serine 149. This *O*-GlcNAcylation event precludes phosphorylation of p53 by the COP9 signalosome (CSN) at a proximal residue, threonine 155, which inhibits the interaction between p53 and components of the ubiquitin-proteasome machinery, leading to slow p53 turnover (Yang et al., 2006). In the case of C/EBPβ, a combination of ETD and collision-induced dissociation (CID) MS approaches identified *O*-GlcNAcylation sites at serine residues 180 and 181, which map to the regulation domain (RD) of the protein. In vitro *O*-GlcNAcylation of C/EBPβ prevented a priming phosphorylation event at threonine 188 [a mitogen-activated protein kinase (MAPK) and cyclinA/cyclin-dependent kinase 2 (Cdk2) target site] and subsequent phosphorylations at serine 184 and threonine 179 (both GSK3 sites), which are all required for the DNA-binding activity of C/EBPβ. Treatment of growth-arrested 3T3-L1 pre-adipocytes with the *O*-GlcNAcase inhibitor GlcNAc-thiazoline (GT) resulted in a significant decrease in the DNA-binding activity of C/EBPβ, as determined by chromatin immunoprecipitation (ChIP) analysis, concomitant with a decrease in the protein's phosphorylation. Double mutation of C/EBPβ (serines 180 and 181 to alanine), which completely abolished its *O*-GlcNAcylation, dramatically increased its transactivation activity as measured by a luciferase reporter assay (Li et al., 2009). These observations correlate with a decreased expression of developmental markers in 3T3-L1 cells exposed to GT, indicating that GlcNAcylation of C/EBPβ delays the pre-adipocyte differentiation program.

Finally, recent findings show that, in *Drosophila melanogaster*, *Ogt* is encoded by the Polycomb group (PcG) gene super sex combs (*sxc*), and *O*-GlcNAcylation of PcG proteins is essential for their ability to repress transcription (Gambetta et al., 2009; Sinclair et al., 2009). These findings suggest that *O*-GlcNAc has an additional role in regulating gene expression through an epigenetic mechanism.

Crosstalk through *O*-GlcNAc–phosphate-cycling multifunctional complexes

In addition to the interplay generated by simultaneously or alternatively modifying a particular substrate, *O*-GlcNAc and phosphate regulate each other by direct attachment of either moiety on their own cycling enzymes and by forming active complexes that can include OGT, *O*-GlcNAcase, kinases and/or phosphatases (Fig. 3A).

A functional complex composed of protein phosphatase 1-β (PP1β), protein phosphatase 1-γ (PP1γ) and OGT was isolated from rat brain extracts subjected to tandem immunopurification and microcystin-Sepharose chromatography. This active complex was able to dephosphorylate and subsequently *O*-GlcNAcylate a synthetic phospho-peptide in vitro, illustrating a yin-yang mechanism by which *O*-GlcNAc- and phosphate-cycling enzymes can be collectively targeted to a substrate for rapid and simultaneous modification. Because PP1β and PP1γ strongly associate with actin and tubulin in neuronal cells, and neurofilaments are known to be *O*-GlcNAcylated (Dong et al., 1993; Dong et al., 1996), this complex might be capable of mediating cytoskeletal organization and/or dynamics in cells of the brain (Wells et al., 2004).

Further studies demonstrated that OGT, PP1, *O*-GlcNAcase and Aurora kinase B all coexist in a functional complex during late M phase in HeLa cells. This supra-macromolecular complex targets several proteins, including the intermediate-filament protein

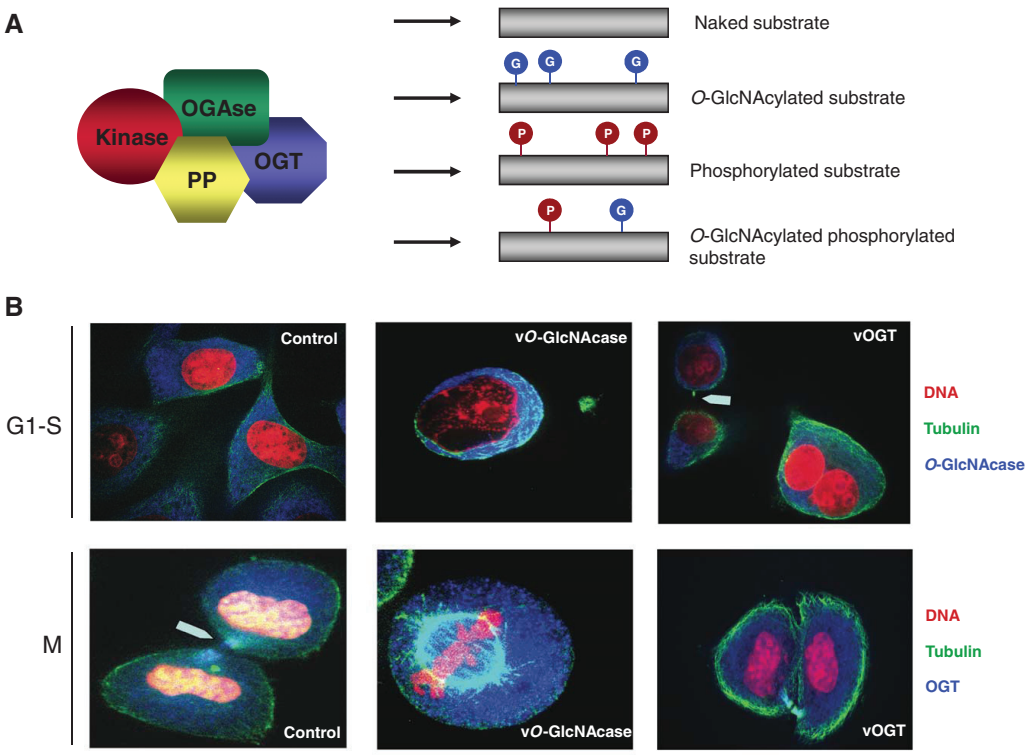

Fig. 3. Multifunctional enzymatic complexes regulate the interplay between *O*-GlcNAc and phosphate in many cellular processes, including the cell cycle. (**A**) A supra-macromolecular complex formed of OGT, *O*-GlcNAcase, kinase(s) and phosphatase(s) (PP) regulates the crosstalk between *O*-GlcNAcylation and phosphorylation of a naked protein, allowing its rapid and dynamic modification by *O*-GlcNAc, phosphate or both. The possibility of such molecular diversity within the same substrate allows the fine-tuning of protein activity in response to cellular changes. Note that dephosphorylation and removal of *O*-GlcNAc are catalyzed by the same complex; these events must be tightly regulated to avoid futile cycles. (**B**) Perturbations of a mitotic complex formed by OGT, *O*-GlcNAcase, Aurora kinase B and PP1 by adenoviral overexpression of OGT (vOGT) or *O*-GlcNAcase (v*O*-GlcNAcase) in HeLa cells results in disruption of cellular and nuclear structure, and in aneuploidy (upper panel), as well as in defective cytokinesis with a mitotic-exit phenotype (lower panel). White arrows point at midbody. This figure has been modified from Slawson et al. (Slawson et al., 2005).

vimentin, to regulate their post-translational status during mitosis. Manipulating global *O*-GlcNAcylation levels by overexpression of OGT or *O*-GlcNAcase resulted in decreased phosphorylation of vimentin at the Aurora-kinase-B target site (serine 82). Disrupting *O*-GlcNAc cycling by treating cells with the *O*-GlcNAcase inhibitor GT decreased phosphate stoichiometry at the same site, but also inhibited vimentin phosphorylation at serine 71, a known target site of Rho kinase; this shows the interplay between the enzymes involved in the mitotic complex and an additional kinase (Slawson et al., 2008). At least one *O*-GlcNAc site has been mapped for vimentin at serine 54 (Wang et al., 2007), and the overall *O*-GlcNAcylation status of the protein was affected when cells were exposed to the Aurora-kinase-B inhibitor ZM (Slawson et al., 2008). The interplay between these phosphorylation and *O*-GlcNAcylation events might regulate the properties of vimentin as a cytoskeletal protein by modulating its disaggregation from filaments and its proper distribution to daughter cells after mitosis (Slawson et al., 2008). The variations in mitotic phosphorylation of vimentin and of other proteins that are induced by perturbations in *O*-GlcNAcylation have been shown to have a negative impact on cell division (Fig. 3B). Pharmacological disruption of global *O*-GlcNAcylation correlates with delays in S- and G2/M-phase progression, and consequent growth arrest. Adenoviral overexpression of *O*-GlcNAcase disrupts cell morphology and nuclear organization, inducing a mitotic-exit phenotype, whereas increased expression of OGT results in defective cytokinesis and aneuploidy (Slawson et al., 2005). These data confirm that the *O*-GlcNAc–phosphate relationship controls the progression of cell division at different stages of the cell cycle.

A recent study clearly established that the crosstalk between *O*-GlcNAc and phosphate can directly regulate kinase activity. Upon ionomycin stimulation, the *O*-GlcNAcylation of calcium/calmodulin-dependent kinase IV (CaMKIV) rapidly decreases (within 2 minutes), returning to basal levels after 10 minutes. This event directly opposes the time-dependent activating phosphorylation of CaMKIV at threonine 200, a target site of the upstream kinase CaMKK. This interplay was further demonstrated by mutation of threonine 200 to alanine, which increased the *O*-GlcNAc content twofold compared with that of the wild-type protein. By combining a new chemo-enzymatic labeling protocol, followed by BEMAD (β-elimination followed by Michael addition with dithiothreitol), at least five *O*-GlcNAc sites in CaMKIV were identified: threonine 57/serine 58, serine 137, serine 189, serine 344/345 and serine 356. The mutation of serine 189 to alanine significantly increased CaMKIV phosphorylation at threonine 200, indicating opposite reciprocal relationships at different sites; however, the threonine 57/serine 58 to alanine mutant showed a marked reduction in phosphorylation at threonine 200, suggesting a complex site-specific crosstalk between *O*-GlcNAc and phosphate. In a three-dimensional model of a predicted CaMKIV structure,

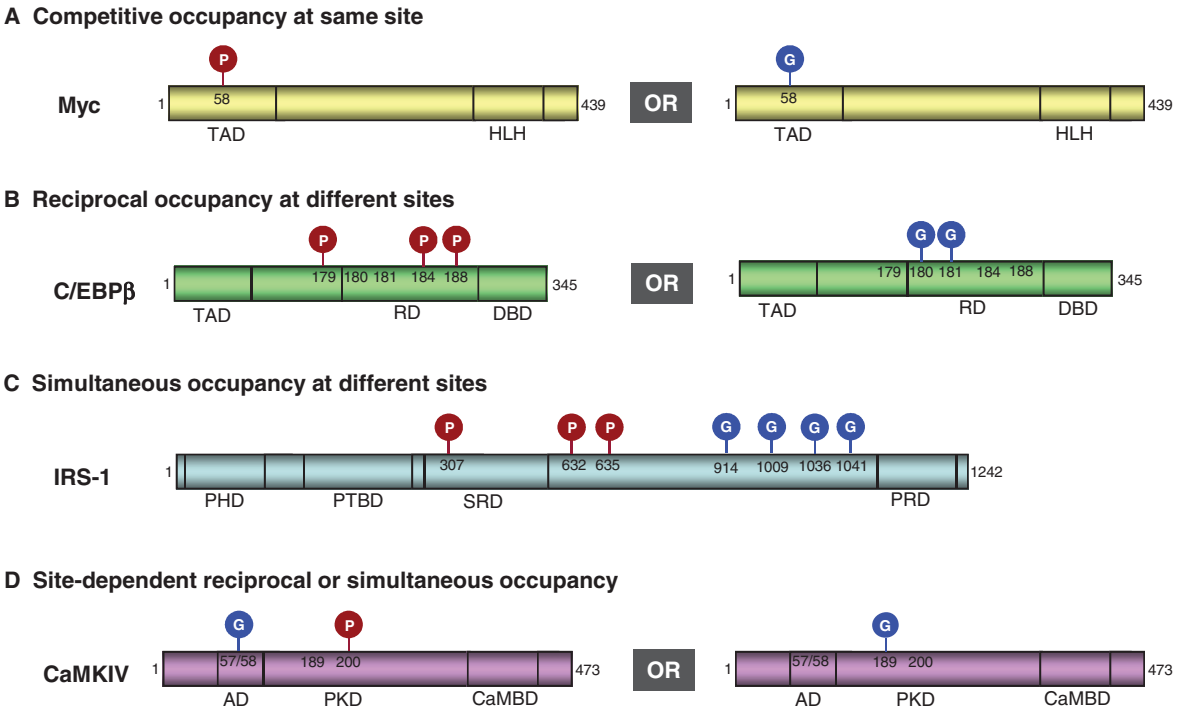

Fig. 4. Four different types of *O*-GlcNAc–phosphate crosstalk on protein substrates. (**A**) Alternative and competitive occupancy at the same amino acid residue, such as in the transactivation domain (TAD) of the transcription factor Myc, which can be either *O*-GlcNAcylated or phosphorylated at threonine 58. (**B**) Alternative and reciprocal occupancy at different sites, such as in the regulatory domain (RD) of the transcription factor C/EBPβ, which is either phosphorylated at threonine 179, serine 184 and threonine 188, or *O*-GlcNAcylated at serines 180 and 181. (**C**) Simultaneous occupancy at different sites, such as in IRS-1, which is both *O*-GlcNAcylated at multiple residues in the C-terminal domain (which contains several docking sites for SH2-domain-containing proteins) and phosphorylated at serines 307, 632 and 635 (which are known to attenuate the insulin signaling pathway). (**D**) Site-dependent reciprocal or simultaneous occupancy, such as in CaMKIV, which exists either as an *O*-GlcNAc–phospho active protein (at residues threonine 57/serine 58 for *O*-GlcNAc and threonine 200 for phosphate) or as an *O*-GlcNAcylated inactive protein (at serine 189). AD, activation domain; CaMBD, calmodulin-binding domain; DBD, DNA-binding domain; HLH, helix-loop-helix domain; PHD, pleckstrin-homology domain; PKD, protein kinase domain; PRD, proline-rich domain; PTBD, phospho-tyrosine-binding domain; SRD, serine-rich domain.

serine 189, threonine 57 and serine 58 all localized within the catalytic cleft of the enzyme, and kinase activity assays using recombinant CREB as a substrate demonstrated that mutating serine 189 to alanine results in a constitutively active protein (independent of stimuli), whereas mutating threonine 57/serine 58 to alanine renders the kinase completely inactive, even though it is *O*-GlcNAcylated at levels similar to the wild-type protein (Dias et al., 2009). These observations suggest that a complex site-specific *O*-GlcNAc–phosphate interplay at serine 189 and threonine 200 controls the basal catalytic activity of CaMKIV, whereas a more dynamic crosstalk between threonine 57/serine 58 and threonine 200 plays an important role in stimulated catalysis.

O-GlcNAc–phosphate interplay and neurodegenerative disease

The crosstalk between *O*-GlcNAc and phosphate is particularly extensive in proteins that are important for neuronal homeostasis, indicating a crucial role in brain function and pathogenesis. In mammalian Neurofilament M (NF-M), which is a highly phosphorylated protein important for axonal growth and maintenance, the major sites of *O*-GlcNAcylation have been mapped to the N-terminal head domain (threonine 48) and the tail domain (threonine 431) (Dong et al., 1993). *O*-GlcNAc and phosphate regulate each other reciprocally at the tail domain of NF-M, and hyperphosphorylation and accumulation of NF-M, concomitantly with its decreased *O*-GlcNAcylation, is a characteristic of brain tissue in patients with Alzheimer's disease (AD) (Deng et al., 2008) and of spinal-cord tissue of a rat model of amyotrophic lateral sclerosis (ALS) (Ludemann et al., 2005). Abnormal *O*-GlcNAcylation and phosphorylation of neurofilaments is also observed in chromatolytic motor neurons of patients with Werdning-Hoffmann disease (Chou and Wang, 1997). In the clathrin-assembly protein AP-3 (AP-180), *O*-GlcNAc and phosphate occur simultaneously on the central structural domain (Murphy et al., 1994). In neocortical regions of the brain in patients with AD, *O*-GlcNAcylation of AP-3 is significantly reduced, which correlates with an increased density of neurofibrillary tangles (NFTs) (Yao and Coleman, 1998b) and defects in synaptic-vesicle recycling (Yao and Coleman, 1998a). Hyperphosphorylation of residues within an *O*-GlcNAcylated peptide of the axonal guidance collapsing response mediator protein-2 (CRMP-2) is linked to its presence in NFTs that are associated with AD (Khidekel et al., 2007). *O*-GlcNAcylation of the cytoplasmic domain of the β-amyloid precursor protein (APP) (Griffith et al., 1995) might affect its phosphorylation and further proteolytic processing, which can lead to the formation of toxic peptides that are found in amyloid plaques of neurons in patients with AD. A recent study demonstrated that ataxin-10, the protein product of the gene affected in spinocerebellar ataxia type 10 (SCA10; an inherited progressive degenerative disease of the central nervous system), selectively associates with

OGT and increases its activity in the brain (Marz et al., 2006). This suggests that the loss of such OGT-targeting proteins during neurodegenerative disease underlies the hypo-*O*-GlcNAcylation of substrates in the diseased brain.

Ogt mutation specifically induced in neurons of mice resulted in increased phosphorylation of the microtubule-associated protein tau (O'Donnell et al., 2004). Extensive research has demonstrated that abnormal hyperphosphorylation of tau promotes its aggregation into the NFTs that are characteristic of AD brain. Early studies showed that, similar to many other cytoskeletal proteins, tau in the bovine brain was extensively modified by *O*-GlcNAc, with an average stoichiometry and site occupancy that paralleled its modification by phosphate (Arnold et al., 1996). Treatment with PUGNAc in metabolically active rat brain slices and in PC12 cells stably overexpressing recombinant human tau resulted in the increased *O*-GlcNAcylation of tau and other proteins, with a concomitant reduction of tau phosphorylation at specific amino acid residues (Liu et al., 2004). Interestingly, not all of the known phosphorylatable serine and threonine residues of tau were affected by these treatments, indicating that *O*-GlcNAcylation negatively regulates tau phosphorylation in a site-specific manner. A comparison of frozen human brain tissue from AD patients with age-matched controls corroborated that, in the brain of patients with AD, *O*-GlcNAcylation levels drop by about 50%, which is accompanied by hyperphosphorylation of tau at specific residues in the cerebrum region. Moreover, soluble tau from diseased brains contains less *O*-GlcNAc than soluble tau from healthy subjects, whereas insoluble aggregates of tau completely lack *O*-GlcNAc (Liu et al., 2009).

An in vivo animal model of starved mice showed that decreased *O*-GlcNAcylation of brain tau correlated with its hyperphosphorylation, suggesting that the reciprocal relationship between the *O*-GlcNAc and phosphate modifications of this protein results from low glucose uptake and/or utilization in the brain. Impaired brain glucose metabolism is known to precede the clinical symptoms of AD, and inhibition of the hexosamine biosynthetic pathway via injection of 6-diazo-5-oxonorleucine (DON) to the lateral ventricles of rat brains is also accompanied by hyperphosphorylation of tau (Liu et al., 2009). This suggests that an early decrease in *O*-GlcNAcylation might be the cause, rather than a consequence, of the accumulation of toxic tau aggregates. In a recent study, it was shown that exposure of PC12 cells to thiamet-G (a newly synthesized, highly selective and potent inhibitor of *O*-GlcNAcase) increases global *O*-GlcNAcylation while simultaneously decreasing tau phosphorylation at sites that are relevant in the pathology of AD (Yuzwa et al., 2008). Because thiamet-G can cross the blood-brain barrier, the results obtained with cells in culture were reproduced in total brain homogenates and in the hippocampus of rats previously treated with oral doses of the inhibitor. These exciting observations support the idea that attenuating tau phosphorylation in the brain by raising *O*-GlcNAc levels might be a therapeutic tool for slowing or preventing the manifestations of AD.

Conclusions and perspectives

Collective evidence has allowed us to propose at least four different types of interplay between *O*-GlcNAc and phosphate on substrates (Fig. 4). First, these modifications can competitively and alternatively occupy the same serine or threonine residue of a protein (as in Myc). Second, *O*-GlcNAc and phosphate can competitively and alternatively occupy different sites, which can be adjacent or distant (as in C/EBPβ). Third, they can simultaneously occupy different sites (as in IRS-1). Fourth, there can be complex crosstalk involving both site-specific alternative and simultaneous occupancy of *O*-GlcNAc and phosphate moieties (as in CaMKVI).

O-GlcNAc and phosphate also regulate each other by generating large functional complexes that consist of OGT, *O*-GlcNAcase, kinases and phosphatases. These complexes can rapidly modify a target substrate in a hierarchical manner (that is, they can mediate dephosphorylation prior to *O*-GlcNAcylation); however, they must be tightly regulated to avoid futile cycles. Additionally, each modification regulates directly the activities of the other's cycling enzymes.

O-GlcNAc–phosphate crosstalk participates in nearly every biological aspect of protein function: changes in subcellular localization (e.g. the translocation of OGT during insulin signaling and the nuclear localization of transcription factors), protein turnover (e.g. p53), enzymatic activity (e.g. CaMKIV), and the DNA-binding and transactivation properties of transcription factors (e.g. C/EBPβ). These changes in the activities of specific proteins play important roles in major cellular processes, as observed in studies of cell-cycle control and cellular responses to decreased glucose metabolism in the senescent brain that precede neurodegeneration.

Further research is necessary to address many unanswered questions regarding the role of *O*-GlcNAc–phosphate crosstalk in signal transduction and transcription. Importantly, what are the structural changes that are induced at a specific residue by one modification versus the other, and how do they affect protein function? Data obtained in studies of model peptides suggest that *O*-GlcNAc induces β-turns in protein structure, whereas phosphorylation opens up the target region (Liang et al., 2006; Wu et al., 1999). Is steric hindrance the mechanism by which alternative occupancy at different sites prevents further modifications? How is site-specific crosstalk regulated on the same protein? How are multifunctional complexes that contain enzymes with opposing activities regulated? Could targeting the *O*-GlcNAc–phosphate interplay represent a strategy for the treatment of cancer or neurodegenerative disease? As we continue to identify additional pathways and processes that are affected by this interplay, it might emerge that the *O*-GlcNAc–phosphate crosstalk is a major post-translational regulator of cellular homeostasis.

We thank the members of the Hart laboratory for their careful reading and suggestions to improve the manuscript. Original research in the Hart laboratory was supported by NIH R01 CA42486, R01 DK61671 and NHLBI Contract N01-HV-28180 to G.W.H. Deposited in PMC for release after 12 months.

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