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O-GlcNAcylation of kinases

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ABSTRACT

Recent evidence indicates that site-specific crosstalk between *O*-GlcNAcylation and phosphorylation and the *O*-GlcNAcylation of kinases play an important role in regulating cell signaling. However, relatively few kinases have been analyzed for *O*-GlcNAcylation. Here, we identify additional kinases that are substrates for *O*-GlcNAcylation using an *in vitro* OGT assay on a functional kinase array. Forty-two kinases were *O*-GlcNAcylated *in vitro*, representing 39% of the kinases on the array. In addition, we confirmed the *in vivo* *O*-GlcNAcylation of three identified kinases. Our results suggest that *O*-GlcNAcylation may directly regulate a substantial number of kinases and illustrates the increasingly complex relationship between *O*-GlcNAcylation and phosphorylation in cellular signaling.

1. Introduction

A hallmark of signal transduction is the dynamic inducible post-translational modification (PTM) of proteins. The most well-studied and understood regulatory PTM is phosphorylation. However, PTM of cytoplasmic and nuclear proteins at serine and threonine residues by β-*N*-acetylglucosamine (*O*-GlcNAc) has been emerging as a fundamental regulatory mechanism involved in cell signaling, metabolism, transcription, and cell division, among others [1,2]. *O*-GlcNAc cycling on proteins, mediated by the enzymes *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase, is dynamically regulated in response to various stimuli and is remarkably similar to phosphorylation. *O*-GlcNAcylation has been detected on myriad proteins, including transcription factors, cytoskeleton proteins, histones, and signal transduction proteins, including a few kinases [3]. Like phosphorylation, *O*-GlcNAcylation can modulate protein function, including enzymatic activity (e.g. CaMKIV) [4], turnover (e.g. p53) [5], protein interactions (e.g. stat5) [6], subcellular localization (e.g. Sp1) [7], DNA affinity, and transcription activity (e.g. PDX-1) [8]. Deletion of OGT is lethal in mice at the embryonic level and at the single-cell level, highlighting the importance of *O*-GlcNAcylation in regulating basic cellular events [9]. Aberrant *O*-GlcNAcylation has been linked to major diseases, including diabetes, cancer, and Alzheimer's disease [10,11].

Many papers have been published regarding the extensive interplay between *O*-GlcNAcylation and phosphorylation. Initial studies demonstrated that *O*-GlcNAc and phosphorylation mainly acted in opposing manners, suggesting a yin-yang relationship [12] . However, recent studies showed that the relationship between these PTMs is more complex than initially thought. Using proteomic approaches, Wang and colleagues [13] demonstrated that increased global *O*-GlcNAcylation, resulting from inhibition of *O*-GlcNAcase, concomitantly decreased a large number of phosphorylation events, as expected. However, *O*-GlcNAcase inhibition also led to an unexpected increase in phosphorylation at many other sites. These results suggest that *O*-GlcNAcylation and phosphorylation not only compete for substrates (at the same or proximal sites), but also that *O*-GlcNAcylation may also regulate phosphorylation cycling enzymes, such as kinases and/or phosphatases. Recently, *O*-GlcNAcylation was shown to directly regulate the kinase activity of CaMKIV toward CREB [4]. *O*-GlcNAcylation of Akt has been related to angiogenesis [14], apoptosis of murine pancreatic β-cells [15], and nuclear localization [16]. PKC isozymes are modified by *O*-GlcNAc, and PKCα activity seems to be regulated by this PTM [17]. It is our hypothesis that not only does phosphorylation regulate the *O*-GlcNAc cycling enzymes, but also that *O*-GlcNAcylation in-turn regulates the activity of many kinases. The human genome reveals that kinases represent about 2% of the genome [18], and until now, just a handful of kinases are described as *O*-GlcNAcylated. In addition, almost all *O*-GlcNAcylated proteins are also known to be phosphorylated, and kinases themselves are often regulated by phosphorylation. Taken together, these observations motivated us to screen for other kinases that may be *O*-GlcNAcylated.

Protein arrays have proven quite useful in research for screening protein activities, protein–ligand or protein–drug interactions, among others [19]. Here, we used a functional human protein array containing 152 kinases as substrate for OGT in order to identify

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kinases that are *O*-GlcNAcylated *in vitro*. We identified 42 kinases that are *O*-GlcNAcylated *in vitro*, representing 39% of kinases analyzed. Of the 110 other kinases, 66 were not good substrates for OGT *in vitro*, while 44 others could not be analyzed due to low abundance or background issues. In addition, we confirmed the presence of *O*-GlcNAc on three of the kinases *in vivo*. These data provide a number of potential targets that may be regulated by *O*-GlcNAcylation and illustrates the increasingly complex relationship between *O*-GlcNAcylation and phosphorylation.

2. Materials and methods

2.1. OGT in vitro labeling

The Panorama Human Kinase v1 Array (HPFM3 Sigma, Saint Louis, USA) was used as substrate for the *in vitro* OGT assay. This array contains a set of 152 full-length human kinases tagged with a biotin-carboxyl carrier protein (BCCP) and a c-Myc tag. First, the slides were blocked with 2% BSA in 50 mM Tris–HCl, pH 7.2 (blocking buffer) for 20 min. The prokaryotic expression vector encoding full-length human OGT [20] was a kind gift of Dr. Suzanne Walker (Harvard Medical School, Boston, MA). Human OGT was expressed and purified as described previously [21]. The *in vitro* OGT assay was performed on the array slides using 2 μg of recombinant OGT and 10 μCi of UDP-[^{3}H]-GlcNAc in 120 μl assay buffer (50 mM Tris–HCl, pH 7.2, 0,1% BSA) covered with cover slips for 2 h at room temperature in a humid chamber. Subsequently, slides were washed with 0.5% NP-40 in assay buffer, followed by incubation with anti-c-Myc-Cy3 conjugate, as suggested by the manufacturer's instructions. The arrays were washed, dried, and scanned for Cy3 (Ex 550 nm/Em 570 nm) on a Typhoon 9400 laser scanner (GE Healthcare, Munich, Germany) to determine protein levels. Then, the slides were exposed to a ^{3}H-sensitive storage phosphor screen (GE Healthcare, Munich, Germany) for one month. The storage phosphor screen was scanned on a Typhoon 9400 laser scanner, and the results were analyzed using Genepix Pro 3.0 software (Molecular Devices Corporation, Sunnyvale, USA). We used several negative spots consisting of BCCP-Myc or beta-galactosidase-BCCP-Myc to normalize the amount of background ^{3}H labeling. We discarded the results of samples with anti-myc-Cy3 signals lower than the lowest negative control spot or when we observe background problems. We considered the sample to be valid only when all four replicate spots displayed the same trend. Kinases are defined as *O*-GlcNAcylated when exhibiting a signal greater than 150% of the *O*-GlcNAcylation signal/protein levels observed on negative controls. The *O*-GlcNAcylation levels (GL) were arbitrarily classified as very high (+++, GL > 3.0); high (++, 3.0 > GL > 2.0); or positive (+, 2.0 > GL > 1.5).

2.2. Cell culture

HEK293A cells were grown in Dulbecco's modified Eagle's medium (25 mm glucose; Mediatech) containing 10% (v/v) fetal bovine serum (Gemini Bio-Products) and penicillin/streptomycin (Mediatech). When indicated, cells were incubated with 10 μm NAG-thiazoline overnight before harvesting.

2.3. Plasmids and cell transfection

HEK293A cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The human ERK-5 and S6LK cDNAs were obtained from the ATCC and subcloned into the pEF-HA mammalian expression plasmid[22]. The PKC-ζ-Flag mammalian expression plasmid was purchased from Addgene.

2.4. Immunoprecipitation and immunoblots

Cells were washed with PBS and collected into lysis buffer (0.5% Nonidet P-40 (Sigma) in 25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 μM PUGNAc with protease cocktail inhibitors). Lysates were sonicated, centrifuged to remove debris, and immunoprecipitations were performed with anti-HA antibody (12CA5 Roche Applied Science) or anti-Flag antibody (Sigma). The immunoprecipitates were washed and then eluted for immunoblot analysis. Samples were separated on SDS–polyacrylamide gels (Bio-Rad), and electroblotted to nitrocellulose (Bio-Rad). The immunoblots were first probed for *O*-GlcNAc RL-2 (Novus). Then, the immunoblots were stripped and reprobed with anti-HA (Covance) or anti-Flag (Sigma) antibodies to detect the protein levels.

3. Results

3.1. Many kinases are in vitro substrates for OGT

The protein array (HPFM3; Sigma) contains a set of 152 full-length human kinases printed in quadruplicate and fused to biotin-carboxyl carrier protein (BCCP). Thus, the printed kinases are not sterically or functionally hindered and are freely available as substrate for OGT. A c-Myc tag is incorporated into all protein kinases on the array slides for quantifying protein levels (Fig. 1A). Using UDP-[^{3}H]-GlcNAc as the donor, we performed an *in vitro* OGT assay directly on the protein array slides to radiolabel any kinases that are good OGT substrates (Fig. 1B). BCCP-myc served as a negative control (yellow box in Fig. 2). We only analyzed the kinases with anti-c-myc-Cy3 signals higher than the lowest negative control spot and all four replicate spots displayed the same trend. As a result, we discarded results from 33 kinases that displayed lower anti-c-myc-Cy3 signals than negative controls (green boxes in Fig. 2), since low protein levels often lead to unreliable results. In addition, we discarded 11 kinases that contained background issues, interfering with proper signal measurement. Out of 108 kinases analyzed, we identified 42 kinases that were *O*-GlcNAcylated (blue boxes in Fig.2), representing 39% of the total assayed. 66 kinases were not good *in vitro* substrates for OGT (red boxes in Fig.2). In our screen, 40 kinases previously unknown to be *O*-GlcNAcylated were identified as substrates for OGT. Two kinases previously shown to be *O*-GlcNAcylated, CaMKIV[4] and casein kinase II[23], were also identified in this assay (Table 1).

3.2. Selected kinases are O-GlcNAcylated in vivo

The *O*-GlcNAcylation levels (GL) of kinases were arbitrarily classified as very high (+++, GL > 3.0); high (++, 3.0 > GL > 2.0); or positive (+, 2.0 > GL > 1.5). To validate the screen, we confirmed the *O*-GlcNAcylation of three selected kinases (one from each GL group), including protein kinase C zeta (PKC-ζ), extracellular regulated kinase 5 (ERK-5), and ribosomal protein S6 kinase-like 1 (S6LK). We over-expressed these kinases in HEK293A cells and treated the cells with the *O*-GlcNAcase inhibitor NAG-thiazoline in order to increase total cellular *O*-GlcNAc levels. As expected, all three kinases immunoprecipitated were confirmed to be *O*-GlcNAcylated *in vivo* and the *O*-GlcNAc levels were responsive to NAG-thiazoline treatment (Fig. 3).

4. Discussion

Dynamic PTMs control virtually all cellular events. *O*-GlcNAcylation and phosphorylation are perhaps amongst the two most abundant regulatory PTMs in cells and share many similarities. Both PTMs occur on Ser/Thr, are extremely dynamic, and can