Published in final edited form as:

Biochem Biophys Res Commun. 2006 December 22; 351(3): 708–712. doi:10.1016/j.bbrc.2006.10.092.

Reciprocal keratin 18 Ser48 O-GlcNAcylation and Ser52 phosphorylation using peptide analysis

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Abstract

Phosphorylation and O-GlcNAcylation of keratin 18 (K18) are highly dynamic and involve primarily independent K18 populations. We used in vitro phosphorylation and O-GlcNAcylation of wild-type, phospho-Ser52, glyco-Ser48, and Ser-to-Ala mutant 17mer peptides (K18 amino acids 40-56), which include the major K18 glycosylation (Ser48) and phosphorylation (Ser52) sites, to address whether each modification blocks the other. The glyco-K18 peptide blocks S52 phosphorylation by protein kinase C, an in vivo K18 kinase, while the phospho-K18 peptide blocks its O-GlcNAcylation. Our findings support the reciprocity of these two posttranslational modifications. Therefore, regulation of protein Ser/Thr phosphorylation and glycosylation at proximal sites can be interdependent and provides a potential mechanism of counter regulation.

Keywords

Intermediate filaments; keratin 18; phosphorylation; O-glycosylation; O-GlcNAcylation; O-GlcNAc transferase

INTRODUCTION

The O-linked β -N-acetylglucosamine (O-GlcNAc) single sugar protein modification, initially described in 1984 [1], is one of many posttranslational modifications, such as phosphorylation, acetylation, methylation and ubiquitination. This unique type of glycosylation is found on a broad range of cytoplasmic and nuclear proteins that include RNA polymerase II, kinases, phosphatases, cytoskeletal proteins, transcription factors, histones and tumor suppressors [2-4]. It has been demonstrated to play a role in diseases such as diabetes, cancer and neurodegeneration [2,3]. Accumulating evidence implicates this glycosylation in the modulation of numerous functions, including protein phosphorylation, degradation,

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subcellular localization, protein-protein interaction and gene transcription [3,4]. Although the O-linked Ser/Thr glycosylation is an abundant and dynamic modification, and in some cases acts reciprocal to phosphorylation [5,6], it is not characterized as well as phosphorylation because of the technical difficulties associated with studying this modification. No clear glycosylation consensus sequence has been defined, but O-GlcNAc-containing sequences are often located at or near known phosphorylation motifs, and most if not all O-GlcNAc-containing proteins are phosphoproteins [7]. A generalized or site-specific reciprocal relationship between O-GlcNAc and phosphorylation has been observed [8,9]. For example, the sites of these two modifications have been mapped to the same residue in some proteins such as the estrogen receptor β , the SV40 T antigen, and c-Myc [4].

Within the intermediate filament (IF) family of cytoskeletal proteins, keratin (K) polypeptide 8, 13, 18 [10,11] and neurofilaments [12-14] undergo O-GlcNAc modification. In epithelial cells, IFs are composed of type I (K9-K20), and type II (K1-K8) keratins. Epithelial cells express at least one type I and one type II keratins, and the two keratin types exist as obligate non-covalent heteropolymers. IFs are highly dynamic, and their assembly and disassembly are regulated by phosphorylation [15]. A major K18 O-GlcNAcylation site is Ser48 [16], which is proximal to a major Ser52 phosphorylation site [17]. K18 Ser52 phosphorylation is highly dynamic during the S/G2/M phases of the cell cycle, in association with filament reorganization [17]. Previous analysis by 2D gels showed that K18 phosphorylation and O-GlcNAcylation involve independent K18 populations, suggesting that these two K18 modifications may regulate each other [11].

Competition of phosphorylation and O-GlcNAcylation at adjacent sites, where the respective sites are located within a few residues of each other is another potential mechanism of regulation. In the case of keratins, suggested evidence for this comes from analysis of keratins isolated from livers of mice fed the hepatotoxin griseofulvin which causes a decrease in K18 glycosylation at its known glycosylation sites (Ser29,30,48) in association with hyperphosphorylation at K18 Ser33,52 [18 and unpublished observations]. In another example, streptozotocin induces an increase in Ser149 O-GlcNAcylation in association with a decrease in p53 Thr155 phosphorylation [6]. Whether, this “neighbor” modulatory effect takes place in both directions is poorly understood.

To understand any potential interplay between the two major K18 modifications, we synthesized a panel of fluorescently-tagged 17-mer peptides that include or exclude the O-GlcNAc and phosphate modifications [5FAM-⁴⁰GSGSRISVS(O-GlcNAc)RSTS(PO4)FRGG] and their corresponding modified Ser-to-Ala mutants. Peptide phosphorylation and O-GlcNAcylation were monitored using capillary electrophoresis and ³H-GlcNAc incorporation into the peptides. Our results demonstrate that K18 S48 O-GlcNAcylation inhibited its adjacent phosphorylation at S52; and conversely K18 S52 phosphorylation prevented O-GlcNAcylation at S48. These findings suggest that K18 S48 O-GlcNAcylation and S52 phosphorylation may negatively regulate each other in cells.

MATERIALS AND METHODS

Reagents and K18 peptides

Recombinant protein kinase C (PKC) ϵ (Cat#14-518) and the PKC lipid Activator (Cat # 20-133) were purchased from Upstate (Dundee, UK); and other reagents were from Sigma-Aldrich (St. Louis, MO). The 5FAM-tagged fluorescent K18 peptides [5FAM-⁴⁰GSGSRISVS(O-GlcNAc)RSTS(PO4)FRGG; Table 1], that include or exclude the O-GlcNAc and phosphate modifications, were synthesized and purified using standard methods. The FAM-labeled peptides (green color) were mixed with TAMRA-labeled complex

carbohydrate markers (red color; used as an internal reference) then analyzed by capillary electrophoresis.

Reaction of *in vitro* peptide phosphorylation

Wild-type, mutant or glycosylated peptides (10 μ M) were incubated with 120 nM of PKC ϵ in 20 mM Hepes (pH 7.4) containing 0.03% Triton X-100, 15 mM MgCl₂, 100 μ M ATP and lipid Activator for indicated time periods (37 °C). Negative controls were performed by not including the enzyme or ATP. Reaction mixtures were then subjected to capillary electrophoresis, and the retention time of the internal reference standards was correlated with each keratin peptide. Quantification of peptide products was done using “Dax” software (Van Mierlo Software Consultancy; Eindhoven, Netherlands).

O-GlcNAc transferase assays

Recombinant O-GlcNAc transferase (OGT) was expressed and purified as described [19]. Briefly, *E. coli* BL21 (DE3) was transformed with the pET32-OGT vector for expression as C-terminal His₈ fusions. The culture was grown to stationary phase, chilled to 16 °C, induced with 0.4 mM IPTG for 12 h at 16 °C (OD=1.2). The cell pellet was washed with ice-cold PBS and lysed by sonication in TBS. The clarified lysate was passed over Ni²⁺IDA IMAC agarose, washed with TBS, and eluted with 250 mM imidazole. OGT was concentrated with a buffer exchange to remove imidazole. K18 peptides (2 mM) were incubated with 0.5 μ g of OGT in 50 mM sodium cacodylate (pH 6.5) and 2.5 mM 5'-adenosine monophosphate, 0.5 μ Ci of UDP-[6-³H]GlcNAc for 30 min (20 °C) [9]. The reaction was terminated with 500 μ l of 50 mM formic acid and 500 mM NaCl, followed by peptide purification using a Sep-Pak C18 cartridge (Waters Corp.; Milford, MA). The cartridges were prewashed with 4 ml of methanol, 4 ml of 50 mM formic acid, the reaction was then loaded onto the cartridge, and the cartridge was washed with 4 ml of 50 mM formic acid, 10 ml of 50 mM formic acid containing 0.5 M NaCl, and 4 ml of distilled H₂O [20]. The peptides were eluted from the cartridge directly for scintillation counting.

RESULTS AND DISCUSSION

PKC ϵ phosphorylates K18 peptides at Ser52

K18 phosphorylation and O-GlcNAcylation involve independent K18 populations as determined using 2D gel analysis [11], which raises the hypothesis that these two K18 modifications may regulate each other. In order to address potential interplay between K18 phosphorylation and O-GlcNAcylation, we synthesized a panel of K18 peptides (Table 1) that alter one or both of the two major posttranslational modification sites, and analyzed their elution profiles using capillary electrophoresis. As shown in Figure 1, the synthesized phosphopeptides (pWT and pgWT) were well-separated from the non-phosphorylated peptides (WT or gWT), respectively, indicating that our separation conditions were suitable to monitor the *in vitro* experiments that address the phosphorylation and glycosylation of the K18 peptides.

Previous studies supported a potential role for PKC in keratin phosphorylation [21-24]. For example, PKC ϵ is a likely *in vivo* K8/K18 kinase as supported by co-immunoprecipitation, comparison of *in vivo* and *in vitro* labeled K18 phosphopeptides, and modulation of keratin phosphorylation using pharmacologic inhibition or activation of PKC [15,21,22]. Based on these prior data, we carried out *in vitro* phosphorylation of the K18 WT and the phospho-mutant (S50/52A) peptides using PKC ϵ . The kinase phosphorylates the majority of the WT K18 peptide (Figure 2A) but does not appear to phosphorylate the phospho-mutant peptide (Figure 2B) at its remaining three serines (Table 1). No phospho-product was generated by the K18 WT peptide when ATP or the kinase were omitted from the reaction mix (not shown). The generated phosphopeptide peak disappears, in association with emergence of a peak

corresponding to the WT peptide after incubation of the product with lambda-phosphatase (not shown). These results indicate that K18 S52 in vivo phosphorylation by PKC can be recapitulated in vitro using a purified peptide.

O-GlcNAc modification of K18 Ser48 inhibits PKC ϵ -mediated phosphorylation of K18 Ser52

The K18 S52 phosphorylation site is proximal to S48, a major in vivo O-GlcNAcylation site. Given that the in vivo K18 S52 phosphorylation can be modeled in vitro using the WT K18 peptide, we asked whether S48 glycosylation in the gK18 glycopeptide interferes with K18 S52 phosphorylation. gK18 in vitro phosphorylation by PKC ϵ (Figure 3A) is not as complete as the phosphorylation of the K18 WT peptide (Figure 2A). As shown in Figure 3B, only 47% of gWT was phosphorylated as compared with 73% of the non-glycosylated WT peptide. This indicates an inhibitory role of K18 S48 O-GlcNAcylation on S52 phosphorylation. The small peak highlighted by an asterisk is an unidentified product.

Ser52 phosphorylation blocks O-GlcNAc modification of K18 at Ser48

We then addressed whether K18 S52 phosphorylation has any effect on K18 S48 O-GlcNAcylation using in vitro O-GlcNAcylation mediated by OGT and measured by ^3H -GlcNAc incorporation into K18 peptides. As shown in Figure 4, glycosylation of the phospho-K18 peptide was significantly blocked as compared with the WT peptide (average of 3146 dpm as compared 6040 dpm, respectively). The S50/52A peptide served as a better substrate as compared with WT K18, and as expected O-GlcNAcylation of the S48A peptide was markedly less than the WT K18 peptide (Figure 4).

Taken together, the use of several K18 protein-mimic peptides indicates that K18 glycosylation at Ser48 and phosphorylation at the proximal residue Ser52 reciprocally hinder each other. To our knowledge, this is the first direct demonstration of reciprocity for neighboring phospho/glyco sites in vitro. It would be difficult to carry out this experiment using intact recombinant K18 WT and mutant proteins due to their relative insolubility, and the confounding variables of other potential Ser/Thr sites that may be in vitro but not in vivo targets. A similar demonstration for in vitro reciprocity of phosphorylation and O-GlcNAcylation was demonstrated for RNA polymerase II tandem repeats, but in this case it is not clear if the in vitro phosphorylation involved the identical glycosylation sites [9]. Our results suggest that neighboring glycosylation and phosphorylation motifs may in some contexts counter regulate each other. Such “neighbor” posttranslational modulating effect, which might apply for in vivo K18 regulation, can potentially extend to other proteins where O-GlcNAcylation and phosphorylation occur within Ser/Thr-rich domains.

Acknowledgements

This work was supported by the NIH grant DK52951 and the Department of Veterans Affairs Merit Award (MBO); and NIH grant DK61671 and HD13563 (GWH). MBO declares that he had a financial consultative arrangement with CellBio Sciences in the past.

Abbreviations

5-FAM, 5-carboxyfluorescein; IF, intermediate filaments; K, keratin; O-GlcNAc, O-linked β -N-acetylglucosamine; OGT, O-GlcNAc transferase; g, glyco; p, phospho; PKC, protein kinase C.

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Figure 1. Capillary electrophoresis elution profiles of non-phosphorylated and phosphorylated K18 peptides

FAM-labeled peptides including WT, pWT, gWT and pgWT were diluted to 1 nM with electrophoresis running buffer and individually analyzed. Each of the four panels shows two tracings. For each panel, the upper tracing (highlighted by horizontal arrows) represents the internal standard tracing which includes two TAMRA-labeled complex carbohydrate species (vertical arrows) whose migration is used as an internal reference. Note that all of the phosphopeptides are well separated from their non-phosphorylated counterparts.

Figure 2. In vitro phosphorylation of the K18 WT and mutant peptides with purified PKC ϵ :

A) The WT peptide was incubated at 37 °C with PKC ϵ and its activator for 0-1080 min. The kinase reaction was stopped by mixing with electrophoresis sample buffer followed by analysis by capillary electrophoresis. As noted in Figure 1 legend, each panel shows two tracings (upper tracing, controls; lower tracing, keratin peptide). Note that the WT peptide (migration position highlighted by an arrow in the upper panel) converts to a newly generated product peak (migration position highlighted by an arrow in the lower panel), which has identical migration to that of the pWT peptide shown in Figure 1 (also confirmed by a mixing experiment, not shown). B) The mutant K18 peptide (where the two potential phosphorylation sites Ser50 and Ser52 are modified to Ala) was subjected to in vitro phosphorylation then analysis as in Panel

A. Note that there is no evidence of generation of a phospho-product that involves the remaining three serines.

Figure 3. Glycosylation of S48 interferes with PKC ϵ -mediated phosphorylation

A) The K18 glyco-peptide (upper panel, migration position highlighted by a thin arrow) was subjected to in vitro phosphorylation followed by capillary electrophoresis. The phospho-peptide product (lower panel, thick arrow) has an identical retention time to the pgWT control peptide shown in Figure 1. The identity of the peak highlighted by an asterisk is unknown. B) Graphic representation of the PKC ϵ -mediated phosphorylation of the K18 WT and gWT peptides. Quantification of the starting material and product peptides was carried out by measuring the area of the peaks using the DAX software as described in Materials and Methods. The rates represent an average of three independent experiments.

Figure 4. Comparison of OGT-mediated glycosylation of WT, pWT and mutant K18 peptides
Equal amounts of K18 peptides were incubated with purified OGT in the presence of [^3H]-UDP-GlcNAc as described in Materials and Methods. The incorporated ^3H -GlcNAc (in DPM) was measured and compared among the K18 peptides. * p value < 0.05 as compared with WT (n= 3). Note that phosphorylation of the K18 peptide blocks its glycosylation.

Table 1

Amino acid sequences of synthesized K18 peptides

Name of K18 peptide	Amino acid sequence (17-mers)
Wild type (WT)	5FAM- ⁴⁰ GSGSRISVSRSTSFRGG
Phospho-mutant (S50A/52A)	5FAM- ⁴⁰ GSGSRISVSRATAFRGG
Glyco-mutant (S48A)	5FAM- ⁴⁰ GSGSRISVARSTSFRGG
Phospho-WT (pWT)	5FAM- ⁴⁰ GSGSRISVSRST ^p SFRGG
Glyco-WT (gWT)	5FAM- ⁴⁰ GSGSRISV ^g SRSTSFRGG
Phospho-glyco-WT (pgWT)	5FAM- ⁴⁰ GSGSRISV ^g SRST ^p SFRGG