Identification of Potential Substrates of *N*-acetylglucosamine Kinase by a Proteomic Approach

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Post-translational *O*-GlcNAc modification (*O*-GlcNAcylation) of serine or threonine is a new protein modulation mechanism. In contrast to the classical glycosylation, *O*-GlcNAcylation occurs in a one-step transfer of *O*-GlcNAc on both nuclear and cytoplasmic proteins. In contrast to the general consensus that *O*-GlcNAc is a final modification, a recent paper (J Proteome Res. 2011 10:2725 - 2733) showed the presence of *O*-GlcNAc-P on a synaptic assembly protein AP180. This finding raises a fundamental question about its prevalence. To address this question, we used proteomics to identify those proteins that were phospho-signal enriched by GlcNAc kinase (NAGK). Comparison of pDsRed2-NAGK_{WT}-transfected HEK293T cell extract with pDsRed2-NAGK_{D107A}-transfected control culture revealed 15 phospho-signal increased spots. Excluding those spots that had no detectable amount of protein expression yielded 7 spots, which were selected for ID determination. Among these, two duplicate spots (two HSP90 β and two ENO1 spots) were shown to be *O*-GlcNAcyated, two (dUTP nucleotidohydrolase mitochondrial isoform 2, glutathione *S*-transferase P) were not known to be involved in *O*-GlcNAcylation, and one (heat shock protein gp96 precursor or grp94) was a glycoprotein. The increase in the phospho-levels of *O*-GlcNAc by NAGK strongly indicates that these proteins are phosphorylated on *O*-GlcNAc. Our present data support the idea that *O*-GlcNAc is not a terminal modification.

Key words : ENO1, HSP90 β , *O*-GlcNAc-P, *O*-GlcNAc kinase, proteomics

Introduction

Addition of a single β -*N*-acetylglucosamine monosaccharide moiety to serine and threonine side chains of a polypeptide backbone through an *O*- β -glycosidic attachment (*O*-linked β -*N*-acetylglucosamine; *O*-GlcNAc) was first discovered by Torres and Hart [26]. Since then, *O*-GlcNAc has been found on a wide range of nuclear and cytoplasmic proteins involved in almost all cellular processes [14, 15, 21, 31].

The addition of *O*-GlcNAc (*O*-GlcNAcylation) 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 [12]. The reverse reaction, removal of *O*-GlcNAc from

proteins, is catalyzed by a neutral β -*N*-acetylglucosaminidase (*O*-GlcNAcase or OGA) [6, 8]. The addition and removal reactions of *O*-GlcNAc moieties are sometimes very rapid and dynamic and inducible in response to cellular changes [18] (for reviews, see Copeland *et al.* [3], Hanover [13], Hart *et al.* [15]). Proteomic advances that involve the selective enrichment *O*-GlcNAcyated proteins from complex mixtures, followed by special mass spectrometric (MS) methods, have allowed the mapping and quantification of multiple *O*-GlcNAcylation sites [19, 28]. Most recently, Click chemistry followed by LC-MS/MS afforded the identification of around 1500 *O*-GlcNAc proteins from a single cell line [10].

The primary function of *O*-GlcNAcylation appears to be the modulation of cellular processes in response to nutrients and to cellular stress. Firstly, the overall catalytic activity of OGT is regulated linearly by a wide range of intracellular concentrations of UDP-GlcNAc [20]. In turn, the concentration of UDP-GlcNAc fluctuates proportionally in response to the flow of nutrients (glucose, glutamine, energy) through the hexosamine biosynthetic pathway [22]. Thus, the *O*-GlcNAcylation of substrates represents a 'metabolic sensor' that modulates protein function to adjust to the nutri-

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tional status of the cell. Secondly, *O*-GlcNAcylation 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 [30]. In addition to promoting cell survival, *O*-GlcNAcylation modulates diverse protein functions, including protein-protein interactions, protein turnover, subcellular localization and changes in activity. Comparing to the effect of phosphorylation, emerging evidence suggests that, by analogy to an electrical circuit, if phosphorylation events represent “microswitches,” which turn on or turn off protein activity, *O*-GlcNAcylation represents a “rheostat” tuning the pathways and processes to accommodate nutrient status and cellular stress [14]. *O*-GlcNAcylation differs from classical complex glycosylation at least two points of view. First, *O*-GlcNAcylated proteins are nucleocytoplasmic, whereas the classical glycosylation occurs on secreted extracellular proteins and on the extracellular segments of integral membrane proteins. Second, *O*-GlcNAcylation is a single molecule (i.e., GlcNAc) addition and the reaction is reversible. In contrast, the classical glycol-moieties are very complex requiring a series of addition reactions which occur in a cotranslational or post-translational manner.

Until recently, the sugar moiety *O*-GlcNAc was thought to be a terminal modification, that is, the *O*-GlcNAc is not additionally modified in any way. However, Graham *et al.* [9] showed that a synaptic assembly protein AP180 purified from rat brain contains a phosphorylated *O*-GlcNAc (*O*-GlcNAc-P) within a highly conserved sequence. This finding opens a possibility for the phosphorylation of *O*-GlcNAc by GlcNAc kinase (NAGK), which is known, so far, to phosphorylate the monomer GlcNAc. In an effort to identify potential *O*-GlcNAc-P proteins we applied proteomics to search proteins, of which phospho-levels are increased by NAGK. In this report we show that, in comparison with control cultures, NAGK-overexpressed HEK293T cells had many phospho-enriched proteins. Surprisingly, some of these proteins were known to be *O*-GlcNAcylated, strengthening the possibility for many more *O*-GlcNAc-P proteins, i.e., *O*-GlcNAc-P^{om}.

Materials and Methods

Preparation of bacterially expressed NAGK

The original murine NAGK clone was a kind gift of Dr.

Stephan Hinderlich (Beuth Hochschule für Technik Berlin, Department of Life Sciences and Technology, Berlin, Germany). Site-directed PCR mutagenesis, cloning, bacterial expression and purification will be published elsewhere (Lee *et al.*, in preparation). Briefly, point mutated NAGK genes were produced by an overlap extension PCR method [17] and inserted into a pRSET vector. The vectors transformed into *E. coli* BL21 (DE3)-pLysS cells (Invitrogen; Carlsbad, CA, USA) and the enzymes were purified through a Ni-nitrilotriacetic acid column (MagneHis™ Ni-Particles) (Promega; Madison, WI, USA). The enzymatic activity was assayed as described elsewhere [1, 27].

Cell culture and transfection

HEK293T cells were cultured at 37°C and 5% CO₂ in D-MEM medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, USA) and 10% fetal bovine serum. Cells were transfected with the respective DsRed2 plasmids (pDsRed2-NAGK_{WT} or -NAGK_{D107A}) using Clontech CalPhos™ Mammalian Transfection Kit (BD Bioscience, Palo Alto, CA, USA) according to the manufacturer's instructions.

PUGNAc treatment and preparation of the cytosolic fraction

Before treatment with *O*-(2-Acetamid-2-deoxy-D-glucopyranosylidene) amino *N*-phenyl carbamate (PUGNAc; Sigma A7229, MO, USA), media were changed to fresh D-MEM without serum. Cells were treated with 50 µM PUGNAc for 2 hr, washed with HBSS, and harvested. After brief centrifugation, the cell pellet was suspended in 200 µl of Nuclei EZ lysis buffer (Sigma NUC-101, MO, USA), and set on ice for 5 min. After centrifugation at 15,000 rpm for 5 min, the supernatant, which is the cytosolic proteins, was collected. To cytosolic proteins (160 µl) were added methanol (640 µl), chloroform (160 µl), and H₂O (480 µl). After vortexing, the solution was centrifuged at 15,000 rpm for 5 min at 4°C, and the upper layer was discarded without disturbing the interface layer. Methanol (450 µl) was added to the remaining solution, mixed, and the mixture was centrifuged at 15,000 rpm for 5 min at 4°C. The pellet containing the cytosolic proteins was air-dried for 5 min.

2 Dimensional polyacrylamide gel electrophoresis (2D PAGE)

IPG dry strips (4-10 NL IPG, 24cm, Genomine, Korea)

were equilibrated for 12-16 hr with 7 M urea, 2 M thiourea containing 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol, 1% pharmalyte and respectively loaded with 200 µg of sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences) following manufacturer's instruction. For IEF, the voltage was linearly increased from 150 to 3,500 V during 3 hr for sample entry followed by constant 3,500 V, with focusing complete after 96 kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50 mM Tris-HCl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20 x 24 cm, 10-16%). SDS-PAGE was performed using Hoefer DALT 2D system (Amersham Biosciences) following manufacturer's instruction. 2D gels were run at 20°C for 1,700 Vh. And then 2D gels were silver stained as described by Oakley *et al.* (1980) but fixing and sensitization step with glutaraldehyde was omitted.

ProQ diamond Phosphoprotein staining

Gels were fixed with 40% ethanol and 10% acetic acid for 1 hr, and rehydrated in 5% ethanol and 5% acetic acid (30 min x 3 times). After rinsing several times in distilled water, the gels were stained with ProQ diamond phosphoprotein staining solution (Invitrogen) for 1 hr. After Gels were destained with ProQ diamond phosphoprotein destaining solution (Invitrogen) for 1 hr, images were acquired using a Diversity 2D Gel Imaging System (Syngene Inc., Cambridge, UK) equipped with Cy3 emission filter.

Image analysis

Quantitative analysis of digitized images was carried out using the PDQuest (version 7.0, BioRad) software according to the protocols provided by the manufacturer. Quantity of each spot was normalized by total valid spot intensity. Protein spots were selected for the significant expression variation deviated over two fold in its expression level compared with control.

Peptide Mass Fingerprinting (PMF)

For protein identification by peptide mass fingerprinting, protein spots were excised, digested with trypsin (Promega, Madison, WI), mixed with a cyano-4-hydroxycinnamic acid

in 50% acetonitrile/0.1% TFA, and subjected to MALDI-TOF analysis (Microflex LRF 20, Bruker Daltonics) as described before [7]. Spectra were collected from 300 shots per spectrum over m/z range 600-3000 and calibrated by two point internal calibration using trypsin auto-digestion peaks (m/z 842.5099, 2211.1046). Peak list was generated using Flex Analysis 3.0. Threshold used for peak-picking was as follows: 500 for minimum resolution of monoisotopic mass, 5 for S/N. The search program MASCOT, developed by The Matrixscience (<http://www.matrixscience.com>), was used for protein identification by peptide mass fingerprinting. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. PMF acceptance criteria is probability scoring.

In vitro Phosphorylation and autoradiography

The reaction mixture [final volume of 150 µl containing 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 2 mM ATP (disodium salt), 60 µCi of γ -³²P-ATP (10 µCi/µl; 3000 Ci/mmol; Perkin Elmer, Massachusetts, USA), 0.38 µg of purified NAGK protein (7.04 U/mg) and 30 µg of TCA-precipitated rat heart extract] was incubated at 37°C for 2 hr. The reaction mixture was either TCA-precipitated again or directly separated in 6% SDS-PAGE, and air-dried. An X-ray film was exposed for 5 days at -30°C.

Results and Discussion

Since Graham *et al.* [9] showed that the Thr-310 of AP180 is modified by *O*-GlcNAc or *O*-GlcNAc-P, but not phosphorylation alone, the general thought that the sugar moiety *O*-GlcNAc is not further modified is being challenged. The *O*-GlcNAc-P could have been transferred at one-step. However, there is currently no evidence for the existence of UDP-GlcNAc-P which would potentially be used in a similar way that UDP-GlcNAc is used by OGT to add *O*-GlcNAc to proteins. Therefore, it is more likely that *O*-GlcNAc-P is modified stepwise, *O*-GlcNAc and then phosphorylation to *O*-GlcNAc-P by NAGK. This two-step modification raises a critical question on the phosphorylation of *O*-GlcNAc, which is linked to the hydroxyl group of serine or threonine. The GlcNAc linked to a peptide chain is different from free ones at least in two ways. Firstly, the moiety may be situated

in a molecular space that would restrict its availability to NAGK. This steric hindrance may set a limit on the substrate *O*-GlcNAc, suggesting that not all *O*-GlcNAc could serve as substrates for NAGK. Secondly, the configuration of *O*-GlcNAc would be another critical criteria for being appropriate for the substrate. Applying saturation transfer difference (STD) NMR experiments on human NAGK, Blume *et al.* [2] found that NAGK exhibits a high β -anomeric preference with the drastically reduced binding affinity for sugars other than GlcNAc. The *O*-GlcNAc is linked to a serine or threonine in β -configuration, the right steric configuration for NAGK. Therefore, a reasonable assumption is that some select *O*-GlcNAc may serve as substrates for NAGK.

Proteins are phosphorylated by NAGK *in vitro*

To prove our assumption, we first tested the possibility of protein phosphorylation by NAGK. To this end, we used heat-inactivated heart extracts as substrate. To eliminate the possibility of phosphorylation by any other kinase in the extract, the heart extract was boiled for 5 min and proteins were precipitated by TCA to eliminate free monomer GlcNAc. The heat-inactivated extracts had no enzyme activities when the extract was incubated in the NAGK reaction buffer (data not shown). This enzymatically inert extract was used as a substrate for the phosphorylation reaction using bacterially expressed NAGK. We used γ - 32 P-ATP to increase detection sensitivities. The autoradiograph of the reaction products, which are separated in a 6% SDS-gel, was shown in Fig. 1. The radioisotope incorporation rate was

Fig. 1. An autoradiograph showing *in vitro* phosphorylation of rat heart extracts by NAGK. Rat heart extracts were used directly (- TCA) or after precipitation with TCA (+ TCA), and incubated in a reaction buffer containing γ - 32 P-ATP and bacterially expressed murine NAGK. The reaction mixture (30 μ g of substrate/lane) was separated in 6% SDS-PAGE, and air-dried. An X-ray film was exposed for 5 days at -30°C . The isotope-labeled band at 50 kDa and on the top of the gel were indicated with arrows.

very low requiring long film-exposure time (5 days). Also, there were no distinct bands labeled with the isotope except at ~ 50 kDa position and on top of the gel. This result indicates that the protein phosphorylation by NAGK is not efficient *in vitro*. The low efficiency could represent the *in vivo* situation, or our *in vitro* reaction condition is not optimal to represent the *in vivo* conditions. Nonetheless, a relatively strong labeled band was present at ~ 50 kDa position, and some smearing signals throughout the lane was evident. The relatively strong signal at the ~ 50 kDa size may represent actin, which is known to have *O*-GlcNAc [16]. This result indicates strongly that NAGK can phosphorylate proteins.

Overexpression of NAGK in HEK293T cells increase the phospho-signals of some proteins

Being encouraged by the *in vitro* phosphorylation results, we set out to identify cellular *O*-GlcNAc-P proteins. The overall strategy is schematically shown in Fig. 2. To optimize the phosphorylation reaction we took advantage of cell cultures, where the phosphorylation reaction takes place in the cell. To this end we transfected the cultured HEK293T cells

Fig. 2. The strategy used in this work to identify *O*-GlcNAc-P proteins. Cultures of HEK293T cells were transfected with the pDsRed2-NAGK_{WT} or -NAGK_{D107A}, and treated with PUGNAc (50 μ M) for 2 hr before harvest. Cell extracts were separated either in an 1D or 2D SDS-PAGE. The gels were stained with ProQ diamond phosphostaining solution. The spots in the 2D-gel that exhibit increased phospho-signals were selected for protein ID determination.

with plasmids that would produce DsRed2-tagged wild-type NAGK (pDsRed2-NAGK_{WT}) or a mutant NAGK (pDsRed2-NAGK_{D107A}) in the transfected cells. The latter was confirmed to have near to zero enzyme activity (manuscript in preparation). To increase the substrate (*O*-GlcNAc) concentration we treated the cultures with PUGNAc (100 μ M), a potent and nontoxic inhibitor of OGA, for 2 hr before harvest [11]. About 70%-confluent cultures were transfected with above mentioned plasmids at the efficiency of ~50% and further incubated for 2 more days. The cytosolic fractions were used for phospho-signal analysis using the ProQ diamond stain. The phospho-stain images of one-dimensional (1D) SDS-gel were shown in Fig. 3. Several distinct bands could be identified that show phospho-signals (Fig. 3, arrows). However, the signals were generally smeared throughout the lane. In comparison with the control culture, the WT-NAGK overexpressed one did not show any apparently stronger band with phospho-signal.

Since it the 1D SDS-gel did not show any protein bands with differential phospho-signals between control and sample cultures, we employed 2D-PAGE, which is more sensitive. The overall images of Coomassie Brilliant Blue R-250 (CBB) stains of pDsRed2- NAGK_{D107} or -NAGK_{WT} were shown in Fig. 4A (upper panels). To visualize the phospho-signals the same gels were stained with ProQ Diamond

Fig. 3. One-dimensional (1D) SDS-PAGE showing phospho-signal profiles. Culture extracts of HEK293T cells, which were transfected with either pDsRed2-NAGK_{WT} (WT) or -NAGK_{D107A} (D107), were separated in a 12% SDS-gel and stained with ProQ diamond phosphoprotein staining solution. Some distinctive bands were marked with arrows. Note that increase or decrease between the two cultures is not apparent with the smear background. Molecular sizes were shown at far left in kDa.

phosphostain solutions. The overall phospho-signal intensity was slightly higher in pDsRed2-NAGK_{WT}-transfected cultures than the pDsRed2-NAGK_{D107A}-transfected one (Fig. 4A,

Fig. 4. (A) Two-dimensional (2D) SDS-PAGE showing phospho-signal profiles. Culture extracts as per Fig. 3 were separated in 2D SDS-gels, and stained with either Coomassie Brilliant Blue (CBB) or ProQ diamond phosphoprotein staining solution (ProQ). Some spots exhibiting apparent increase in phospho-signals were marked with arrowheads. Molecular sizes (kDa) and isoelectric points (pI) were shown at far left and bottom, respectively.

(B) Enlarged images of the selected 7 spots. Digitized images were analysed with the PDQuest (version 7.0, BioRad) software. Seven protein spots (arrows) were selected for the significant expression variation deviated over two fold in its expression level compared with control.

lower panels). Some spots with increased phospho-signals could be identified with naked eyes (arrowheads). However, when quantitative analysis of digitized images was carried out using the PDQuest (version 7.0, BioRad) software, we could find 31 spots that exhibited either increased or decreased phospho-signals (Table 1). Among them 15 spots showed increased phospho-signals. Eliminating some spots showed drastic increase or decrease in the protein amounts (CBB stain), we selected 7 out of the remaining spots (underlined with broken lines). The CBB and ProQ stain images were shown in Fig. 4B. Again, increase in the phospho-signal was evident in the naked eyes for 5 spots (704, 808, 1701, 7504, 8501), and the other 2 spots (5105, 5202) were difficult to notice the signal difference with the naked eyes.

Since the digitized images revealed significant increases in the signal, we carried out protein identification of these 7 spots.

The NAGK-mediated phospho-signal increased spots are *O*-GlcNAc proteins

Among the 7 spots, the ID's of 6 spots could be determined by peptide mass fingerprinting (PMF) of the trypsin peptides by MS and the search program MASCOT (Table 2). To our surprise 4 of them were GlcNAcylated, and one (spot# 5105) was glycosylated. To strengthen the data credibility the 4 spots were duplicates of the same protein ID's (704 and 1701; HSP90 and 7504 and 8501, enolase 1). Definite confirmation for the presence of *O*-GlcNAc-P requires ver-

Table 1. Quantitative comparison of the protein spots

Spot#	MW	PI	CBB		ProQ	
			D107A	WT	D107A	WT
703	81.46	4.08	-	1056.93	-	723.97
704*	82.14	4.14	1644.87	805.13	617	2212.47
706	82.25	4.19	748.61	181.49	1117.66	-
707	82.88	4.30	1039.79	-	978.48	-
805	90.03	4.08	-	2202.06	-	1453.98
808*	90.74	4.13	1526.6	836.26	173.72	384.97
1701*	83.57	4.58	4065.79	6523.5	4553.08	10151.07
1703	81.51	4.46	1414.92	-	1197.5	-
1704	81.82	4.37	1012.39	-	1030.66	-
2303	32.57	4.93	144.94	78.42	54.06	393.76
3501	49.02	5.01	-	6350.9	-	275.58
4107	13.13	5.58	2632.73	1188.27	143.7	-
4108	14.33	5.62	-	295.07	1166.8	1022.57
4404	36.31	5.51	1076.68	15.47	416.43	-
5105*	15.38	5.61	563.2	301.01	150.13	310.87
5202*	19.66	6.23	1240.7	328.87	133.7	207.24
5302	35.51	5.88	598.37	396.11	387.53	161.29
5703	76.35	5.73	1457.33	-	37.03	-
6304	34.82	6.99	742.78	1395.43	766.95	82.9
6306	34.86	6.81	3753.41	2.87	1253.78	490.46
6510	44.61	6.97	2849.26	780.22	640.16	393.76
6511	44.98	6.73	3918.6	62.77	978	-
6701	67.46	6.99	2675.1	8173.21	789.74	538.83
6702	67.59	6.79	2484.96	-	510.28	-
7104	14.39	7.38	347.01	199.34	447.07	94.7
7303	33.59	8.00	59.32	348.01	239.5	1458.53
7504*	44.74	7.82	1190.17	2264.38	-	373.04
7801	117.83	7.65	-	7380.43	-	4237.29
8305	31.45	8.16	-	62.29	-	471.62
8501*	44.44	8.03	3131.49	2154.29	-	240.85
9305	31.26	9.63	-	246.79	-	367.79

*The seven spots selected for ID determination. -, Non-detetable.

Table 2. The protein ID and O-GlcNAc modification

Spot#	Protein ID	O-GlcNAcylation
704	HSP 90-beta	+
808	heat shock protein gp96 precursor	*
1701	HSP 90-beta	+
5105	dUTP nucleotidohydrolase, mitochondrial isoform 2	?
5202	glutathione S-transferase P	?
7504	Chain A, Crystal Structure Of Human Enolase 1	+
8501	Chain A, Crystal Structure Of Human Enolase 1	+

+, O-GlcNAc modified; *, glycosylated; ?, not known

ification of m/z 284 in MS spectrum. Unfortunately, due to our limited technical supports we could not show m/z 284. The Ser⁴³⁴ and Ser⁴⁵² of Hsp90 β and the Ser⁴⁶¹ of Hsp90 α are O-GlcNAcylated [24]. Ser⁴⁵² is also a phosphorylation site [4]. Both sites are located in the M-segment of Hsp90 which is thought to be essential for client protein binding. O-GlcNAc modification of enolase 1 (ENO1) was indicated by immunoprecipitation with anti-GlcNAc [29], isolation using helix pomatia agglutinin lectin binding and immunoblotting with anti-GlcNAc and anti-ENO1 antibodies [25]. In addition to its innate glycolytic function, ENO1 plays an important role in several biological and pathophysiological processes including hypoxia tolerance through nonglycolytic mechanisms, progression of tumors, neuroblastoma, and lung cancer (recently reviewed in [5]). Rabbit muscle enolase was reported to be phosphorylated by PKC [23]. O-GlcNAcylation of HSP90 and ENO1 would confer more diverse control over these proteins.

Possible effect of O-GlcNAc-P on signal transduction

Accumulating data suggest that O-GlcNAcylation opposes the effect of phosphorylation [15]. The cycling of two post-translational modifications on the same polypeptide provides the cell with a rapid response mechanism to fine tune protein interactions and functions [15]. It was reported that NAGK is able to phosphorylate O-GlcNAc modified proteins *in vitro* (M. Berger, unpublished observation [2]). However, no phosphorylation of the c-myc Thr58-O-GlcNAc peptide was observed in the NMR experiments [2]. They speculated that two spacious prolines surrounding the threonine with the bound O-GlcNAc may hinder the binding of the O-GlcNAc to NAGK. However, the presence of O-GlcNAc-P was indeed verified on AP180 [9]. One thing to note is that our proteomic approach revealed only 7 proteins which were increased in phospho-signals by NAGK even with highly sensitive digitized analysis. It is possible

that very inefficient phosphorylation is the reason. However, this possibility is very low because the reaction took place in the cell. A more reasonable interpretation would be that it well represents the *in vivo* situation. As we assumed in the initial stage of this work only some select O-GlcNAc moieties in appropriate 3D molecular environments without steric hindrance would serve as substrates for NAGK, and the present data support this assumption. The fact that two duplicate spots (two HSP90 β and two ENO1), which were spatially separate in the 2D-PAGE, were selected is a strong indication for both the reliability of our procedure and for steric limitation on the O-GlcNAc. Addition of another post-translational modification (i.e., O-GlcNAc-P) to the pre-existing cycle of O-GlcNAcylation and phosphorylation would provides the cell with a rapid response mechanism for generating enormous molecular diversity to fine tune cellular signal transductions at the analog level. Therefore, our present data support that O-GlcNAc is not a terminal modification.

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초록 : 프로테오믹스를 이용한 N-아세틸글루코사민 인산화효소 기질단백질의 동정

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단백질 번역 후 *O*-GlcNAc 수식은 단백질 조절의 새로운 기전으로 대두되고 있다. 전통적인 당수식과 달리 *O*-GlcNAc 수식은 단 한번의 *O*-GlcNAc 전달로 이루어지며, 핵 및 세포질단백질 모두에 수식될 수 있다. *O*-GlcNAc은 이 분자를 끝으로 하는 최종수식으로 생각되어 왔으나, 최근의 논문(J Proteome Res. 2011 10:2725-2733)은 AP180 단백질에 *O*-GlcNAc-P가 존재함을 보고하였다. 이 논문은 *O*-GlcNAc-P가 일반적인 단백질수식인지에 대한 중요한 질문을 던진다. 이에 답하고자 저자들은 HEK293T 세포에 *O*-GlcNAc 인산화효소 NAGK를 DsRed2에 연결한 DsRed2-NAGK_{WT} 혹은 효소활성이 없는 돌연변이 NAGK를 표현하는 DsRed2-NAGK_{D107A}를 표현시키고, 단백질 추출물을 얻어 2D-PAGE로 분리한 후 인산화 정도를 측정하여, NAGK_{WT}에 의하여 인산화가 증가되는 15개의 단백질 스폿을 선별하였다. 이 가운데 7개 스폿을 동정한 결과 2개의 스폿은 *O*-GlcNAc 수식 단백질인 HSP90 β , 다른 2개의 스폿도 *O*-GlcNAc 수식 단백질인 ENO1로 동정되었으며, 나머지(dUTP nucleotidohydrolase mitochondrial isoform 2, glutathione *S*-transferase P, grp94)는 *O*-GlcNAc 수식 여부를 아직 모르는 단백질이었다. NAGK에 의하여 *O*-GlcNAc 단백질의 인산화가 증가된다는 사실은 *O*-GlcNAc이 인산화되어 *O*-GlcNAc-P로 수식됨을 시사하며, 따라서 본 연구의 결과는 *O*-GlcNAc이 최종 수식이 아님을 지지한다.