Dynamic Interplay between O-Linked N-Acetylglucosaminylation and Glycogen Synthase Kinase-3-dependent Phosphorylation*

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O-GlcNAcylation on serine and threonine side chains of nuclear and cytoplasmic proteins is dynamically regulated in response to various environmental and biological stimuli. O-GlcNAcylation is remarkably similar to O-phosphorylation and appears to have a dynamic interplay with O-phosphate in cellular regulation. A systematic glycoproteomics analysis of the effects of inhibiting specific kinases on O-GlcNAcylation should help reveal both the global and specific dynamic relationships between these two abundant post-translational modifications. Here we report the O-GlcNAc perturbations in response to inhibition of glycogen synthase kinase-3 (GSK-3), a pivotal kinase involved in many signaling pathways. By combining immunoaffinity chromatography and SILAC (stable isotope labeling with amino acids in cell culture)-based quantitative mass spectrometry, we identified 45 potentially O-GlcNAcylated proteins. Quantitative measurements indicated that at least 10 proteins had an apparent increase of O-GlcNAcylation upon GSK-3 inhibition by lithium, whereas surprisingly 19 other proteins showed decreases. O-GlcNAcylation changes on a subset of the proteins were confirmed by follow-up experiments. By combining a new O-GlcNAc peptide enrichment method and β-elimination followed by Michael addition with DTT, we also mapped the O-GlcNAc site (Ser-55) of vimentin, which showed an apparent increase of O-GlcNAcylation upon GSK-3 inhibition by lithium, whereas surprisingly 19 other proteins showed decreases. O-GlcNAcylation changes on a subset of the proteins were confirmed by follow-up experiments. By combining a new O-GlcNAc peptide enrichment method and β-elimination followed by Michael addition with DTT, we also mapped the O-GlcNAc site (Ser-55) of vimentin, which showed an apparent increase of O-GlcNAcylation upon GSK-3 inhibition. Based on the MS data, we further investigated potential roles of O-GlcNAc on host cell factor-1, a transcription co-activator, and showed that dynamic regulation of O-GlcNAcylation on host cell factor-1 influenced its subcellular distribution. Taken together, these data indicated the complex interplay between phosphorylation and O-GlcNAcylation that occurs within signaling networks. Molecular & Cellular Proteomics 6: 1365–1379, 2007.

O-GlcNAc is a dynamically regulated post-translational modification (PTM), in which a β-N-acetylglucosamine moiety is attached to hydroxyl side chains of serine or threonine residues of proteins by O-GlcNAc-transferase (1) and removed by β-N-acetylglucosaminidase (O-GlcNAcase) (2). O-GlcNAc is ubiquitous on nuclear and cytoplasmic proteins in all multicellular eukaryotes (3). Dynamic O-GlcNAcylation plays critical roles in signal transduction (4), transcriptional control (5, 6), cell cycle regulation (7), protein degradation (8), neurodegeneration (9), and stress responses (10). Abnormally regulated O-GlcNAcylation has been found in diseases such as diabetes (11) and Alzheimer disease (12).

The role of O-GlcNAcylation in signal transduction is at least in part related to its competitive interplay with O-phosphorylation. Some of the known O-GlcNAc sites are the same as or adjacent to phosphorylation sites. For example, O-GlcNAc is reciprocal to O-phosphorylation on the C-terminal domain of RNA polymerase II (13), Thr-58 of c-Myc (14), Ser-16 of murine estrogen receptor-β (15), endothelial nitric-oxide synthase (16), and SV40 large T antigen (17). The “yin-yang” hypothesis (18) postulates that, on at least some proteins, O-GlcNAc functions in the signaling cascades in a manner directly competitive with O-phosphorylation. Previous studies showed that globally increased phosphorylation levels induced by okadaic acid (a phosphatase inhibitor) led to decreased O-GlcNAcylation in certain cell lines (19). On the other side, globally increased O-GlcNAc levels induced by a competitive O-GlcNAcase inhibitor, O-(2-acetamido-2-deoxy-o-glucopyranosylidene)-amino-N-phenylcarbamate (PUGNAc), led to insulin resistance in adipocytes and reduced activation of Akt by phosphorylation (4).

Although O-GlcNAc has been recognized as a regulator for stimulus responses similar to O-phosphorylation, its specific relationships and global interplay with O-phosphorylation re-
main largely unknown. Due to the very limited number of O-GlcNAc sites that have been mapped so far, a consensus sequence for O-GlcNAcylation remains elusive. However, many known O-GlcNAc attachment sites are similar or identical to those used by serine/threonine kinases for phosphorylation, particularly by the proline-directed kinases. We hypothesized that inhibition of a specific kinase would cause a perturbation to O-GlcNAcylation. We chose to inhibit GSK-3 for the following reasons. 1) Several of the mapped O-GlcNAc sites are also sites for GSK-3 phosphorylation. 2) GSK-3 is as an evolutionarily conserved Ser/Thr kinases, and it is one of the few kinases that are constitutively active in resting cells. 3) GSK-3 is a key component of many intracellular signaling pathways in most of which O-GlcNAc-modified proteins are involved. 4) GSK-3 has a wide array of substrates, including cytoskeletal proteins, metabolic enzymes, cell cycle regulators, and many transcription factors (for reviews, see Refs. 20 and 21).

Recent advances in mass spectrometry have made MS-based quantitative proteomics a valuable tool for characterizing signaling pathways (for a review, see Ref. 22). Its application includes not only identifying novel components in the pathways but also delineating signaling cascades. MS-based quantitative proteomics generally involves either metabolically incorporating or chemically labeling a mass tag (usually stable isotopes) into proteins of interest. Stable isotope labeling with amino acids in cell culture (SILAC) has been successfully used to profile the complex phosphoproteome, such as identifying substrates of tyrosine kinases in the insulin signaling pathway (23) and modeling a Her2/neu signaling network (24). Most previous work has focused on the study of phosphorylation. Based upon these advances, we used the SILAC method to profile the complex O-GlcNAc proteome and its dynamic regulation upon stimulation of cells by certain stimuli.

Here we used lithium to selectively inhibit GSK-3 in cells and then isolated the O-GlcNAc subproteome by O-GlcNAc-specific immunopurification. Using SILAC and a mass spectrometric approach, we successfully identified 45 potentially O-GlcNAcylated proteins. Quantitative measurements indicated that 10 proteins showed increased O-GlcNAcylation and 19 showed decreased O-GlcNAcylation upon GSK-3 inhibition. Combining a newly developed glycoprotein detection method and \( \beta \)-elimination followed by Michael addition with DTT (BEMAD), we also mapped one O-GlcNAc site (Ser-55) on the intermediate filament protein vimentin. Finally we investigated the potential roles of O-GlcNAc on a transcription co-activator, host cell factor-1 (HCF-1), which showed significantly decreased O-GlcNAcylation upon GSK-3 inhibition. Taken together, these results established this approach as an efficient and reliable method to globally monitor meaningful changes of O-GlcNAcylation in response to acute biological stimuli and to delineate the interplay between O-GlcNAc and O-phosphorylation in specific signaling pathways.

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**MATERIALS AND METHODS**

**Reagents and Antibodies—**All chemicals were obtained from Sigma except as noted. General anti-O-GlcNAc antibody (CDT110.6) is a mouse IgM monoclonal antibody antigen-purified from ascites (25). Rabbit polyclonal antibodies against HCF-1, N18 (HCF-1\(_N\)) and H12 (HCF-1\(_H\)), were kind gifts from Dr. Winship Herr (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). Mouse monoclonal anti-GSK-3\(\beta\) was obtained from BD Transduction Laboratories. Rabbit polyclonal anti-HSP90\(\alpha\) was from Abcam (Cambridge, MA). Mouse monoclonal anti-vimentin, anti-\(\alpha\)-tubulin, and rabbit polyclonal anti-actin were from Sigma. Mouse monoclonal anti-Rb, anti-lamin A/C, and anti-\(\beta\)-catenin were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against phospho-GSK-3\(\beta\) (Ser-9) was from Cell Signaling Technology, Inc. (Danvers, MA). Mouse polyclonal anti-heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 was from GeneTex (San Antonio, TX). Mouse monoclonal anti-NF45 was obtained from Abnova Corp. All horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences except that fluorescently labeled secondary antibodies were from Molecular Probes (Eugene, OR). Alkaline phosphatase was from New England Biolabs (Ipswich, MA).

**Cell Culture and Treatments—**COS7 cells were grown in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) supplemented with 10% (v/v) dialyzed fetal bovine serum and 1% (v/v) penicillin-streptomycin. Before lithium or AR-A014418 (Calbiochem) treatment, cells were grown to 80% confluence and serum-starved for 18 h. For PUGNAC treatment, 200 \(\mu\)M PUGNAC (Carbogen) was added to medium during serum starvation and resupplemented at the 9th h. GSK-3\(\beta\) knockdown was performed with GSK-3\(\beta\) siRNA reagents (Upstate, Charlottesville, VA) following the manufacturer’s instructions.

**Peptide Synthesis—**A 19-amino acid peptide (CMASAVSPANL-PAVLQPR) containing the N-terminal 18-amino acid peptide of HCF-1 (as underlined, antigen used to generate N18 antibody (26)) was synthesized by the Synthesis and Sequencing Core Facility (Department of Biological Chemistry, The Johns Hopkins University School of Medicine).

**Immunoprecipitation and Western Blotting—**For O-GlcNAc immunoprecipitation, antigen-purified CTD110.6 was bound and cross-linked to agarose-conjugated anti-IgM (Sigma) by using dimethyl pimelimidate and borate. Small scale O-GlcNAc immunoprecipitation was performed in Eppendorf tubes, and the bound O-GlcNAc proteins were eluted by boiling in 2× Laemmli sample buffer. Large scale O-GlcNAc enrichment for SILAC experiments was performed in a column with a 6-mi bed volume, and bound proteins were eluted by 1 M GlcNAc. Immunoprecipitations with other antibodies were performed following the antibody manufacturers’ instructions. For all Western blots, membranes were incubated overnight with primary antibody diluted in TBS containing 0.1% (v/v) Tween 20 and 3% (w/v) bovine serum albumin. Films were developed with ECL reagents and Hyperfilm (Amersham Biosciences).

**Two-dimensional Gel Electrophoresis—**COS7 extracts were soaked overnight into 11-cm IPG Ready-Strips with pH range 3–10 (Bio-Rad) and then subjected to isoelectric focusing for 7 h. Upon completion, the IPG strips were incubated for 10 min each in equilibration buffers (375 mM Tris, 6 M urea, 2% SDS, 20% glycerol) containing 130 mM DTT or 135 mM iodoacetamide. The strips were then sealed on top of a 10% SDS-polyacrylamide gel with 10% agarose and electrophoresed at 100 V.

**Confocal Microscopy and Image Processing—**COS7 cells were plated on Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY) coated with poly-D-lysine. Upon harvest, cells were washed twice with cold PBS containing 1 mM Mg\(^{2+}\) and then fixed with 3% (w/v) paraformaldehyde. After quenching in PBS/Mg\(^{2+}\) containing 100 mM glycine, the cells were permeabilized in PBS/Mg\(^{2+}\) containing...
0.5% (v/v) Triton X-100 for 10 min and washed twice in PBS/Mg\(^{2+}\). The slide was blocked for 1 h by PBS/Mg\(^{2+}\) containing 5% (w/v) BSA and incubated for 3 h at room temperature with primary antibody diluted in the same buffer containing 3% (w/v) BSA. After extensive washing, the slide was incubated for 1 h with fluorescently labeled secondary antibody. After extensive washing, the slide was stained by propidium iodide (1 μg/ml) for 30 s. Cells were washed and mounted. Fluorescent images were taken by using an UltraView confocal microscope equipped with a Zeiss lens at the Microscopy Core Facility (Department of Cell Biology, The Johns Hopkins University School of Medicine). Cytoskeletal fluorescence was quantitated using IPLab software (BD Biosciences).

**Silac, Trypsin Digestion, and LC-MS/MS—Preadapted COS7 cells** were grown in Dulbecco’s modified Eagle’s medium (4.5g/liter glucose, free of arginine) supplemented with 10% diized fetal bovine serum, 1% penicillin-streptomycin, and 86 mg/liter L-[\(^{13}C_6\)]-arginine (Cambridge Isotope Laboratories, Andover, MA). A control sample was grown in the same medium but with regular L-arginine. After treatment, cells were harvested and Nuc/Cyt preparation was performed. Equal amounts (10 mg) of Nuc/Cyt lysates from both control and lithium-treated samples were pooled together, and O-GlcNAc enrichment was performed by immunoaffinity chromatography. Eluted O-GlcNAc proteins were precipitated by TCA. Pellets were boiled in 2× sample buffer, resolved on a 15 × 15 cm SDS-polyacrylamide gel, and stained with Coomassie G-250. The gel lane was cut into slices, washed, reduced by 0.1 M DTT, alkylated by 55 mM iodoacetamide, and digested overnight by trypsin (Promega, Madison, WI). Tryptic peptides were extracted by 5% formic acid, 50% acetonitrile; dried down using a speed vacuum; resuspended in 1% formic acid; and subjected to LC-MS/MS using a QSTAR Pulsar mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with an on-line autosampler and a reverse-phase nano-HPLC system (Agilent 1100 series, Agilent Technologies, Palo Alto, CA). The analytic column had an inner diameter of 75 μm and was packed with a 10 cm of C\(_{18}\) resin (Vydac, Columbia, MD). The flow rate in the column was set at 200 nl/min.

**Mapping Sites of O-GlcNAcylation**—Immunoprecipitated vimentin was in-gel digested and then treated by alkaline phosphatase for 2 h. Enzymatic labeling, biotinylation, strong cation exchange, and avidin enrichment of O-GlcNAc-modified peptides were performed sequentially as shown in Fig. 5A. BEMAD treatment of avidin-enriched peptides was essentially as described earlier (27). Mutated Gal-T1(Y289L) and UDP-GalNAz (azido-modified galactose) were from the Click-iT GlcNAc enzymatic labeling kit, and biotin alkaline was from the glycoprotein detection kit (both from Molecular Probes). Cation exchange and avidin chromatography (Applied Biosystems) were performed according to the manufacturer’s instructions. BEMAD-treated samples were analyzed by a Finnigan LTO mass spectrometer (ThermoFinnigan) coupled with a nano-2DLC pump (Eksigent Technologies, Dublin, CA). The spray voltage was set at 2.1 kV. Flow rate was 300 nl/min with splitless direct injection. The LTO was programmed to record a full precursor scan (350–1800 \(m/z\)) followed by fragmentation and MS/MS scans of the top eight most intense ions.

**Identification of H12-reactive Protein Bands (HCF-1)—HCF-1 was purified by double immunoprecipitation. 8 mg of COS7 nuclear extract was first immunoprecipitated with O-GlcNAc antibody. Bound proteins were eluted from the column with 0.5 M GlcNAc. The eluant was then immunoprecipitated with HCF-1 antibody (H12). The final eluant was resolved in duplicate by SDS-PAGE. Half of the gel was transferred to a nitrocellulose membrane and blotted with H12 antibody. The other half was stained with Coomassie G-250. Protein bands corresponding to the H12-reactive bands on the Western blot film were cut out and digested with trypsin. The tryptic peptides were resuspended in 1% formic acid and manually loaded into a 7-cm C\(_{18}\) column by a high pressure bomb. The peptides were eluted with a 40-min gradient (running from 5 to 95% acetonitrile) delivered by a Magic 2002 reverse-phase HPLC system (Michrom BioResources, Auburn, CA) and injected into an LCQ classic ion trap mass spectrometer (ThermoElectron, Waltham, MA) equipped with a nanoelectrospray source (Proxeon).**

**Data Processing and Analysis—**Mass spectrometric data from the QSTAR were analyzed by the AnalystQDS software (Agilent Technologies). The generated peak list files were merged into a master file, which was then searched by Mascot (Matrix Science, Boston, MA) against the non-redundant National Center for Biotechnology Information database NCBI (2,273,764 entries; December 15, 2005) and Swiss-Prot (208,005 entries; February 9, 2006). The search parameters were as follows: maximum allowed missed cleavages, 1; fixed modifications, carbamidomethyl (Cys); variable modifications, oxidation (Met), phosphorylation (Ser/Thr), and O-GlcNAc (Ser/Thr); monoisotopic peptide tolerance, ≤0.3 Da; MS/MS tolerance, ≤0.15 Da. -Fold changes of peptides were quantitated and verified both manually and by using MSQuant software. Spectra from the LCQ and LTQ were analyzed by BioWorks software (Version 3.3, Thermo Electron). The database search was performed against NCBI (3,454,046 entries; March 14, 2006) using the same parameters as described earlier except that peptide tolerance was set at ±1.5 Da, MS/MS tolerance was set at ±0.6 Da, and that only DTT (Ser/Thr) was used as variable modification in the case of vimentin site mapping. For all protein identifications, at least two matched peptides were required. In the case of only two peptides matched, one of the peptides was required to have a Mascot score of at least 40. All spectra used for quantitation are available upon request.

**RESULTS AND DISCUSSION**

**Lithium Treatment Inhibits GSK-3 and Is Accompanied by O-GlcNAc Perturbations**—Lithium is known to potently and selectively inhibit GSK-3 (28). Because constitutively active GSK-3 phosphorylates β-catenin and directs it to degradation by the proteasome, lithium treatment leads to accumulation of β-catenin (29). It is also well documented that lithium leads to inhibitory autophosphorylation of GSK-3β on Ser-9 (30). In this study, we used lithium to inhibit GSK-3 in COS7 cells. After starvation in serum-free medium, COS7 cells were treated with 25 mM lithium chloride for up to 2 h. Western blots with antibodies against phospho-GSK-3β (Ser-9) and β-catenin showed gradually increasing amounts of these two molecules in cell lysates (Fig. 1, A and B), reflecting the inhibition of GSK-3 in COS7 cells. To see whether GSK-3 inhibition is accompanied by changes in global O-GlcNAcylation, we selected 1-h lithium-treated and control cell lysates and resolved them by two-dimensional gel electrophoresis. Western blots with O-GlcNAc antibody showed significantly different patterns of O-GlcNAcylation between control and lithium-treated COS7 cells (Fig. 1C, upper panel). As a specificity control for the O-GlcNAc antibody, Western blots with the same antibody but in the presence of 1 M GlcNAc showed very few reactive spots (Fig. 1C, lower panel).**

**O-GlcNAcylation Profiling upon GSK-3 Inhibition**—Quantitative mass spectrometry based on SILAC was used to profile GSK-3 inhibition-induced O-GlcNAcylation changes. In the SILAC-MS experiment, lysates of control and labeled cells are pooled, go through all the experimental procedures as a mix-
ture, and are quantitated in the last MS step. The quantitation accuracy is only limited by the peptide signals observed on the mass spectrometers, which in most cases are of high resolution and accuracy. Moreover quantitation of one single protein is usually the average of quantitation of multiple peptides. So compared with 2-DE-based quantitative methods, which usually have substantial gel-to-gel variation, the SI-LAC-MS method is more unbiased and less prone to human errors.

The overall approach (Fig. 2A) used in this study is a revised form of the SILAC-MS method reported by Ibarrola et al. (23). COS7 cells were either grown in medium supplemented with either normal arginine (unlabeled) or medium with \( \text{L-[^{13}C_6^{15}N_4]} \) arginine (labeled). Each heavy arginine introduces a 10-Da shift in the mass spectrum. After 18 h of serum starvation, heavy isotope-labeled COS7 cells were treated with 25 mM LiCl for 1 h, whereas unlabeled COS7 cells were left untreated as a control. After harvest, Nuc/Cyt preparation was performed. Equal amounts of Nuc/Cyt lysates were pooled and precleared. Then the lysate was subjected to immunoaffinity enrichment using a column packed with immobilized CTD110.6 antibody. Elutant was resolved by SDS-PAGE, stained with Coomassie G-250, and digested with trypsin. A portion of the COS7 lysate, flow-through, and eluant was saved and used to demonstrate that the antibody column effectively enriched O-GlcNAc proteins (Fig. 2B). Tryptic peptides were detected and analyzed by a QSTAR Pulsar hybrid LC-MS/MS system run in a data-dependent acquisition mode. The top three most abundant ions (+2 or +3) in each survey scan were selected for CID. The masses of resulting fragments were analyzed by the TOF analyzer and used to sequence the parental peptides (Fig. 2C, lower panel). After the database search, the extracted ion chromatogram of each peptide of interest (±0.02 Da) was generated (Fig. 2C, upper panel), and peaks were verified by their elution time. Then the MS spectrum containing each selected peptide was then inspected for a companion peptide 10 Da apart (Fig. 2C, middle panel). The relative abundance of unlabeled to labeled peptides was calculated as the ratio of the peak areas.

Using this approach, we identified 45 potential O-GlcNAcylated proteins. All the identified proteins had at least two sequenced different peptides, and in the case of only two matched peptides, one of the peptides was required to have a Mascot score of at least 40. If 32 had been used as the
FIG. 2. Schematic for SILAC experiment and sample MS spectra. A, workflow of the SILAC experiment. B, O-GlcNAcylated proteins were highly enriched by immunooaffinity chromatography. Equal amounts of total proteins (1 μg) from starting material, the flow-through, and eluant were blotted with O-GlcNAc antibody. C, extracted ion chromatography (XIC), ion pairs, and MS/MS sequencing (b ions and y ions as indicated) of a sample vimentin peptide, FADLSEAANR. 1D, one-dimensional; WB, Western blot.
### TABLE I

**GSK-3 inhibition is accompanied with differentially regulated O-GlcNAcylation: a list of identified proteins and fold changes**

Red, significantly increased O-GlcNAcylation; blue, significantly decreased O-GlcNAcylation. Numbers in square brackets are reference citations.

| Protein name                                      | Accession       | Matched Peptide | Fold change | Peptide for quantification | O-GlcNAc? |
|---------------------------------------------------|-----------------|-----------------|-------------|----------------------------|-----------|
| 1. Heat shock protein 27KD                        | NP_00103295.1   | 5               | 3.14        | 4                          | Yes *     |
| 2. Tubulin beta                                   | NP_001060.1     | 15              | 2.37        | 10                         | Yes [65]  |
| 3. Chaperone protein HSP90 beta                   | AAF82792.1      | 6               | 2.0         | 4                          | Yes [27]  |
| 4. Heat shock cognate 71KD protein 8              | P19378          | 7               | 2.0         | 4                          | Yes [65]  |
| 5. Vimentin                                       | NP_003371.1     | 20              | 1.61        | 15                         | Yes †     |
| 6. Elongation factor-I alpha                      | NP_001393.1     | 6               | 1.49        | 4                          | Yes [27]  |
| 7. DNA-binding protein A (cold shock domain protein A) | Q62764       | 5               | 1.43        | 4                          |           |
| 8. Y-box transcription factor                     | P27817          | 3               | 1.42        | 3                          |           |
| 9. Arin beta                                      | NP_001092.1     | 18              | 1.42        | 12                         |           |
| 10. Lactase dehydrogenase A                       | Q9IE24          | 3               | 1.38        | 2                          |           |

* No change/non-significant change in O-GlcNAcylation

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* Whelan, S., and Hart, G., unpublished data.
† Zeidan, Q., and Hart, G., unpublished data.
‡ This study.
cutoff score, then the number of identified proteins would be around 100. Some of the identified proteins were already known to be O-GlcNAcylated, whereas some were novel (Table I). 10 identified proteins showed apparent major increases in O-GlcNAcylation (ratio ≥ 1.3) upon LiCl treatment. 19 proteins showed major decreases (ratio ≤ 0.7) (Table I and Fig. 3A). In these experiments, we were unable to quantitate O-GlcNAc changes on four proteins due to lack of an arginine residue in the sequenced peptides. To confirm the SILAC quantitation, we next immunoprecipitated a subset of the identified proteins (hnRNP C1/C2, vimentin, NF45, and HSP90β) and performed Western blotting with anti-O-GlcNAc antibody. The apparent changes in O-GlcNAcylation were in agreement with the SILAC data (Fig. 4), indicating the reliability of MS quantitation. Moreover Western blots with O-GlcNAc antibody in the presence of competing GlcNAc yielded no signal (Fig. 4), confirming that the proteins were indeed O-GlcNAcylated. In summary, the follow-up experiments showed proof of the effectiveness of the overall approach in identifying potential O-GlcNAc proteins and quantitating O-GlcNAcylation perturbations. Compared with the 2-DE Western blot data (Fig. 1C), it seemed that only a portion of proteins that underwent changes in their O-GlcNAcylation upon GSK-3 inhibition were actually identified and quantitated with the

Fig. 3. Identification and quantitation. A, summary of -fold changes quantitated by SILAC. B, functional distribution of identified proteins.
SILAC-MS approach. This is not unexpected considering the limitations of immunoaffinity enrichment using O-GlcNAc antibody. The O-GlcNAc moieties of some O-GlcNAcylated proteins are buried when the proteins fold or multimerize (31). These proteins can be recognized by O-GlcNAc antibody when the proteins are fully denatured (such as in Western blotting) but cannot be pulled down by immunoprecipitation when they are in native states.

The 45 identified O-GlcNAc proteins fell into a variety of function groups, including cytoskeletal proteins, heat shock or chaperone proteins, RNA processing enzymes, transcription factors, ribosomal proteins, and metabolic enzymes (Fig. 3B). It is notable that proteins involved in RNA processing and transcriptional regulation represent the largest portion of the identified proteins. 11 members (C1/C2, U, R, A1, U1, K, H3 isoform b, D isoform b, A/B, A2/B1, and D-like) of the hnRNP superfamily were found to have decreased O-GlcNAcylation upon GSK-3 inhibition with -fold changes from 0.34 to 0.78. The hnRNP superfamily was first recognized as a group of chromatin-associated RNA-binding proteins. Accumulating evidence suggests that hnRNPs are involved in a variety of cellular events, such as transcription control, gene recombination, pre-mRNA splicing, mRNA trafficking, mRNA turnover, and telomere length maintenance (for reviews, see Refs. 32 and 33). Many hnRNP members are multifunctional. More interestingly, one single hnRNP can sometimes perform opposing functions. hnRNP D recognizes AU-rich motifs in the 3′-untranslated regions of cytokine and oncoprotein mRNAs and accelerates their degradation (34). In contrast, hnRNP D was also reported to stabilize the human α-globin mRNA (35), suggesting that hnRNP D is involved in both stabilization and decay of mRNAs. Another example is hnRNP K, which can act as either a transcription activator or a repressor: it binds the upstream pyrimidine-rich region of c-myc gene and promotes transcription (36), but it is also reported to be a repressor of CCAAT/enhancer-binding protein β-mediated gene activation (37). It is possible that PTMs of hnRNPs are vital for the reciprocal functions of hnRNPs. The prevailing decreases of O-GlcNAcylation on hnRNPs may indicate a major switch of hnRNP functions upon GSK-3 inhibition. To date, knowledge about the PTMs of hnRNPs remains very limited. Idriss et al. (38) showed that reversible phosphorylation of hnRNP A1, which functions in mRNA transport and splicing, regulates its binding to RNA. Ser-87 of hnRNP D was reported to be phosphorylated by cAMP-dependent protein kinase, and this “priming phosphate” enabled the subsequent phosphorylation of Ser-83 by GSK-3β. Phosphorylation of Ser-87 enhances the transactivator activity of hnRNP D, whereas phosphorylation of Ser-83 inhibits such activity, indicating the importance of PTMs to the function of hnRNP D (39). Currently hnRNP G is the only hnRNP that is known to be O-GlcNAcylated (exact site unknown) (40). Recent work documented that hnRNP G is also phosphorylated on Ser-208 (41). In the present study, hnRNP C1/C2 showed the most significant ratio (0.34) of decrease of O-GlcNAcylation upon GSK-3 inhibition, whereas total expression levels were equal (Table I, Fig. 3A, and Fig. 4A). Stone et al. (42) reported that hnRNP C1/C2 had both basal and hydrogen peroxide-inducible
phosphorylation catalyzed by casein kinase II and at least one other unknown Ser/Thr kinase. Phosphorylation of hnRNP C1/C2 regulated its binding to pre-mRNA. To elucidate the interplay of O-GlcNAc and O-phosphorylation on hnRNP C1/C2 (whether they work synergically or reciprocally), further investigation will be required, including mapping the O-GlcNAcylation site(s) and verifying whether hnRNP C1/C2 is a direct substrate of GSK-3.

Mapping O-GlcNAc Sites on Vimentin—Besides monitoring global O-GlcNAc changes, the approach described in this study also helps predict novel O-GlcNAc-modified proteins because immunoaffinity highly enriched O-GlcNAc proteins (Fig. 2B). It should be noted that due to limitations of the immunoaffinity method (specificity, indirect binding, etc.), all the identifications can only be considered as “potential” O-GlcNAc proteins before additional experiments confirm the existence of O-GlcNAc. A simple method is to perform O-GlcNAc immunoblotting with the appropriate antibody control (Fig. 4), whereas more unambiguous evidence comes from mapping the O-GlcNAc sites. Here we modified the protocols of newly developed commercial kits used for glycoprotein detection and combined them with BEMAD for O-GlcNAc site mapping. In detail, trypsin-digested peptides were first dephosphorylated, and then a mutant H9252-1,4-galactosyltransferase (Gal-T1) transferred a GalNAz to the O-GlcNAc moiety on the modified peptides. Reaction of biotin alkaline with the azido group attached a biotin tag to the GalNAz. After removing excess biotin reagent with strong cation exchange, O-GlcNAc peptides were isolated from naked peptides by avidin chromatography. The purified fraction was then subjected to BEMAD reaction, which replaced the GlcNAc-GalNAz-biotin moiety with a DTT. Mapping of the DTT site assigned the original O-GlcNAc sites (Fig. 5A). Using this method, we identified at least one O-GlcNAc site (Ser-55) on the intermediate filament protein vimentin (Fig. 5, B and C). Ser-55 resides in the hyperphosphorylated head domain of vimentin (Fig. 5D).
Phosphorylation has been extensively studied for vimentin and found to be essential for its polymerization and reorganization in cells upon external stimulation and during mitosis (for a review, see Ref. 43). So far, no phosphorylation has been reported for Ser-55. But Ser-56 is known to be dynamically phosphorylated by at least two different kinases (44, 45). Previous evidence showed that adjacent phosphorylation and phosphorylated by at least two different kinases (44, 45). In this study, we observed increased O-GlcNAcylation of Ser-55 upon GSK-3 inhibition (Table I and Fig. 4B). Combining the facts that LiCl inhibition of GSK-3 promotes cell activation and differentiation in low serum medium (46) and that Ser-56 phosphorylation cycles rapidly in response to agonists (44, 45), it is tempting to think that the increase of vimentin O-GlcNAcylation correlates with changes in vimentin phosphorylation and the overall vimentin network.

Mapping O-GlcNAc sites has been one of the major obstacles in studying protein O-GlcNAcylation. First, O-GlcNAc proteins are very substoichiometric. Second, O-GlcNAc is extremely labile. Under conditions of the SILAC experiment described in this study, probably only a very few, if any, O-GlcNAc peptides can be observed because although O-GlcNAc proteins have been enriched O-GlcNAc peptides are still substoichiometric in the pool of naked peptides. It was observed that the presence of naked peptides suppressed the ionization of O-GlcNAc peptides by at least 10-fold in the standard electrospray ionization. In addition, O-GlcNAc can be lost even at the ESI interface (47, 48). So far, almost all the O-GlcNAc site mapping studies involve using a large amount of starting materials (usually overexpressed recombinant proteins) and enrichment of O-GlcNAc at the peptide level (27, 49–51). Here we tested a method to enrich derivatized O-GlcNAc peptides by avidin affinity chromatography. Because the derivatized O-GlcNAc moiety is still labile and can be easily lost during CID before site information can be obtained, we combined this enrichment method with BEMAD reaction during which the labile GlcNAc-GalNAz-biotin moiety was replaced with non-labile DTT. Sites of DTT attachments can be easily identified by ion trap mass spectrometer. Using this method, we successfully mapped one vimentin O-GlcNAc site with a relatively small amount of starting material. We expect to apply this method to site mapping of more complex samples in the future.

O-GlcNAcylation of HCF-1 Is Dynamically Regulated and Is Influenced by Lithium Treatment—The SILAC-MS experiment demonstrated an apparent decrease (ratio of 0.22) of O-GlcNAcylation on HCF-1 (also known as HCF-C1 or C1 factor) upon LiCl treatment. HCF-1 is a family of polypeptides with molecular mass ranging from 68 to 180 kDa. The polypeptides result from site-specific proteolysis of a full-length precursor containing more than 2000 amino acids. After proteolysis, the stable N- (HCF-1α) and C-terminal (HCF-1β) fragments of HCF-1 remain non-covalently associated (52). HCF-1 is a chromatin-bound protein and was initially discovered as a cellular cofactor involved in the activation of herpes simplex virus immediate early gene expression (53). The functions of HCF-1 remain elusive. Previous work has been focused on its function as a transcription co-activator (54, 55), its roles in cell cycle progression (56), and its functions in pre-mRNA splicing (57). Little is known about the PTMs of HCF-1 except that phosphorylation of HCF-1 was reported on an HCF-1 homologue in Caenorhabditis elegans (CeHCF), and phosphorylation on HCF-1 was found to cycle rapidly during the cell cycle (56). HCF-1 interacts directly with O-GlcNAc-transferase and is heavily O-GlcNAcylated. O-GlcNAc modifications on HCF-1 have been used to partially purify HCF-1 (58), although nothing is known about the regulation or potential function(s) of O-GlcNAc on this protein. To confirm our SILAC-MS data about HCF-1, we immunoprecipitated HCF-1 with O-GlcNAc antibody and used Western blotting with anti-HCF-1C antibody (H12) to document that at least two HCF-1 C-terminal fragments significantly decreased O-GlcNAcylation upon LiCl treatment (Fig. 6A). The top band in Fig. 6A, which appeared to be the full-length HCF-1, showed an apparent increase of O-GlcNAcylation. But it was not reproducible. In contrast, Western blotting with HCF-1N antibody (N18) showed no significant change in band intensities (data not shown). Reverse immunoprecipitation demonstrated consistent results (Fig. 6B). To rule out the possibility that bands shown in Fig. 6A were the result of nonspecific binding, we purified larger amounts of HCF-1 from nuclear extract by double immunoprecipitations (see “Materials and Methods”). Samples were resolved in duplicate by SDS-PAGE. Half of the gel was transferred and blotted with H12 antibody. The other half was stained with Coomassie Blue. Western blotting showed the same pattern of bands (data not shown) as in Fig. 6A. We then trypsin-digested the two protein bands that showed the most significant decrease of O-GlcNAc (as marked in Fig. 6A by * ) and identified them by ion trap mass spectrometer. Database searches showed that they both corresponded to HCF-1 polypeptides (Fig. 6C). Next we treated COS7 cells with PUGNAc, an inhibitor of O-GlcNAcase, the enzyme that catalyzes the removal of O-GlcNAc. As shown in Fig. 6D, PUGNAc strongly increased O-GlcNAcylation on HCF-1, whereas adding LiCl after PUGNAc treatment attenuated the increase. The slight difference of band patterns shown in Fig. 6D was simply due to using a different percentage gel. Taken together, our results indicated that HCF-1 polypeptides carried cycling O-GlcNAc modifications, and these modifications were down-regulated by LiCl treatment.

Decrease of O-GlcNAcylation on HCF-1 Correlates with Cytoplasmic Accumulation of HCF-1—The subcellular localization of HCF-1 is predominantly nuclear. After site-specific proteolysis, the N- and C-terminal fragments remain non-covalently associated through two sets of interaction modules that contain fibronectin type 3 repeats (59). HCF-1C possesses a consensus nuclear localization signal located at the C terminus. Previous studies demonstrated that HCF-1C is
capable of recruiting and maintaining the majority of HCF-1 in the nucleus via its nuclear localization signal. More recent studies suggest that at least a fraction of HCF-1 shuttles between the cytoplasm and nucleus and can carry the viral transcriptional factor VP16 into the nucleus of herpes simplex virus-1-infected cells. Based on this information, we were interested to see whether O-GlcNAc influenced the subcellular localization of HCF-1. We treated cells with 25 mM LiCl for up to 2 h. Nuclear and cytoplasmic fractions were separated for each time point. To verify appropriate fractionation, we probed the fractions for α-tubulin and lamin A/C. Consistent with desired fractionation, lamin A/C was only detected in the nuclear fractions, whereas the majority of α-tubulin was in the cytoplasmic fractions. Using the same fractions, we analyzed the subcellular distribution of HCF-1. Immunoblotting with N18 antibody indicated an increasing amount of HCF-1 in the cytoplasm along the time course (Fig. 7A). Blotting with N18 antibody in the presence of excess N18 peptide (the antigen used to produce the N18 antibody) yielded no signal, confirming the specificity of the experiment. Because the majority of HCF-1 was still nuclear, the amount change of HCF-1 in the nuclei, if any, was not noticeable.

For immunofluorescence experiments, COS7 cells were serum-starved in the absence or presence of 200 μM PUGNAc, stimulated by LiCl for 2 h, and then fixed. Confocal images showed increased cytoplasmic staining by N18 antibody in LiCl-treated samples (Fig. 7B). As described earlier, LiCl caused decreased O-GlcNAcylation on some HCF-1 polypeptides. We postulate that cytoplasmic accumulation of HCF-1 is at least partially related to O-GlcNAcylation of HCF-1 because cells pretreated with PUGNAc, which increased O-GlcNAcylation on HCF-1 (Fig. 6D), did not yield cytoplasmic accumulation of HCF-1 upon LiCl treatment. Consistent with the visual interpretation, quantitative assessment of the confocal images confirmed that LiCl treatment/GSK-3 inhibition may affect expression of a number of genes by modulating the subcellular localization of HCF-1.

Decrease of O-GlcNAcylation on HCF-1 Is a Consequence of GSK-3 Inhibition—Although LiCl has been widely used as an inhibitor of GSK-3, there is argument that LiCl is likely to have other effects. Therefore, we wanted to confirm that the effect of LiCl in reducing HCF-1 O-GlcNAcylation was via inhibition of GSK-3. A more specific GSK-3β inhibitor, AR-A014418 (64), was used to treat serum-starved cells. Similar immunoprecipitation and Western blotting were performed as described earlier. Both direct and reverse immunoprecipitation indicated that AR-A014418 caused similar decreases of O-GlcNAcylation on HCF-1 (Fig. 8A). Moreover more HCF-1 polypeptides showed reduction of O-GlcNAcylation (Fig. 8A).
Fig. 7. Changes in O-GlcNAcylation on HCF-1 is accompanied with cytoplasmic accumulation of HCF-1. A, COS7 cells were treated by 25 mM LiCl for the indicated time. Cytoplasmic and nuclear fractions per treatments were separated by SDS-PAGE and blotted by HCF-1 antibody (N18) in the presence or absence of competing peptides (20 mM). Western blots with anti-α-tubulin and -lamin A/C were used as fractionation and loading controls. B, confocal images confirmed the cytoplasmic accumulation of HCF-1. COS7 cells were serum-starved for 18 h in the presence or absence of 200 μM PUGNAc and then treated with 25 mM LiCl for 2 h. Cells were fixed and stained by the indicated primary antibodies. DNA was stained by propidium iodide (PI). Green is HCF-1, Red is DNA. Arrowheads point to cytoplasmic HCF-1. C, quantitation of the confocal images (based on 60 cells per treatment). N18, synthetic peptide used to generated HCF-1 antibody (N18).
HCF-1-interacting proteins, which are also known to be O-GlcNAcylated (58). To further verify that GSK-3β inhibition could reduce O-GlcNAcylation of HCF-1, we used siRNA to knock down the GSK-3β level. As shown in Fig. 8B (left panel), siRNA significantly reduced the total level of GSK-3β. Here immunoblots against tubulin and Rb were used as loading and specificity controls. O-GlcNAc immunoprecipitation from cell lysates followed by HCF-1 blotting showed that less HCF-1 was pulled down from the GSK-3β siRNA-treated sample (Fig. 8B, right panel), which mimicked the effect of LiCl. Taken together, the data clearly indicated that LiCl reduced the O-GlcNAcylation of HCF-1 at least partially via inhibition of GSK-3β.

Conclusions—The results in the current study established the overall approach as a rapid and reliable method to globally identify and profile meaningful O-GlcNAc perturbations in response to not only specific kinase inhibition but also other biological stimuli. Because this approach generally cannot distinguish between changes in O-GlcNAcylation and protein expression levels, in some cases, especially when cells are treated for a longer period of time, simple follow-up experiments are required to check possible changes in specific protein expression levels.

Enrichment of the low abundance regulatory proteins that carry even lower stoichiometric O-GlcNAc addition is a major obstacle of studying O-GlcNAcylation. Using a pan-specific O-GlcNAc-specific antibody was proved to be an effective way to enrich O-GlcNAc proteins in the current study. However, antibodies always have recognition site accessibility issues due to steric hindrance; suffer from low affinities, especially those with small carbohydrate specificity; and arouse specificity concerns. For the same reason, proteins eluted from immunoaffinity chromatography can only be considered as potential O-GlcNAcylated proteins before further evidence is obtained to prove the existence of O-GlcNAc. As such, combining the SILAC-MS-based mass spectrometric approach with improved O-GlcNAc enrichment methods may be one of the future directions to improve O-GlcNAcomics.

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