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Simultaneously Identifying and Distinguishing Glycoproteins with O-GlcNAc and O-GalNAc (the Tn Antigen) in Human Cancer Cells

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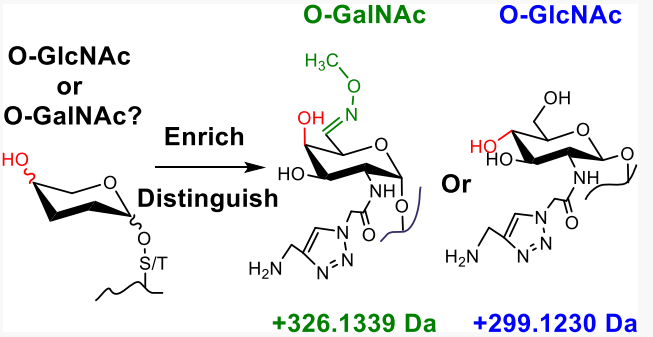
ABSTRACT: Glycoproteins with diverse glycans are essential to human cells, and subtle differences in glycan structures may result in entirely different functions. One typical example is proteins modified with O-linked *β*-*N*-acetylglucosamine (O-GlcNAc) and O-linked *α*-*N*-acetylgalactosamine (O-GalNAc) (the Tn antigen), in which the two glycans have very similar structures and identical chemical compositions, making them extraordinarily challenging to be distinguished. Here, we developed an effective method benefiting from selective enrichment and the enzymatic specificity to simultaneously identify and distinguish glycoproteins with O-GlcNAc and O-GalNAc. Metabolic labeling was combined with bioorthogonal chemistry for enriching glycoproteins modified with O-GlcNAc and O-GalNAc. Then, the enzymatic reaction with galactose oxidase was utilized to specifically oxidize O-GalNAc, but not O-GlcNAc, generating the different tags between glycopeptides with O-GlcNAc and O-GalNAc that can be easily distinguishable by mass spectrometry (MS). Among O-GlcNAcylated proteins commonly identified in three types of human cells, those related to transcription and RNA binding are highly enriched. Cell-specific features are also revealed. Among glycoproteins exclusively in Jurkat cells, those involved in human T-lymphotropic virus type 1 (HTLV-1) infection are overrepresented, which is consistent with the cell line source and suggests that protein O-GlcNAcylation participated in the response to the virus infection. Furthermore, glycoproteins with the Tn antigen have different subcellular distributions in different cells, which may be attributed to the distinct mechanisms for the formation of protein O-GalNAcylation.



INTRODUCTION

With the development of mass spectrometry (MS)-based proteomics, global and site-specific analysis of protein modifications becomes possible,[1−7] advancing our understanding of protein functions and cellular activities. However, comprehensive analysis of protein glycosylation is still extraordinarily challenging because of the diversity of glycan structures and the wide dynamic range of glycoprotein abundances.[8−10] Additionally, using bottom-up proteomics, glycopeptides are identified based on the molecular weights of the parent ions measured in full MS and the fragments in tandem MS. However, when glycopeptides possess glycans with the same chemical composition but different conformations, they may not be easily distinguished by MS.

Two types of protein O-glycosylation, i.e., O-GlcNAcylation (O-linked *β*-*N*-acetylglucosamine (O-GlcNAc)) and O-GalNAcylation (O-linked *α*-*N*-acetylgalactosamine (O-GalNAc)), are typical examples. The modified groups of O-GlcNAc and O-GalNAc have the exact same molecular weight, and both of them are bound to the hydroxyl group of the serine and threonine residues. It has been widely reported that proteins modified with O-GalNAc, i.e., the Tn antigen, are upregulated in many cancer cells and tissues, and its abundance is tightly related to cancer progression and metastasis.[11,12] Protein O-GlcNAcylation is a reversible and highly dynamic modification, and it is catalyzed by O-GlcNAc transferase (OGT) and is removed by O-GlcNAcase (OGA). This modification is crucial for many cellular events such as transcription regulation, signal transduction, and cell cycle control.[13−15] Because MS cannot readily distinguish these two types of important protein glycosylation, previously reported O-GlcNAcylated proteins from MS-based proteomics may also contain glycoproteins with the Tn antigen.

To distinguish and simultaneously profile glycoproteins with O-GlcNAc and the Tn antigen in human cancer cells, we developed a method to enrich glycopeptides with O-GlcNAc and the Tn antigen and then to distinguish them benefitting from the specificity of an enzymatic reaction prior to MS analysis. Cells were treated with a sugar analogue containing an azido group, which allowed for the selective enrichment of

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glycoproteins using bioorthogonal chemistry. After proteins were digested, the enriched glycopeptides with O-GlcNAc and O-GalNAc were distinguished by the specific enzymatic oxidation of GalNAc using galactose oxidase (GAO). The oxidized GalNAc was further derivatized by methoxyamine to generate a 27 Da mass difference with the unreacted O-GlcNAc, which enabled us to unambiguously distinguish these two types of important protein glycosylation by MS. This integrative and effective method was employed to globally and site-specifically analyze glycoproteins with O-GlcNAc and O-GalNAc in three types of cancer cells. In the biological duplicate experiments, 537 O-GlcNAcylated proteins and 103 glycoproteins modified with the Tn antigen were site-specifically identified. The O-GlcNAcylated proteins identified in all three cell lines were highly enriched with transcription and RNA binding functions. However, among O-GlcNAcylated proteins only in Jurkat cells, those involved in human T-lymphotropic virus type 1 (HTLV-1) infection were over-represented, suggesting that O-GlcNAcylation may play a role in regulating the cellular response to the retrovirus. Glycoproteins in the spliceosome and with the nuclear localization signal (NLS) are also enriched. For glycoproteins with the Tn antigen, while many of them in Jurkat cells were highly enriched in the extracellular exosome, those in MCF7 and K562 cells were mainly found in the endoplasmic reticulum (ER). This method can be extensively applied to study glycoproteins with O-GlcNAc and the Tn antigen in the biochemical and biomedical research fields, advancing our understanding of protein glycosylation.

EXPERIMENTAL SECTION

Cell Culture, Cell Lysis, and Protein Digestion. Jurkat, MCF7, and K562 cells (from American type culture collection (ATCC)) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) (Thermo) in a humidified incubator with 5.0% CO_2 at 37 °C. When cells reached ∼50% confluency, the medium was switched to DMEM containing 10% dialyzed FBS, and *N*-azidoacetylgalactosamine-tetraacylated (Ac_4GalNAz) (Click Chemistry Tools) was added to a final concentration of 250 μM. The cells were further cultured for 48 h, and then 50 μM of (1*Z*)-*δ*-lactone-2-(acetylamino)-2-deoxy-*N*-[[(phenylamino)carbonyl]oxy]-D-gluconimidic acid (*Z*-PUGNAc) (Cayman Chemicals) was added 2 h before cell harvest.

Cells were washed with phosphate-buffered saline (PBS), harvested by scraping, and centrifuged at 500*g* for 3 min and washed 3 times with ice-cold PBS. Cell pellets were lysed on an end-over-end rotor for 45 min at 4 °C in a lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH = 7.4, 150 mM NaCl, 0.5% sodium deoxycholate (SDC), 25 units/mL benzonase, and 1 tablet/10 mL ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor). The lysate was centrifuged at 4600*g* for 10 min, and the resulting supernatant was transferred to a new tube to perform the copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC) reaction. Briefly, the lysate was first added with 250 μM photocleavable (PC)-biotin−alkyne (Click Chemistry Tools), 1 mM $CuSO_4$, 5 mM Tris(3-hydroxypropyltriazolylmethyl) amine (THPTA), and 5% dimethyl sulfoxide (DMSO). After being mixed thoroughly, both freshly prepared sodium L-ascorbate and aminoguanidine hydrochloride were added to the same final concentration of 15 mM to initiate the reaction. The reaction tube was covered with aluminum foil and rotated for 2 h at room temperature. The reaction was quenched, and proteins were purified using the methanol−chloroform protein precipitation method. Proteins were digested with trypsin (protein/trypsin = 100:1) in a digestion buffer (50 mM HEPES pH = 8.6 and 1.6 M urea) at 37 °C for 16 h.

Glycopeptide Enrichment. After protein digestion, the solution was acidified by the addition of trifluoroacetic acid (TFA) to a final pH value of ∼2. Peptides were desalted using a tC18 Sep-Pak cartridge (Waters). Then, purified peptides were incubated with the high-capacity NeutrAvidin agarose resins (Thermo) at room temperature for 1 h. The sample was transferred to a spin column and washed 10 times using 100 mM PBS. The NeutrAvidin agarose resins with enriched glycopeptides were transferred to a glass vial, and glycopeptides were then eluted through the cleavage of the photocleavable linker with the UV radiation at 350 nm for 1 h at room temperature and 30 min for the second elution. The eluted peptides were desalted again, followed by being lyophilized and stored at −80 °C for further oxidation and labeling.

Optimization of Oxidation and Labeling Conditions. To optimize the reaction conditions in a real sample matrix, a synthetic glycopeptide (VGVT(*α*-O-GalNAc)ETP-NH_2) with the Tn antigen was added into peptides digested from a Jurkat whole cell lysate. For the oxidation reaction, 50 pmol of the synthetic glycopeptide with the Tn antigen and about 25 μg Jurkat peptides were oxidized in 40 μL oxidation solution containing 1 U galactose oxidase (GAO) (Innovative Research), 1.6 U horseradish peroxidase (HRP) (Sigma-Aldrich), 20 U catalase (CAT) (Sigma-Aldrich), 50 mM sodium phosphate (pH = 7.0), and various concentrations of DMSO for 1 h at 37 °C. After the oxidation, we added a 40 μL labeling solution containing 0.2 M methoxyamine hydrochloride, 0.1 M sodium acetate (NaOAc), and 0.1 M *p*-phenylenediamine to a final volume of 80 μL, and the labeling reaction lasted for 1 h at room temperature. The samples were desalted, lyophilized, and analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

To optimize the labeling reaction conditions, 50 pmol of the glycopeptide and ∼25 μg Jurkat peptides were oxidized in 40 μL oxidation solution containing 1 U GAO, 1.6 U HRP, 20 U CAT, 50% of DMSO, and 50 mM sodium phosphate (pH = 7.0) for 1 h at 37 °C. After the oxidation, TFA was added to adjust pH to 2−3 and the peptides were desalted and lyophilized. Then, dried peptides were treated with 80 μL labeling solution (0.2 M methoxyamine hydrochloride, 0.1 M NaOAc and 0.1 M *p*-phenylenediamine, pH = 4.5) for different periods (0, 1, 2, and 4 h) at room temperature. Furthermore, different concentrations of the labeling reagent (0.2, 0.4, and 0.8 M methoxyamine hydrochloride) were also examined.

Oxidation and Labeling of the Tn Antigen after NeutrAvidin Enrichment. The enriched peptides were oxidized with a 40 μL oxidation solution containing 1 U GAO, 1.6 U HRP, 20 U CAT, 50% DMSO, and 50 mM sodium phosphate (pH = 7.0) for 1 h at 37 °C. After the oxidation, the peptides were desalted and lyophilized. Then, dried peptides were treated with 80 μL labeling solution (0.2 M methoxyamine hydrochloride, 0.1 M NaOAc, and 0.1 M *p*-phenylenediamine) for 2 h at room temperature. The labeled peptides were further purified by a stage-tip and eluted into three fractions using 20, 50, and 80% acetonitrile (ACN) with 1% HOAc, respectively.

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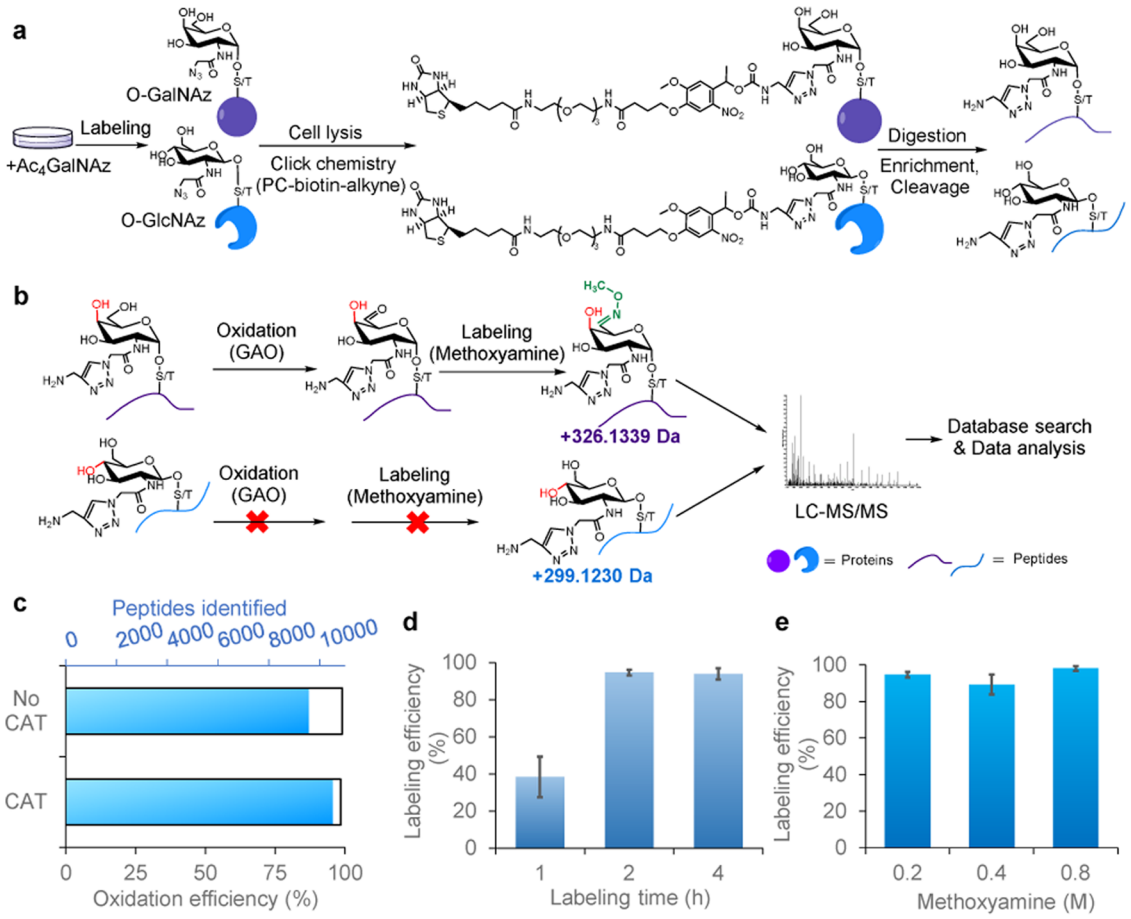

Figure 1. Experimental procedure for simultaneous enrichment and analysis of glycoproteins with O-GlcNAc and O-GalNAc, and optimization of experimental conditions for oxidation and labeling of Tn-bearing glycopeptides. (a) Principle of the enrichment of glycoproteins with O-GlcNAc and the Tn antigen by integrating metabolic labeling, bioorthogonal chemistry, and a cleavable linker. (b) Experimental design for the differentiation of glycopeptides with O-GlcNAc and O-GalNAc benefitting from the specificity of the enzymatic reaction. (c) Effect of the addition of catalase (CAT) on the oxidation efficiency and the number of peptides identified. (d, e) Effect of various reaction times (d) and concentrations of methoxyamine (e) on the aldehyde labeling.

LC-MS/MS Analysis. The peptide samples were dissolved in a solvent containing 5% ACN and 4% formic acid (FA), and 4 μL of the solution was loaded onto a microcapillary column packed with C18 beads (Magic C18AQ, 3 μm, 200 Å, 75 μm × 16 cm, Michrom Bioresources) using a Dionex WPS-3000TPLRS autosampler (UltiMate 3000 thermostatted Rapid Separation Pulled Loop Wellplate Sampler). Peptides were separated by reversed-phase high-performance liquid chromatography using an UltiMate 3000 binary pump with a 120 min gradient of 1−22, 2−30, and 5−45% ACN (with 0.125% FA), respectively, for the three fractions. Peptides were detected with a data-dependent Top15 method in a hybrid dual-cell quadrupole linear ion trap—Orbitrap mass spectrometer (LTQ Orbitrap Elite, ThermoFisher, with Xcalibur 3.0.63 software). For each cycle, one full MS scan (resolution: 60 000) in the Orbitrap cell at the automatic gain control (AGC) target of 1×10^6 was followed by up to 15 MS/MS recorded in the Orbitrap cell with high mass accuracy and high resolution for the most intense ions. The selected ions were excluded from further analysis for 90 s. Ions with singly or unassigned charges were not fragmented and sequenced. MS/MS scans were activated by higher-energy collision dissociation (HCD) at 34% normalized collision energy, and fragments were detected in the Orbitrap cell.

Database Search and Data Filtering. The resulting raw files with MS/MS spectra were searched against the human (*Homo sapiens*) protein database (downloaded from Uniprot) using Byonic. The following parameters were used during the search: 20 ppm precursor mass tolerance; 0.025 Da product ion mass tolerance; two missed cleavages; variable modifications: oxidation of methionine (+15.9949 Da), glycosylation on serine, threonine, and cysteine with modified O-GlcNAc (HexNAc (1) +96.0436 Da) or O-GalNAc (HexNAc (1) +123.0545 Da). To ensure the high quality of the glycopeptide identification, additional filters including |log prob| >4, score >300, and PEP 1D <0.001 were implemented.[2,16]

Glycosylation Site Localization and Quality Control. The confidence of glycosylation site localizations was determined by LuciPHOr2, which is a posttranslational modification (PTM) site localization tool using tandem mass spectrometry spectra.[17] The glycosites with the false localization rate (FLR) <0.05 were considered as confidently localized. All identified *S*-glycosylated peptides were removed.

Bioinformatic Analysis. Well-localized sites were selected to perform the motif analysis using online software (pLogo).[18] Information of protein domains was extracted from the InterPro database, Uniprot database, and the online prediction software SUPERFAMILY.[19−21] Phosphorylation, acetylation,

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methylation, and ubiquitination sites were extracted from PhosphoSitePlus and were compared with well-localized O-GlcNAcylation sites identified in the current experiments.[22] Protein clustering was performed using the database for annotation, visualization, and integrated discovery (DAVID).[23] Information of human transcription factors was extracted from the human transcription factor database.[24] Protein secondary structures and solvent accessibilities were predicted by NetSurfP-2.0.[25] Protein subcellular localizations were extracted from subcellbarcode.org.[26]

RESULTS AND DISCUSSION

Principle of Enriching Glycopeptides with O-GlcNAc and O-GalNAc. Integration of metabolic labeling and bioorthogonal chemistry is very powerful to study proteins and their modifications. In this work, an unnatural sugar analogue is employed to feed cells for labeling glycoproteins.[27−33] The sugar analogue carrying a small chemical group (i.e., the azide group) is exploited by glycosyltransferases to modify glycoproteins, and then the chemical group in labeled glycoproteins can serve as a handle to enrich them for MS analysis. Here, we chose *N*-azidoacetylgalactosamine-tetraacylated (Ac_4GalNAz) to label glycoproteins.[27,31] With the presence of UDP-galactose 4′-epimerase (GALE), UDP-GalNAz and UDP-GlcNAz are interconvertible in human cells, and thus both O-GlcNAcylated and O-GalNAcylated proteins can be labeled. After cell lysis, labeled glycoproteins were conjugated with a photocleavable (PC) biotin−alkyne tag through bioorthogonal chemistry. Then, proteins were digested, and glycopeptides carrying the biotin tag were enriched with the NeutrAvidin resins. The enriched glycopeptides were released through the cleavage of the PC linker under the UV light,[34−36] and an amine group was left on the glycans, facilitating MS detection of glycopeptides (Figure 1a).

Enzymatic and chemical reactions were further performed to distinguish glycopeptides with O-GlcNAz or O-GalNAz. Galactose oxidase (GAO) is a metalloenzyme that catalyzes the oxidation of the alcohol group of galactose and GalNAc to aldehyde. The stringent regioselectivity of this enzymatic reaction allows specific oxidation of the hydroxyl group at C6 of galactose and GalNAc but not glucose or GlcNAc. Like many enzymatic reactions, this reaction is highly efficient.[37−39] Using the synthetic glycopeptide with the Tn antigen, we found that the oxidation is close to completion. Glycopeptides with O-GalNAc were oxidized with GAO, and the resulting aldehyde groups were further coupled with methoxyamine, which is more stable. High selectivity of GAO resulted in tagging only glycopeptides with the Tn antigen, which was confirmed by the synthesized glycopeptides with O-GlcNAc and O-GalNAc in our previous work.[39] Eventually, the different mass tags on glycopeptides with O-GlcNAc or O-GalNAc (299.1230 vs 326.1339 Da) enable us to unambiguously distinguish them during MS analysis (Figure 1b).

Optimizing the Experimental Conditions for the Oxidation and Tagging of the Tn Antigen. The oxidation of galactose and GalNAc with GAO has been extensively reported.[40,41] Based on previous work, horseradish peroxidase (HRP) was used to facilitate the enzymatic oxidation because it consumes one of the products, H_2O_2, and to activate GAO, which may be deactivated during the reaction. Catalase (CAT) was also exploited to rapidly transform H_2O_2 to H_2O and O_2, which pushed the reaction further toward the completion according to Le Chatelier's principle. Together with CAT and HRP, GAO worked very well in oxidizing the sugars, but when it was applied to oxidize glycopeptides, it was reported that only less than 20% of oxidized glycopeptides were recovered because the production of hydroxyl radicals and hydrogen peroxide could damage the peptide backbone.[42,43] To overcome this issue, DMSO was introduced as a radical scavenger to protect glycopeptides previously.[39] DMSO has been widely used to quench hydroxyl radicals in food and environmental chemistry.[44,45]

Inspired by the previous results, parallel experiments were performed to further optimize the oxidation efficiencies and minimize the oxidative damage to peptide backbones. The labeling of the resulting aldehyde using methoxyamine was also optimized. We spiked the synthetic glycopeptide with the Tn antigen into digested peptides from a Jurkat whole cell lysate. The synthetic glycopeptide with the Tn antigen allowed us to accurately examine the effect of the reaction conditions on the oxidation and tagging efficiencies. The purpose of using digested peptides from a Jurkat whole cell lysate as background was to evaluate the effect of peptides being damaged by radicals from the oxidation reaction and the scavenging effect of DMSO.

First, we evaluated the effect of CAT on the protection of peptides during the oxidation reaction in the presence of DMSO and checked whether it could have a negative effect on the oxidation of GalNAc. The oxidation efficiency was evaluated by comparing the intensity of the oxidized synthetic Tn-bearing glycopeptide with that of the nonoxidized form. With or without CAT, the oxidation efficiencies remained very high and were close to 100%, as shown in Figure 1c. However, more peptides were identified with CAT than without CAT (10519 vs 9584 on average from the triplicate experiments). It clearly demonstrates that besides DMSO, the addition of CAT can further protect peptides during oxidation.

Next, we optimized the conditions for tagging the aldehyde group using methoxyamine. Using aniline as a catalyst, the oxime formation was dramatically accelerated under pH = 4.5.[46,47] Some previous studies demonstrated that the addition of the acetate group[48] and using substituted aniline with higher pK_a further accelerated the reaction.[47,49] Therefore, a mixture of methoxyamine, sodium acetate, and p-phenylenediamine was employed to study the optimal conditions for a quick and complete reaction. The yield for the 2 h reaction is much higher than that for 1 h, and a longer time (4 h) did not result in much difference (Figure 1d). Therefore, the reaction time was set to 2 h and compared the different concentrations of methoxyamine. No significant changes were found with the increase of methoxyamine concentration (Figure 1e). Later on, 0.2 M methoxyamine was used to tag the aldehyde group.

Identifying and Distinguishing Glycopeptides Modified with O-GlcNAc and the Tn Antigen. This method was applied to simultaneously identify glycopeptides with O-GlcNAc and the Tn antigen in three types of human cancer cell lines (Jurkat, MCF7, and K562). One example of identified glycopeptide modified with O-GlcNAc or the Tn antigen is displayed in Figure 2a,b, respectively. The O-GlcNAcylated peptide, S#NPVIPISSSNHSAR (# as O-GlcNAcylation site), was confidently identified with score = 439.04, |log prob| = 7.78, and a mass accuracy of 0.03 ppm using Byonic.[50] This peptide is from CCR4-NOT transcription complex subunit 4 (CNOT4), which has E3 ubiquitin ligase activity, promoting ubiquitination and degradation of targeted proteins. An example of the Tn-bearing glycopeptide, SEALPT@DLPAP-

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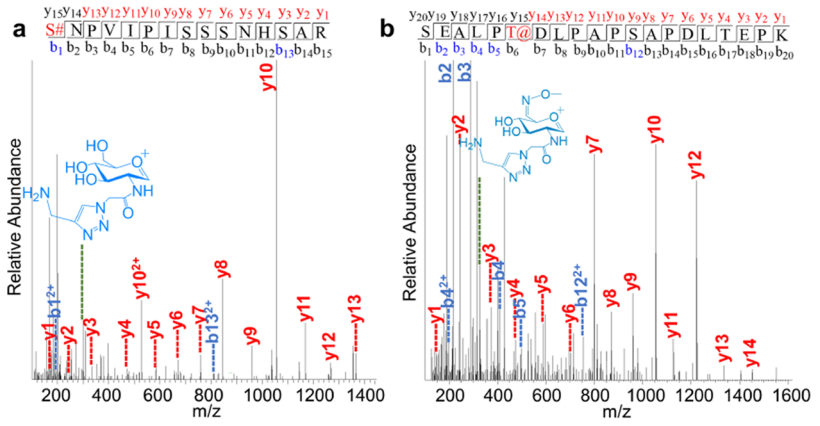

Figure 2. Comparison of tandem mass spectra identified with glycopeptides modified with O-GlcNAc or the Tn antigen. (a) An example tandem mass spectrum of the identified O-GlcNAcylated peptide. (b) An example of the tandem mass spectrum of the identified Tn-bearing glycopeptide.

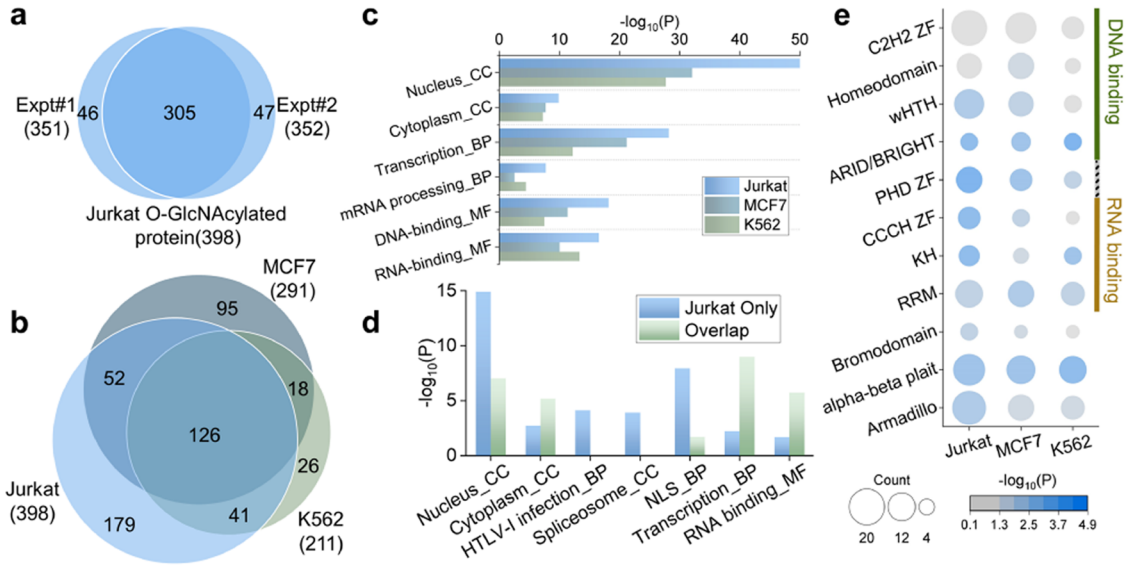

Figure 3. Analysis results for identified O-GlcNAcylated proteins. (a) Results of O-GlcNAcylated proteins identified from Jurkat cells in the duplicate experiments. (b) Comparison of the O-GlcNAcylated proteins identified in three types of cells. (c) Comparing the protein clustering results for O-GlcNAcylated proteins in different cell lines based on cellular compartment (CC), biological process (BP), and molecular function (MF). (d) Comparison of the O-GlcNAcylated proteins identified in all three cell lines (overlap) and those only identified in Jurkat cells (Jurkat only). (e) Comparing the domains among the O-GlcNAcylated proteins identified in different types of cells.

SAPDLTEPK (@ refers to the Tn-modified glycosite), was identified with a score of 808.90, |log prob| = 15.91, and a mass accuracy of 0.42 ppm (Figure 2b). This peptide belongs to glucosidase 2 subunit β (PRKCSH), which is mainly localized in the ER and is involved in the *N*-glycan metabolic pathway. In tandem mass spectra from higher-energy collision dissociation (HCD), intact glycan fragments could be present, serving as direct evidence for the presence of the glycans on glycopeptides. In the example of the O-GlcNAcylated peptide (Figure 2a), a fragment of unoxidized HexNAc was detected with an m/z of 300.1300 Da. In comparison, a fragment of oxidized HexNAc tagged with methoxyamine (m/z = 327.1412 Da) appeared in the spectrum of the Tn-bearing glycopeptide (Figure 2b). This difference of the glycan fragments provided another proof of the current method being able to unambiguously distinguish glycopeptides modified with O-GlcNAc or the Tn antigen.

Analysis of O-GlcNAcylated Proteins Revealed Cell-Specific Features. The numbers of O-GlcNAcylated proteins identified in the biological duplicate experiments from Jurkat cells are included in Figure 3a. In total, 398 O-GlcNAcylated proteins were identified, and the reproducibility of the experiments was reasonably high based on the overlap of glycoproteins identified in the duplicate experiments. Furthermore, 291 and 211 O-GlcNAcylated proteins were identified from MCF7 and K562 cells, respectively (Figure S1). All identified O-GlcNAcylated peptides are listed in Table S1, and the comparison of O-GlcNAcylated proteins among these three types of cells is shown in Figure 3b. In total, 537 O-GlcNAcylated proteins were identified.

Glycoproteins with O-GlcNAc from different types of cells were clustered using the database for annotation, visualization, and integrated discovery (DAVID),[23] and some results are shown in Figure 3c. Based on the cellular compartment, proteins in the categories of nucleus and cytoplasm were highly enriched, which were consistent with previous reports.[51,52] O-GlcNAcylated proteins were overrepresented in many biological processes, including transcription, and mRNA process-

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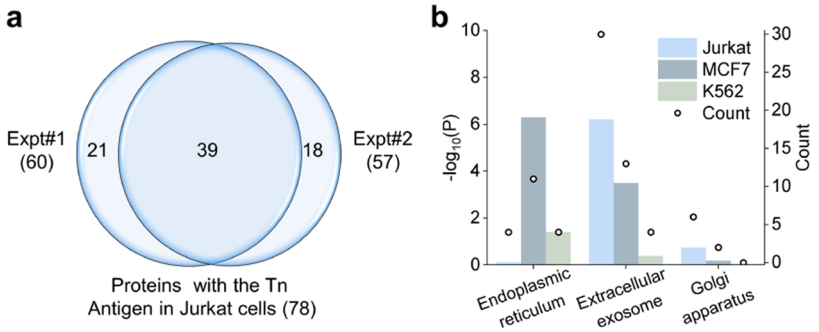

Figure 4. Identification of glycoproteins modified with the Tn antigen. (a) Results for the identification of glycoproteins with the Tn antigen in Jurkat cells in the duplicate experiments. (b) Distribution of the Tn-modified glycoproteins in the secretory pathway in three types of cells.

ing. Interestingly, although the $-\log_{10}(P)$ value of O-GlcNAcylated proteins associated with transcription in K562 cells was much lower than that in MCF7 cells, its $-\log_{10}(P)$ value of glycoproteins involved in mRNA processing was higher. Similarly, O-GlcNAcylated proteins in K562 that were involved in RNA binding had a higher $-\log_{10}(P)$ value compared with that related to DNA binding, which was the opposite to those in the other two cell lines. As expected, these results indicate that among the well-known functions of O-GlcNAcylated proteins, they vary in different types of cells.

To further compare the functions of O-GlcNAcylated proteins in a specific cell line with their general functions, the O-GlcNAcylated proteins identified exclusively in Jurkat cells (Jurkat only, 179 glycoproteins) were compared with those commonly identified in all three cell lines (overlap, 126). First, the glycoproteins identified only in Jurkat cells had higher enrichment in the nucleus, but it is the opposite for those in the cytoplasm (Figure 3d). Interestingly, O-GlcNAcylated proteins involved in HTLV-1 infection were found to be highly overrepresented only in Jurkat cells. Human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus that causes adult T-cell leukemia. As the Jurkat cell line was generated from the peripheral blood of a patient with T-cell leukemia, it is reasonable that those O-GlcNAcylated proteins participated in the response to the viral infection. Some glycoproteins were also enriched in the spliceosome and with the nuclear localization signal (NLS) among *O*-glycoproteins only in Jurkat cells. This could be due to O-GlcNAcylation being involved in the regulation of spliceosome activity and the entry of proteins to the nucleus in the Jurkat cells. In comparison, the O-GlcNAcylated proteins identified in all three cell lines were highly overrepresented with transcription and RNA binding functions. This is no surprise as the participation of O-GlcNAcylation in these activities was well established.

The modification of O-GlcNAc on transcription factors is important for the regulation of gene expression, and it was reported that over 25% of O-GlcNAcylated proteins are involved in transcriptional regulation.[51] Specifically, O-GlcNAcylation of transcription factors (TFs) affects protein activity and stability and mediates protein−protein interaction, DNA binding, and nucleocytoplasmic shuttling of TFs. Eventually, it modulates gene expression.[53] The DNA binding domain is a signature for TFs and is pivotal for their functions. For example, FOXA1, a TF with tumor-suppressive activity in breast cancer, was identified in this work. O-GlcNAcylation of FOXA1 may result in destabilization of the protein and, in turn, promote cancer invasiveness.[54,55] The DNA binding domains from O-GlcNAcylated TFs identified from three cell lines were extracted and compared (Figure 3e). The results showed different patterns for the DNA binding domains. For example, Cys2−His2 zinc finger (C2H2 ZF) was not enriched in any cell line. Homeodomain was overrepresented in the glycoproteins from MCF7 cells. Similarly, winged helix−turn−helix was highly enriched in glycoproteins from Jurkat and MCF7 cells. Some DNA domains were overrepresented in O-GlcNAcylated proteins from all three cell lines, including ARID/BRIGHT and plant homeodomain zinc finger (PHD ZF, also binds to RNA). The DNA binding domains have distinct patterns in O-GlcNAcylated TFs, suggesting that O-GlcNAcylation has different functions in regulating transcriptional events in different types of cells.

O-GlcNAcylation is also critical for the functions of some RNA binding proteins.[56] The RNA binding domains were compared for the O-GlcNAcylated proteins in three cell lines, and different patterns were observed as well (Figure 3e). Bromodomain recognizes acetylated lysine residues and could be essential for gene transcription by interacting with histones.[57] This domain was highly enriched among O-GlcNAcylated proteins in Jurkat cells. $\alpha-\beta$ plaits is a nucleotide binding domain, and it was overrepresented in O-GlcNAcylated proteins from all three cell lines. Furthermore, armadillo repeat is a protein binding domain that is critical for signal transduction, cytoskeletal regulation, and protein degradation.[58] It was highly enriched among the glycoproteins from Jurkat cells, suggesting that some O-GlcNAcylated proteins could be involved in these processes.

In three types of cells, 850 unique well-localized O-GlcNAcylation sites were identified (Table S2), and the well-localized sites from the glycoproteins identified commonly in all three cell lines (55 glycoproteins) are compared in Figure S2. It is well known that there are extensive crosstalks between protein O-GlcNAcylation and other modifications, especially phosphorylation.[59−63] It was found that many well-localized O-GlcNAcylation sites (i.e., 575/850) are in proximity (±10 amino acid residues) to the reported phosphorylation sites. Furthermore, 181, 94, and 74 O-GlcNAcylation sites are near the sites of ubiquitination, methylation, and acetylation, respectively (Figure S3). Around two-thirds of the O-GlcNAcylation sites being located on the same site or close to at least one phosphorylation site further corroborate that these two types of important modifications have a strong correlation, and they work together to regulate protein activities. Motif analyses were performed for the well-localized O-GlcNAcylation sites, and the detailed analysis is included in Figure S4 and the Supporting Information.

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Different Subcellular Localizations among Glycoproteins with Tn Antigen from Different Cell Lines. In this work, glycopeptides with O-GlcNAc and the Tn antigen were simultaneously enriched and then distinguished using the enzymatic reaction prior to MS analysis. From the biological duplicate experiments, 78 Tn-modified glycoproteins were site-specifically identified in Jurkat cells (Figure 4a). Twenty-eight and 16 Tn-modified glycoproteins were found in MCF7 and K562 cells, respectively (Figure S5). These results are in good agreement with some previous reports, in which Jurkat cells were known to have more proteins modified with the Tn antigen.[64,65] Unique glycopeptides identified with the Tn antigen from three cell lines are included in Table S3.

To compare the distribution of Tn-bearing glycoproteins in the secretory pathway among three cell lines, the identified glycoproteins were clustered based on cellular compartment (Figure 4b). The Tn-bearing glycoproteins in Jurkat cells were highly enriched in the extracellular exosome but not in the ER. Conversely, the glycoproteins with the Tn antigen from MCF7 and K562 cells were significantly overrepresented in the ER. Six glycoproteins modified with the Tn antigen were found in the Golgi apparatus in Jurkat cells, while only two and none in MCF7 and K562 cells were in the Golgi, respectively. The results demonstrate the variation of the subcellular localization of Tn-modified glycoproteins in different cell lines.

Based on previous research, the expression of glycoproteins with the Tn antigen was majorly attributed to two mechanisms: first, the relocation of polypeptide *N*-acetylgalactosamine-transferases (GalNAc-Ts, responsible for the addition of α-GalNAc to polypeptides) from the Golgi to the ER caused dramatic upregulation of glycoproteins with the Tn antigen in the ER;[66,67] second, the partial or complete loss of the molecular chaperone, i.e., C1GALT1-specific chaperone 1 (Cosmc), which is required for the proper folding of T-synthase, resulted in glycoproteins with the Tn antigen. T-synthase is essential for the elongation of O-GalNAc to produce mucin-type O-glycosylation, and without properly folded T-synthase, the truncated *O*-glycan (the Tn antigen) is produced.[68,69] Therefore, the generation of the Tn antigen in MCF7 and K562 cells could be majorly attributed to the first mechanism, in which GalNAc-Ts translocate from the Golgi to the ER. Conversely, the Tn antigen in the Jurkat cells was generated due to the loss of function of Cosmc in the Golgi, which was reported previously.[68] Therefore, in Jurkat cells, the Tn antigen mostly emerged on glycoproteins in the Golgi and extracellular exosome. The two different mechanisms may also be the reason for the relatively low overlap among the glycoproteins with the Tn antigen identified from three cell lines (Figure S6). These results further demonstrate that the current method is highly effective in distinguishing glycoproteins with O-GlcNAc and O-GalNAc.

CONCLUSIONS

Both O-GlcNAc and O-GalNAc are bound to the side chains of the serine and threonine residues of proteins, but their functions are entirely different. These two glycans are structurally very similar and have identical chemical compositions and molecular weights, which make it extraordinarily challenging to unambiguously distinguish them. Here, we developed a novel method integrating metabolic labeling, enzymatic and chemical reactions, and MS-based proteomics to simultaneously identify and distinguish glycoproteins modified with O-GlcNAc and the Tn antigen. In the biologically duplicate experiments, we simultaneously and site-specifically identified 398, 291, and 211 O-GlcNAcylated proteins and 78, 28, and 16 glycoproteins modified with the Tn antigen in Jurkat, MCF7, and K562 cells, respectively. The O-GlcNAcylated proteins commonly identified in all three cell lines are highly enriched with transcription and RNA binding functions. Further analysis of the O-GlcNAcylated proteins also revealed cell-specific features. For example, among glycoproteins exclusively in Jurkat cells, those involved in HTLV-1 infection are overrepresented, which is consistent with the cell line source and suggests that protein O-GlcNAcylation participated in the response to the virus infection. Glycoproteins in the spliceosome and with the nuclear localization signal (NLS) are also enriched in Jurkat cells. For glycoproteins with the Tn antigen, they have different subcellular distributions in different types of cells, which can be attributed to the distinct mechanisms for the formation of protein O-GalNAcylation. This method can be extensively applied to study glycoproteins with O-GlcNAc and the Tn antigen to advance our understanding of protein glycosylation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c05438.

The results regarding the motif analysis of well-localized O-GlcNAcylation sites; comparison of identified O-GlcNAcylated proteins in the duplicated experiments in MCF7 and K562 cells (Figure S1); overlap of the well-localized O-GlcNAcylation sites identified in three types of cells (Figure S2); the numbers of O-GlcNAcylation sites identified here that were in close proximity to other reported modification sites (Figure S3); motif analysis for all O-GlcNAcylation sites identified here and those sites in close proximity to other modifications (Figure S4); comparison of identified glycoproteins with the Tn antigen in the duplicated experiments in MCF7 and K562 cells (Figure S5); and comparison of the glycoproteins with the Tn antigen identified in three types of human cells (Figure S6) (PDF)

Identified O-GlcNAcylated peptides from the three types of cells in the duplicate experiments (Table S1) (XLSX)

Well-localized O-GlcNAcylation sites identified from the three types of cells (Table S2) (XLSX)

Identified glycopeptides modified with the Tn antigen from the three types of cells in the duplicate experiments (Table S3) (XLSX)

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Notes

The authors declare no competing financial interest.

The raw files generated by MS analyses are available on a publicly accessible website: ftp://massive.ucsd.edu/MSV000088088/.

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