

Enhanced Transfer of a Photocross-linking *N*-Acetylglucosamine (GlcNAc) Analog by an *O*-GlcNAc Transferase Mutant with Converted Substrate Specificity*

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Background: Photocross-linking *O*-GlcNAc (*O*-GlcNDAz) can be used to capture *O*-GlcNAc-mediated protein-protein interactions.

Results: Mutagenesis of the UDP-GlcNAc binding pocket of OGT enhances transfer of a photoreactive GlcNAc analog (GlcNDAz).

Conclusion: OGT(C917A) catalyzes increased enzymatic incorporation of GlcNDAz at sites of protein *O*-GlcNAc modification *in vitro* and in cells.

Significance: Enabling identification of *O*-GlcNAc-mediated protein interactions will provide insights into molecular mechanisms of *O*-GlcNAc function.

O-Linked β -*N*-acetylglucosamine (*O*-GlcNAc) is a post-translational modification of proteins in multicellular organisms. *O*-GlcNAc modification is catalyzed by the *O*-GlcNAc transferase (OGT), which transfers *N*-acetylglucosamine (GlcNAc) from the nucleotide sugar donor UDP-GlcNAc to serine or threonine residues of protein substrates. Recently, we reported a novel metabolic labeling method to introduce the diazirine photocross-linking functional group onto *O*-GlcNAc residues in mammalian cells. In this method, cells are engineered to produce diazirine-modified UDP-GlcNAc (UDP-GlcNDAz), and the diazirine-modified GlcNAc analog (GlcNDAz) is transferred to substrate proteins by endogenous OGT, producing *O*-GlcNDAz. *O*-GlcNDAz-modified proteins can be covalently cross-linked to their binding partners, providing information about *O*-GlcNAc-dependent interactions. The utility of the method was demonstrated by cross-linking highly *O*-GlcNAc-modified nucleoporins to proteins involved in nuclear transport. For practical application of this method to a broader range of *O*-GlcNAc-modified proteins, efficient *O*-GlcNDAz production is critical. Here we examined the ability of OGT to transfer GlcNDAz and found that the wild-type enzyme (wtOGT) prefers the natural substrate, UDP-GlcNAc, over the unnatural UDP-GlcNDAz. This competition limits *O*-GlcNDAz production in cells and the extent of *O*-GlcNDAz-dependent cross-linking. Here we identified an OGT mutant, OGT(C917A), that efficiently transfers GlcNDAz and, surpris-

ingly, has altered substrate specificity, preferring to transfer GlcNDAz rather than GlcNAc to protein substrates. We confirmed the reversed substrate preference by determining the Michaelis-Menten parameters describing the activity of wtOGT and OGT(C917A) with both UDP-GlcNAc and UDP-GlcNDAz. Use of OGT(C917A) enhances *O*-GlcNDAz production, yielding improved cross-linking of *O*-GlcNDAz-modified molecules both *in vitro* and in cells.

O-Linked β -*N*-acetylglucosamine (*O*-GlcNAc)⁴ is a single sugar modification found on both intracellular and extracellular proteins (1–3). This reversible post-translational modification occurs on proteins of diverse functional classes but primarily those that reside in the cytosol and nucleus. Intracellular *O*-GlcNAc is produced by the *O*-GlcNAc transferase (OGT), which catalyzes transfer of GlcNAc from the nucleotide sugar donor UDP-GlcNAc to serine and threonine side chains of proteins (4, 5). The dynamic *O*-GlcNAc modification is removed from proteins by the action of the *O*-GlcNAc hydrolase (OGA) (6). In cells and in organisms, *O*-GlcNAc levels are tightly controlled (7). Dysregulation of *O*-GlcNAc is linked to multiple chronic human diseases, including diabetes, cancer, and neurodegenerative disease, although the molecular mechanisms remain to be fully elucidated (8).

In many cases, *O*-GlcNAc appears to function as a sensor, detecting nutrient state and cell stress, but the molecular details of how *O*-GlcNAc modification affects the activity of modified proteins are only beginning to become clear. Some studies have focused on the interplay between *O*-GlcNAcylation and phos-

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⁴ The abbreviations used are: *O*-GlcNAc, β -*N*-acetylglucosamine; *O*-GlcNDAz, diazirine-modified *O*-GlcNAc; wtOGT, wild-type *O*-GlcNAc transferase; OGT, *O*-GlcNAc transferase; GlcNAc, *N*-acetylglucosamine; GlcNDAz, diazirine-modified GlcNAc; OGA, *O*-GlcNAc hydrolase; UAP1, UDP-*N*-acetylglucosamine pyrophosphatase 1; HPAEC, high performance anion exchange chromatography; CKII, casein kinase II; RIPA, radioimmune precipitation assay.

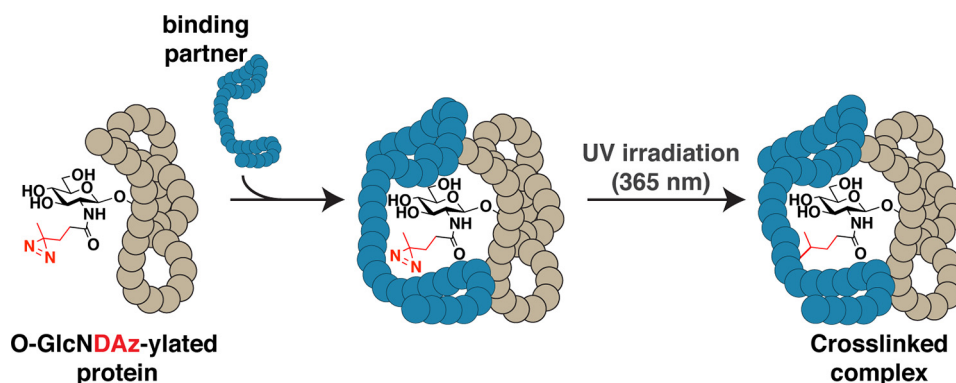


FIGURE 1. Methodology to identify binding partners of O-GlcNAcylated proteins. O-GlcNAcylated proteins are metabolically labeled with O-GlcNDaz in place of the natural modification. UV irradiation of cells activates the diazirine for cross-linking, resulting in a covalent cross-linked complex between an O-GlcNDazylated protein and its binding partner.

phorylation; in certain cases, these modifications compete for the same sites, and they are often observed as reciprocal events, whereas in at least one example, the two modifications appear to be coordinately controlled (9–12). O-GlcNAc modification can promote the stability of proteins (13) and their propensity to associate with specific cellular structures (14, 15). In at least one case, O-GlcNAc modification interferes with recognition between two proteins (16). Although other post-translational modifications, like phosphorylation, are well known to promote protein-protein interactions, less information has been available about whether O-GlcNAc can function in this way. Still, at least two studies show that modification of proteins with O-GlcNAc promotes their interactions with binding partners (17, 18), suggesting that O-GlcNAc may function as a recognition motif. However, identifying protein-protein interactions promoted by O-GlcNAc remains difficult; the modification is substoichiometric, and standard biochemical techniques cannot assess whether O-GlcNAc directly mediates the interaction or plays a more indirect role.

We reported a photocross-linking approach to discover and characterize interactions mediated by the O-GlcNAc modification. In this approach, we append the diazirine, the smallest photocross-linking functional group, onto O-GlcNAc modifications in live cells (19). Upon UV irradiation, the diazirine is activated, generating a highly reactive carbene. This carbene can insert into any neighboring molecule, generating a covalent bond between the O-GlcNAc-modified protein and its binding partner (Fig. 1). If no binding partner is nearby, the carbene is rapidly destroyed by reaction with water. These cross-linked complexes can be analyzed by immunoblot and/or mass spectrometry-based proteomics methods to identify binding partners of O-GlcNAc-modified proteins of interest. To introduce the diazirine onto O-GlcNAc-modified proteins in cells, we use a metabolic labeling method that exploits the GlcNAc salvage pathway of mammalian cells. Cells are cultured with a cell-permeable, diazirine-modified GlcNAc-1-P analog, Ac₃GlcNDaz-1-P(AcSATE)₂. Ac₃GlcNDaz-1-P(AcSATE)₂ is deprotected inside of cells, to release diazirine-modified GlcNAc-1-P (GlcNDaz-1-P). We use cells that overexpress a mutant form of UDP-N-acetylglucosamine pyrophosphatase 1 (UAP1), UAP1(F383G), that can efficiently convert GlcNDaz-1-P to UDP-GlcNDaz, because the endogenous human UAP1

cannot catalyze this transformation. OGT transfers GlcNDaz from UDP-GlcNDaz to cellular proteins, creating diazirine-modified O-GlcNAc, which we call O-GlcNDaz. Using this method, we showed that O-GlcNAc-modified nucleoporins could be photocross-linked to karyopherins that pass through the nuclear pore, effectively creating a snapshot of nuclear transport in action.

The O-GlcNDaz method effectively cross-links a variety of O-GlcNAc-modified proteins, but obtaining sufficient cross-linked material for mass spectrometry analysis remains challenging. We reasoned that maximizing production of O-GlcNDaz would yield more cross-linked material for subsequent steps. Indeed, UDP-GlcNDaz competes with the endogenous nucleotide sugar, UDP-GlcNAc, for OGT-catalyzed transfer onto proteins. Here we examined the ability of OGT to transfer natural GlcNAc and photocross-linking GlcNDaz to peptide substrates using *in vitro* assays. We found that wild-type OGT (wtOGT) exhibits a marked preference for the natural sugar, a feature that probably limits O-GlcNDaz production in cells. Through site-directed mutagenesis, we identified a mutant, OGT(C917A), that exhibits converted substrate specificity, preferentially transferring GlcNDaz rather than GlcNAc. Kinetic characterization of the activity of wtOGT and OGT(C917A) with UDP-GlcNAc and UDP-GlcNDaz confirmed the reciprocal substrate specificities of the two enzymes. We show that OGT(C917A) can be used to increase O-GlcNDaz modification and enhance O-GlcNDaz-mediated cross-linking both *in vitro* and in cells.

Experimental Procedures

Construction of OGT Plasmids—All experiments were conducted using the ncOGT (nucleocytoplasmic OGT) isoform. The pET24b plasmid encoding OGT was provided by Suzanne Walker (Harvard Medical School) and used to express recombinant OGT in *Escherichia coli* (20). The pcDNA/To-Myc-His plasmid encoding OGT was provided by Carolyn Bertozzi (University of California, Berkeley, CA) and used to produce recombinant OGT in mammalian cells (21).

Point mutations were introduced into OGT plasmids using primers designed through the GenScript primer design algorithm. Mutagenesis was performed according to the QuikChange protocol (Stratagene). The following primers

Mutant OGT Prefers GlcNDaz

were used: M501G, 5'-GTGCATCCTCATCATAGTGGGCT-ATATCCTCTTTCTCATGGC-3' (forward) and 5'-GCCATGAGAAAGAGGATATAGCCCACTATGATGAGGATGCAC-3' (reverse); M501V, 5'-GTGCATCCTCATCATAGTGTGCTATATCCTCTTTCTCATGGC-3' (forward) and 5'-GCCATGAGAAAGAGGATATAGCACACTATGATGAGGATGCAC-3' (reverse); C917G, 5'-GACACTCCACTCGGTAAATGGGCACACCACAGGG-3' (forward) and 5'-CCCTGTGGTGTGCCCATTAGCGAGTGGAGTGTC-3' (reverse); C917A, 5'-GACACTCCACTCGCTAATGGGCACACCACAGGG-3' (forward) and 5'-CCCTGTGGTGTGCCCATTAGCGAGTGGAGTGTC-3' (reverse); A942G, 5'-GGAGAGACTCTTGGTTCTCGAGTTGCAGCATCC-3' (forward) and 5'-GGATGCTGCAACTCGAGAACCAAGAGTCTCTCC-3' (reverse). The entire coding sequence of each mutated plasmid was confirmed through sequencing at the University of Texas Southwestern DNA sequencing core facility.

Cell Lines and Cell Culture—HeLa cells, obtained from the ATCC, were cultured in DMEM, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. K562 cells were obtained from ATCC and cultured in DMEM, 10% FBS, and 1% penicillin/streptomycin for routine culturing and for cross-linking experiments.

To produce cells stable expressing UAP1(F383G), K562 cells were infected with the pSIN4-UAP1(F383G) lentivirus (19). Briefly, 2.0×10^5 K562 cells in a 1-ml volume of fresh medium were mixed with 1 ml of the pSIN4-UAP1(F383G) lentivirus. Cells were centrifuged at $514 \times g$ and 32 °C for 30 min. After gentle mixing, centrifugation was repeated at $514 \times g$ and 32 °C for 30 min. After centrifugation, medium was removed by aspiration, and cells were resuspended in 2 ml of fresh medium and transferred to a well of a 6-well plate. Selection with 1.5 $\mu\text{g}/\text{ml}$ puromycin for cells expressing UAP1(F383G) was performed for 2 weeks.

Production of UDP-GlcNDaz in UAP1(F383G)-expressing K562 cells was analyzed by high performance anion exchange chromatography (HPAEC) as described (19). Briefly, K562 cells were cultured with or without Ac₃GlcNDaz-1-P(AcSATE)₂ (>95% purity by ¹H NMR) for 6 h. Cells were harvested by centrifugation, washed once with PBS, and pelleted. Metabolites were released by lysing cells with 80% "super cold" methanol on dry ice. Cellular debris were removed by centrifugation at $20,817 \times g$ and 4 °C for 10 min. The supernatant containing metabolites was transferred to a clean vessel and evaporated under vacuum. The metabolite pellet was resuspended in 40 mM sodium phosphate buffer (pH 7.0) and analyzed by HPAEC using conditions reported previously (19).

Transient Overexpression of Mutant OGT Enzymes in HeLa Cells—OGT and OGT mutants were overexpressed in HeLa cells to identify mutants with improved activity toward UDP-GlcNDaz. HeLa cells (3 million) in Opti-MEM (Invitrogen, 11058-021) were transfected with 24 μg of DNA and 60 μl of Lipofectamine 2000 (Invitrogen, 11668-019). Four h after transfection, Opti-MEM medium was removed, and fresh DMEM was added. After 24 h, cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM DTT, protease inhibitor mixture, and 1 mM phenylmethanesulfonyl

fluoride (PMSF)). Myc-His-tagged OGT (wild-type and mutants) were immunoprecipitated by incubating 1.2 mg of total protein with 1 μl of anti-Myc antibody 9B11 (Cell Signaling, 22768) at 4 °C for 18 h. After overnight incubation, 25 μl of protein G beads (Sigma, P3296) were added and incubated for 3 h at 4 °C. Beads were washed three times with RIPA buffer lacking protease inhibitors and then used for the following glycotransferase activity assays.

Glycosylation reactions were performed to analyze the ability of the OGT (wild type and the indicated mutants) to release UDP via the Promega UDP-GloTM glycosyltransferase assay (V6962). This assay measures UDP release via production of a luminescence signal. Beads with the immunopurified OGT enzymes (wild type and the indicated mutants) were resuspended in 25 μl of transferase buffer (25 mM HEPES, pH 7.2, 10 mM MnCl₂, 1 mM EDTA with 1 \times protease inhibitor mixture (Santa Cruz Biotechnology, Inc., sc-29131) and 1 mM PMSF). Five μl of this resuspension was mixed together with 20 μl of transferase buffer containing OGT substrates to achieve final concentrations of 100 μM casein kinase II (CKII) peptide and 10 μM Ultra-Pure UDP-GlcNAc (provided by Promega) or UDP-GlcNDaz (synthesized as described previously (19), >95% purity by ¹H NMR). Reaction mixtures were incubated at room temperature for 0 or 60 min. UDP-Glo detection reagent was added to quench the glycosylation reaction and develop luminescence. After 1 h at room temperature, luminescence signal was measured using a Synergy Neo BioTek multimode plate reader with detection between 620 and 665 nm (Lance red emission, BioTek, part no. 1035041).

Glycosylation reactions were performed to measure the ability of immunopurified OGT (wild type and mutants) to transfer GlcNAc and GlcNDaz to the CKII peptide. Beads with the immunopurified OGT enzymes (wild type and the indicated mutants) were washed twice with transferase buffer. A 150- μl mixture of CKII peptide (final concentration 100 μM) and equimolar amounts of UDP-GlcNAc and UDP-GlcNDaz (final concentrations 500 μM each) was added to each bead sample. The reaction was allowed to incubate at room temperature for 1 day, followed by 4 °C for 2 days. The reaction was centrifuged to remove beads. The supernatant was analyzed by high performance liquid chromatography (HPLC) using a Dionex HPLC Ultimate 3000 with UV detection at 220 nm. Solvent systems and gradients were described previously (22).

Overexpression of Recombinant OGT and OGT(C917A) in E. coli and Purification—pET24b plasmid encoding OGT (20) or OGT(C917A) was used to produce recombinant enzyme as described previously (22). Briefly, BL21(DE3) competent cells were transformed with each plasmid. A single colony was added to 50 ml of LB containing kanamycin and incubated at 37 °C without shaking for 14 h. Eight ml of the starter culture was used to inoculate 1 liter of LB, which was incubated at 37 °C with shaking at 250 rpm until A₆₀₀ reached 0.6–0.8. After cooling the culture to 20 °C for 30 min, cells were induced with 0.5 mM isopropyl 1-thio- β -D-galactopyranoside for 20 h. Bacteria were harvested by 30-min centrifugation at $1154 \times g$ at 4 °C. Bacteria were lysed in 100 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM Triton X-100, 0.3 mM lysozyme, 1 \times protease inhibitor mixture (Sigma, S8830) on ice for 30 min, followed by sonication for 3

min alternating 10-s pulse on and 30-s pulse off. Insoluble material was removed by centrifugation at $12,000 \times g$ and 4°C for 30 min. Cell lysates were incubated with nickel beads (nickel-nitrilotriacetic acid; Qiagen, 30210) for batch purification. Nickel beads were washed with 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 20 mM β -mercaptoethanol, 20 mM imidazole, and 0.1 mM PMSF. OGT (wild type or mutant) was eluted with 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 20 mM β -mercaptoethanol, 200 mM imidazole, and 0.1 mM PMSF. As an alternative to batch purification, OGT was also purified using a Profinia instrument (Bio-Rad, 620-1010) with an immobilized metal ion affinity chromatography column (Bio-Rad, 732-4610) according to the manufacturer's instructions and using the same buffers as the batch method. Protein purity was estimated by Coomassie staining. wtOGT was $>80\%$ pure, and OGT(C917A) was $>90\%$ pure.

Analysis of Peptide Glycosylation—wtOGT or OGT(C917A) recombinantly expressed in and purified from *E. coli* was used for analysis of peptide glycosylation. The CKII substrate peptide sequence was purchased from GenScript and had the sequence PGGSTPVSSANMMK with N-terminal acetylation and C-terminal amidation. Reactions contained 20 mM Tris-HCl, pH 8.0, 12.6 mM MgCl_2 , 20 mM β -mercaptoethanol, 100 μM CKII peptide, and 1 μM wtOGT or OGT(C917A). UDP-GlcNAc (Sigma, U4375, $>98\%$ purity) and/or UDP-GlcNDaz were included at the concentrations indicated. After incubation at 37°C for 16 h, the enzyme was removed by filtration through a 10,000 molecular weight cut-off Amicon unit (Millipore, UFC5010BK) that was centrifuged at $17,000 \times g$ for 5 min at room temperature. Peptide products were analyzed using a Dionex HPLC Ultimate 3000 with UV detection at 220 nm. Solvent systems and gradients were described previously (22).

Determination of Michaelis-Menten Parameters—Initial rates of reactions catalyzed by wtOGT and OGT(C917A) were obtained using the Promega UDP-GloTM glycosyltransferase assay (V6962) to measure UDP release. Reactions contained 20 mM Tris-HCl, pH 8.0, 12.6 mM MgCl_2 , 20 mM β -mercaptoethanol, and 100 μM CKII peptide. The nucleotide sugar (UDP-GlcNAc (provided by the Promega kit) or UDP-GlcNDaz) concentrations were 0.3, 1, 3, 10, and 30 μM . The enzyme concentration was 25 nM, except in the case of wtOGT with UDP-GlcNDaz, where 50 nM wtOGT was needed to accurately measure activity. Reactions were incubated at 23°C for a time course of 5, 10, 15, 30, or 45 min, and then UDP-Glo detection reagent was added to quench the glycosylation reaction and develop luminescence. After 1 h at room temperature, luminescence signal was measured using a Synergy Neo BioTek multimode plate reader with detection between 620 and 665 nm (Lance red emission, BioTek, part no. 1035041). Each sample was measured twice, and the averaged value was used. For each enzyme/substrate pair, five or six independent experiments were performed. Typically, the increase in luminescence was linear with respect to time between 5 and 45 min, except for the case of OGT(C917A) with UDP-GlcNDaz, where the time-dependent increase in luminescence was linear only to 30 min. Linear data were used to determine initial rates, which were

plotted *versus* substrate concentration. Michaelis-Menten parameters were calculated using GraphPad Prism.

In Vitro Peptide Cross-linking and Analysis—The biotin-tagged CKII peptide was purchased from GenScript and had the sequence biotin-PGGSTPVSSANMMK with C-terminal amidation. Glycosylation and HPLC analysis were performed as described above for the unmodified peptide. After removing the enzyme by filtration, the crude filtrate was incubated with an O-GlcNAc-recognizing antibody (RL2, Thermo, MA1-027) in RIPA buffer for 15 h at 4°C . Samples were placed in an ice water bath under UV irradiation (365 nm, UVP, XX-20BLB lamp) for 15 min or kept on ice in the dark. After irradiation, the samples were immediately lysed in RIPA buffer and then analyzed by immunoblot, using an HRP-conjugated anti-biotin antibody (Cell Signaling, 7075S) to detect antibody cross-linked to biotinylated peptide. Quantification was performed using a ChemiDocTM MP imager (Bio-Rad, 170-8280). Blots were stripped and reprobed to detect the total amount of RL2 antibody. The amount of biotinylated (cross-linked) antibody in each lane was normalized to the total amount of antibody in the same lane. The amount of cross-linking in the sample containing wtOGT and 500 μM UDP-GlcNDaz was arbitrarily set to 1, and cross-linking in other samples was calculated relative to this value.

In-cell Cross-linking—Two million K562 cells stably expressing UAP1(F383G) were nucleofected with 1.5 μg of DNA encoding an OGT plasmid (wtOGT or OGT(C917A)) by following the protocol provided by the Lonza kit V (VCA-1003). After 5 h, cells were resuspended in DMEM. Twenty-four h after nucleofection, cells were cultured with medium containing 6 μl of 50 mM $\text{Ac}_3\text{GlcNDaz-1-P}(\text{AcSATE})_2$ in DMSO to achieve a final concentration of 100 μM $\text{Ac}_3\text{GlcNDaz-1-P}(\text{AcSATE})_2$ or 6 μl of DMSO alone. After 7 h, cells were harvested by centrifugation and resuspended in PBS before irradiation with 365 nm light for 15 min. Cells were lysed in 100 μl of RIPA buffer per 2 million cells and then centrifuged at $20,817 \times g$ for 10 min at 4°C to remove insoluble material. Lysates were separated by 7.5% SDS-PAGE, transferred to polyvinylidene fluoride, and probed for NUP153 (SA-1 Abcam, ab96462). The amount of cross-linked NUP153 was determined by quantifying the indicated region on the blot using a ChemiDocTM MP imager (Bio-Rad, 170-8280). Immunoblots were stripped and reprobed with an antibody against actin (Abcam, ab8227). The amount of cross-linked NUP153 in each sample was normalized to the amount of actin in the same sample to control for sample loading. The amount of cross-linking in the sample containing overexpressed wtOGT was arbitrarily set to 1, and cross-linking in the sample containing OGT(C917A) was calculated relative to this value. Immunoblots were stripped and reprobed with an antibody against Myc antibody 9B11 (Cell Signaling, 22768) for OGT expression.

Immunoprecipitation was performed with the same cell culture conditions; 70 μg of total protein lysate was incubated with 1 μl of mAb414 antibody (Covance, MMS-120R) in a total volume of 200 μl for 14 h at 4°C . Samples were incubated with 10 μl of protein G-Sepharose[®] Fast Flow (Sigma-Aldrich, P3296) for 3 h at 4°C . The beads were washed three times with RIPA buffer. Proteins were eluted by the addition of $2\times$ loading dye

Mutant OGT Prefers GlcNDAz

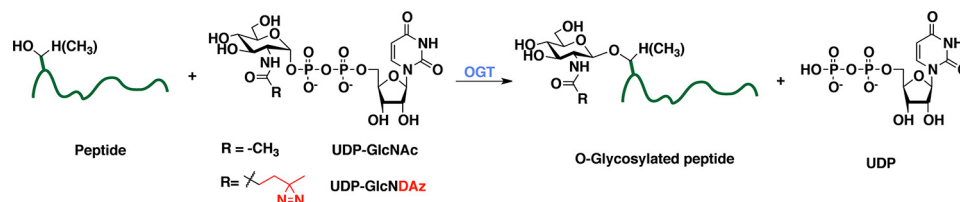


FIGURE 2. OGT glycosylation reactions with natural and unnatural substrates. OGT transfers GlcNAc from the natural substrate, UDP-GlcNAc, to serine or threonine residues of substrate peptides, producing O-GlcNAc-modified peptides. To incorporate O-GlcNDAz on peptides, OGT must transfer GlcNDAz from an unnatural nucleotide sugar, UDP-GlcNDAz.

(100 mM Tris-HCl, pH 6.8, 25 mM EDTA, 0.04 g/ml SDS, 0.4 mg/ml bromphenol blue) containing 20 mM DTT and incubation for 10 min at 90 °C. Samples were analyzed by immunoblot using the SA-1 antibody, as above.

Results

Wild-type OGT Prefers UDP-GlcNAc over UDP-GlcNDAz—We used *in vitro* assays to measure the ability of purified recombinant OGT to transfer GlcNAc and GlcNDAz to substrate peptides (Fig. 2). Recombinant wtOGT was expressed in *E. coli* and purified (22). We incubated the enzyme with a synthetic CKII peptide (22, 23) and either UDP-GlcNAc or UDP-GlcNDAz and evaluated production of the corresponding glycosylated peptides by HPLC analysis. We observed efficient production of both O-GlcNAc-modified CKII and O-GlcNDAz-modified CKII (Fig. 3). Next, we evaluated the nucleotide sugar specificity of wtOGT using a competition assay in which the enzyme was incubated with an equimolar mixture of UDP-GlcNAc and UDP-GlcNDAz. We chose this 1:1 ratio of UDP-GlcNAc to UDP-GlcNDAz because it is similar to the ratio we observe in cells that have been engineered for UDP-GlcNDAz production (19). Under these competitive conditions, we observed that wtOGT preferentially transferred GlcNAc, yielding primarily O-GlcNAc-modified CKII and very little O-GlcNDAz-modified CKII. On average, we observed that about 92% O-GlcNAcylated peptide and about 8% O-GlcNDAzylated peptide were produced under conditions of equimolar UDP-GlcNAc and UDP-GlcNDAz. This result indicated a significant limitation of the current O-GlcNDAz cross-linking protocol that depends on endogenous OGT for O-GlcNDAz production. We hypothesized that altering the substrate specificity of OGT might result in improved O-GlcNDAz production and cross-linking in cells, ultimately yielding more material for proteomics identification of O-GlcNAc binding partners.

Expanding the OGT Active Site to Accommodate the Diazirine of UDP-GlcNDAz—Fortunately, several crystal structures of OGT in complex with substrates, including UDP-GlcNAc, have been reported (20, 24–26). We examined the structure of OGT complexed with UDP-GlcNAc (Protein Data Bank entry 4GZ5) (24) to identify residues that might sterically occlude UDP-GlcNDAz binding. We found that when we modeled an alkyl diazirine extended off of the *N*-acyl chain of GlcNAc, the side chains of Met-501, Cys-917, and Ala-942 were in close proximity to the diazirine group (Fig. 4A). Therefore, we prepared plasmids encoding Myc-tagged OGT mutants M501G, M501V, C917A, C917G, and A942G. We expressed wtOGT and OGT mutants in HeLa cells and isolated

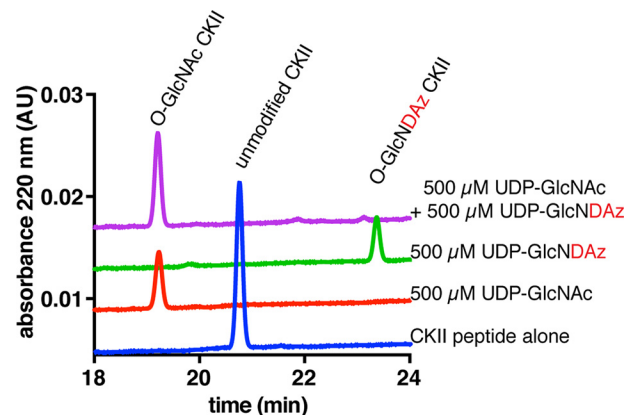


FIGURE 3. wtOGT prefers UDP-GlcNAc to UDP-GlcNDAz. Glycosylated CKII peptides produced using wtOGT. Wild-type OGT (1 μM) was incubated together with CKII peptide (100 μM) and 500 μM UDP-GlcNAc, 500 μM UDP-GlcNDAz, or a mixture of 500 μM UDP-GlcNAc and 500 μM UDP-GlcNDAz. Reaction products were separated by reverse-phase HPLC using UV absorbance (220 nm) for detection. Experiments were performed in triplicate with a single representative trial shown. AU, absorbance units.

the enzymes by immunoprecipitation. First, we assessed whether the OGT mutants were active by incubating them with UDP-GlcNAc and CKII peptide. Release of UDP was measured by a coupled enzyme assay with luminescence detection (UDP-Glo). All mutants were capable of releasing UDP from UDP-GlcNAc (Fig. 4B). We used the same assay to evaluate whether the mutants were capable of utilizing UDP-GlcNDAz as a donor. Four of the five mutants (M501G, C917A, C917G, and A942G) demonstrated activity with UDP-GlcNDAz, with the M501G and C917A mutants yielding the greatest release of UDP (Fig. 4C). Next, we used a competition assay to evaluate the ability of immunopurified wtOGT and each OGT mutant to transfer GlcNAc and GlcNDAz to a peptide substrate. Immunopurified wtOGT or OGT mutant was incubated with CKII peptide and an equimolar mixture of UDP-GlcNAc and UDP-GlcNDAz. Production of O-GlcNAc- and O-GlcNDAz-modified CKII was evaluated by HPLC. To our astonishment, OGT(C917A) preferentially incorporated O-GlcNDAz on CKII, with little O-GlcNAc-modified CKII detected (Fig. 4D). We constructed a model of OGT containing a mutation of Cys-917 to Ala and observed that this mutant may accommodate the diazirine extension while still providing structure to the protein and not introducing excess space in the binding pocket around the *N*-acyl group of GlcNDAz (Fig. 4E). Based on this model, we chose to further characterize the ability of recombinant OGT(C917A) to transfer GlcNDAz onto peptides.

OGT(C917A) Prefers UDP-GlcNDAz over UDP-GlcNAc—We evaluated the ability of purified OGT(C917A) to transfer GlcNAc and GlcNDAz to a peptide. Recombinant

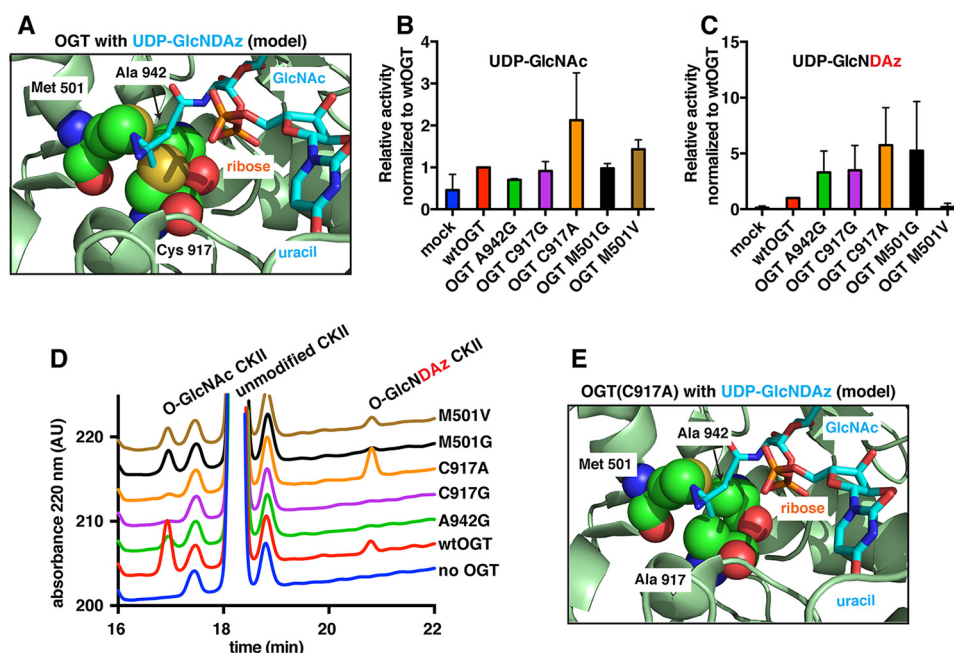


FIGURE 4. Mutagenesis of OGT to accommodate diazirine modification to UDP-GlcNAc. *A*, a model of human OGT (Protein Data Bank entry 4GZ5) (24) complexed with UDP-GlcNDAz substrate shows that the diazirine from the *N*-acyl group is in close proximity to positions 501, 917, and 942. *B* and *C*, immunopurified OGT (wild type or the indicated mutant) was incubated together with 100 μ M CKII peptide and 10 μ M UDP-GlcNAc (*B*) or 10 μ M UDP-GlcNDAz (*C*). Release of UDP was analyzed by UDP-Glo assay. Averaged values from two independent experiments are shown, with error bars representing the S.E. *D*, immunopurified OGT (wild type or the indicated mutant) was incubated with CKII peptide and an equimolar concentration of UDP-GlcNAc and UDP-GlcNDAz. Products were analyzed by HPLC. Data shown are a single trial representative of three independent experiments. *E*, a model of OGT(C917A) with UDP-GlcNDAz reveals space available to accommodate the extended diazirine functional group. AU, absorbance units.

OGT(C917A) was expressed in *E. coli* and purified. We incubated the enzyme with CKII peptide and either UDP-GlcNAc or UDP-GlcNDAz and evaluated production of the corresponding O-GlcNAc- or O-GlcNDAz-modified peptides by HPLC analysis (Fig. 5). As expected, OGT(C917A) was capable of transferring both GlcNAc and GlcNDAz. Next, we performed a competition experiment, incubating OGT(C917A) with CKII peptide and an equimolar mixture of UDP-GlcNAc and UDP-GlcNDAz. Recombinant OGT(C917A), like the immunopurified OGT(C917A), transferred primarily GlcNDAz to the peptide, with very little O-GlcNAc-modified peptide detected (Fig. 5). On average, we observed about 8% O-GlcNAcylated peptide and about 92% O-GlcNDAzylated peptide under conditions of equimolar UDP-GlcNAc and UDP-GlcNDAz. This result confirmed that OGT(C917A) prefers UDP-GlcNDAz over UDP-GlcNAc.

Kinetic Parameters Reveal That wtOGT and OGT(C917A) Have Different Substrate Specificities—To further characterize the nucleotide sugar preferences of wtOGT and OGT(C917A), we determined the Michaelis-Menten parameters for each enzyme with UDP-GlcNAc and with UDP-GlcNDAz (Fig. 6 and Table 1). The CKII peptide was used as the acceptor substrate in this analysis. Values obtained for wtOGT with UDP-GlcNAc were similar to those reported by others (20). As predicted from our competition analysis, GlcNDAz was transferred less efficiently than GlcNAc (Fig. 6, A–C), resulting in a 5-fold decrease in k_{cat} and a greater than 2-fold increase in K_m for the modified substrate. We examined the kinetics of OGT(C917A) with UDP-GlcNAc and UDP-GlcNDAz substrates. OGT(C917A) exhibited reduced activity with the natural substrate, UDP-GlcNAc, as compared with wtOGT (Fig. 6,

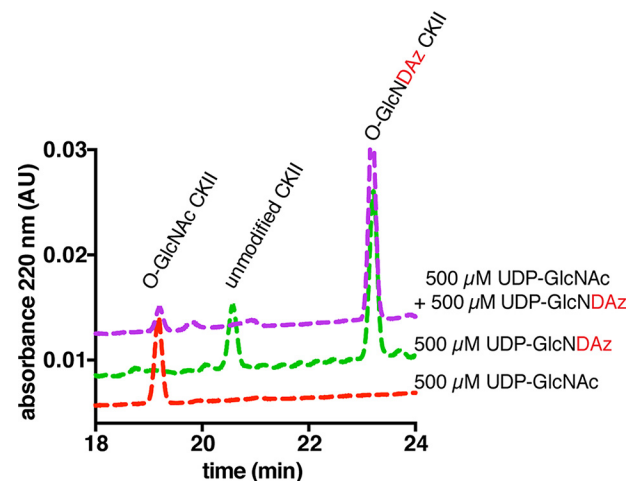


FIGURE 5. OGT(C917A) prefers UDP-GlcNDAz to UDP-GlcNAc. Glycosylated CKII peptides produced using OGT(C917A). OGT(C917A) (1 μ M) was incubated together with CKII peptide (100 μ M) and 500 μ M UDP-GlcNAc, 500 μ M UDP-GlcNDAz, or a mixture of 500 μ M UDP-GlcNAc and 500 μ M UDP-GlcNDAz. Reaction products were separated by reverse-phase HPLC using UV absorbance (220 nm) for detection. Experiments were performed in triplicate with a single representative trial shown. AU, absorbance units.

A and *D*). This difference in activity could be attributed to a 3-fold reduction in k_{cat} as well as greater than 7-fold increase in K_m (Table 1). To our delight, we found that OGT(C917A) transferred GlcNDAz efficiently, with both an increased k_{cat} and decreased K_m as compared with the natural UDP-GlcNAc substrate (Fig. 6, D–F). Indeed, introduction of the C917A mutation resulted in an enzyme whose catalytic efficiency (k_{cat}/K_m) with UDP-GlcNDAz was about 4 times the value observed for wtOGT with the natural substrate, UDP-GlcNAc. Overall,

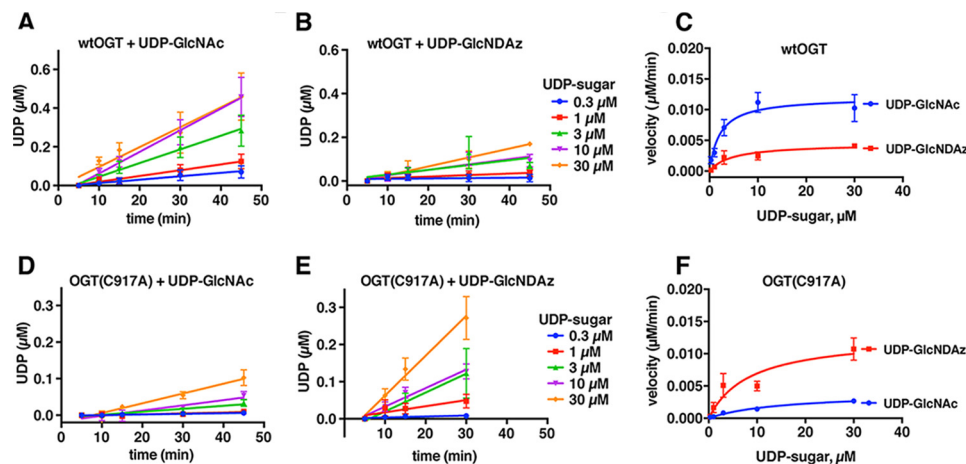


FIGURE 6. **wtOGT and OGT(C917A) have reversed nucleotide sugar specificities.** A, initial rates of UDP production by wtOGT with various concentrations of UDP-GlcNAc (0.3, 1, 3, 10, and 30 μM). B, initial rates of UDP production by wtOGT with various concentrations of UDP-GlcNDaz (0.3, 1, 3, 10, and 30 μM). C, velocities of wtOGT with UDP-GlcNAc (blue circles) and UDP-GlcNDaz (red squares). D, initial rates of UDP production by OGT(C917A) with various concentrations of UDP-GlcNAc (0.3, 1, 3, 10, and 30 μM). E, initial rates of UDP production by OGT(C917A) with various concentrations of UDP-GlcNDaz (0.3, 1, 3, 10, and 30 μM). F, the velocities of OGT(C917A) with UDP-GlcNAc (blue circles) and UDP-GlcNDaz (red squares). Averaged values for Michaelis-Menten analysis were obtained from at least five independent experiments. Error bars, S.E.

TABLE 1

Kinetics of wtOGT and OGT(C917A) with UDP-GlcNAc and UDP-GlcNDaz

Initial rates of reaction (shown in Fig. 6) were analyzed to determine enzyme velocities. Kinetic parameters were obtained by non-linear regression analysis, fitting the data to the Michaelis-Menten equation. Parameters are expressed as the mean ± S.E. as determined from at least five independent experiments.

Enzyme	UDP-GlcNAc			UDP-GlcNDaz		
	k_{cat} min^{-1}	K_m $μM$	k_{cat}/K_m $μM^{-1} min^{-1}$	k_{cat} min^{-1}	K_m $μM$	k_{cat}/K_m $μM^{-1} min^{-1}$
Wild type	$0.48 ± 0.05$	$2.1 ± 0.8$	$0.22 ± 0.09$	$0.09 ± 0.01$	$4.7 ± 2.3$	$0.019 ± 0.009$
C917A	$0.16 ± 0.02$	$15.4 ± 4.6$	$0.010 ± 0.003$	$0.51 ± 0.14$	$8.5 ± 6.3$	$0.060 ± 0.047$

the kinetic analysis shows that OGT(C917A) transfers GlcNDaz more efficiently than GlcNAc.

Increased GlcNDaz Transfer Leads to Enhanced Cross-linking in Vitro—Encouraged by the observation that OGT(C917A) prefers UDP-GlcNDaz over UDP-GlcNAc, we investigated whether the altered substrate preference led to enhanced O-GlcNDaz-mediated cross-linking. For our initial analysis, we used a cell-free model system in which we evaluated O-GlcNDaz-mediated cross-linking between glycosylated CKII peptide and an O-GlcNAc-recognizing antibody, RL2. To facilitate detection of the cross-linked complex, we incorporated a biotin tag in the acceptor CKII peptide. Glycosylation of the biotinylated CKII peptide was similar to what we observed for the unmodified CKII peptide (Figs. 3 and 5), with wtOGT preferentially transferring GlcNAc and OGT(C917A) preferentially transferring GlcNDaz (Fig. 7A). Indeed, when the biotinylated CKII peptide was incubated with wtOGT and a 1:1 mixture of UDP-GlcNAc and UDP-GlcNDaz, about 84% of the peptide was glycosylated with O-GlcNAc. In contrast, incubation of the peptide with OGT(C917A) and a 1:1 mixture of UDP-GlcNAc and UDP-GlcNDaz resulted in about 77% of the peptide glycosylated with O-GlcNDaz (Fig. 7B). After removing wtOGT or OGT(C917A) from the reaction mixture, the resulting peptides were incubated with RL2 antibody and cross-linked by application of UV radiation. Cross-linking between the peptide and RL2 antibody was revealed by immunoblot analysis, where we detected anti-biotin reactivity appearing at the molecular weight corresponding to the light chain of RL2 antibody, as we have observed previously (19). As expected, reaction mixtures

containing only UDP-GlcNAc yielded no O-GlcNDaz-modified peptide and no cross-linking to RL2 antibody, whereas reaction mixtures containing UDP-GlcNDaz produced O-GlcNDaz-modified peptide that cross-links to RL2 (Fig. 7C). Notably, when the reaction mixtures contained equimolar UDP-GlcNAc and UDP-GlcNDaz, cross-linking was observed when OGT(C917A) was used but not when wtOGT was used (red boxes in Fig. 7C). Furthermore, even when UDP-GlcNDaz was the only nucleotide sugar used, more cross-linking was observed from reaction with OGT(C917A) than from reaction with wtOGT (Fig. 7D). These results show that the C917A mutation leads to increased O-GlcNDazylation, which in turn produces a higher level of O-GlcNDaz-mediated cross-linking.

Increased GlcNDaz Transfer Leads to Enhanced Cross-linking in Cells—Next, we tested whether the C917A mutation to OGT could lead to increased O-GlcNDaz cross-linking in cells. Our O-GlcNDaz cross-linking method relies on the F383G mutant of UAP1 to catalyze in-cell production of UDP-GlcNDaz from Ac₃GlcNDaz-1-P(AcSATE)₂ (Fig. 8A). We prepared K562 cells stably expressing UAP1(F383G) and then cultured them with Ac₃GlcNDaz-1-P(AcSATE)₂. The lysate was analyzed by HPAEC, revealing robust production of UDP-GlcNDaz (Fig. 8B). The UAP1(F383G)-expressing K562 cells were nucleofected with plasmid DNA encoding wtOGT or OGT(C917A) and then cultured with Ac₃GlcNDaz-1-P(AcSATE)₂ for 7 h. The intact cells were subjected to UV irradiation and then lysed. We analyzed the lysates by immunoblot, probing for NUP153, a protein that is known to be heavily O-GlcNAc-modified and for which we have previously

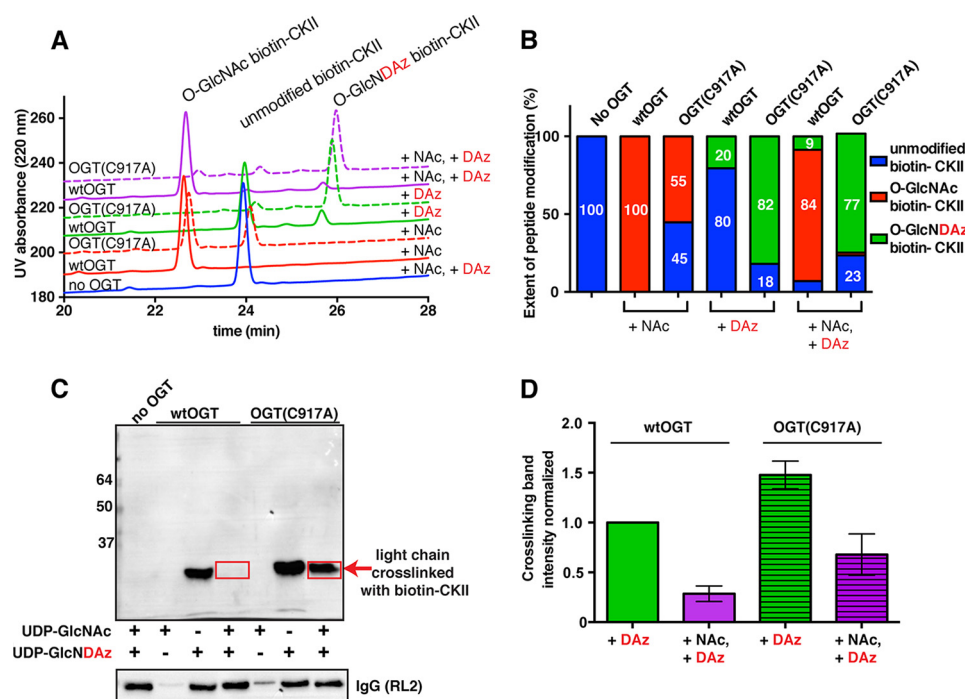


FIGURE 7. OGT(C917A) yields increased peptide cross-linking *in vitro*. *A*, biotinylated CKII peptide was glycosylated by wtOGT or OGT(C917A) using UDP-GlcNAc alone (+ NAc), UDP-GlcNDaz alone (+ DAz), or an equimolar mixture of UDP-GlcNAc and UDP-GlcNDaz (+ NAc + DAz). Reaction products were analyzed by HPLC. *B*, quantification of the ratios of unmodified, O-GlcNAc-modified, and O-GlcNDaz-modified peptides produced in *A*. *C*, peptides produced in *A* were incubated with RL2 antibody, UV-irradiated, and analyzed by immunoblot using an anti-biotin antibody. *D*, average RL2-peptide cross-linking intensities for replicate experiments as in *C*. Cross-linking intensity in the sample containing wtOGT and UDP-GlcNDaz was arbitrarily set to 1, and other samples were normalized relative to this value. Three independent experiments were performed, with error bars representing S.E.

observed O-GlcNDaz-mediated cross-linking (19). In cells transfected with both wtOGT and OGT(C917A), we observe increased anti-NUP153 reactivity at higher apparent molecular weight (Fig. 8C). For both wild-type and mutant OGT, the increases depend on both UV irradiation and the inclusion of Ac₃GlcNDaz-1-P(AcSATE)₂. Therefore, we believe that these bands correspond to O-GlcNDaz cross-linked forms of NUP153. We performed the analysis four times, each time quantifying the reactivity in the cross-linked region and normalizing these values relative to an actin loading control. In each experiment, we observed more cross-linked material in the cells transfected with OGT(C917A) as compared with cells transfected with wtOGT. The increases ranged from 18 to 94% (Fig. 8D). We also investigated whether overexpression of OGT(C917A) would allow us to isolate more cross-linked material from cells. Again, UAP1(F383G)-expressing K562 cells were nucleofected with DNA encoding wtOGT or OGT(C917A) and then cultured with Ac₃GlcNDaz-1-P(AcSATE)₂ and UV-irradiated. We immunoprecipitated nucleoporins using mAb414 antibody, which recognizes multiple NUPs, including NUP153. Immunoblot analysis using anti-NUP153 revealed substantially more cross-linked NUP153 in the immunoprecipitate from cells transfected with OGT(C917A) as compared with cells transfected with wtOGT (Fig. 8E). Because the overexpression levels of wtOGT and OGT(C917A) were comparable (Fig. 8F), we conclude that increased O-GlcNDaz production by OGT(C917A) leads to enhanced O-GlcNDaz-mediated cross-linking in cells and enables isolation of more cross-linked material for downstream analysis.

Discussion

C917A Mutation to OGT Reverses UDP-GlcNAc/UDP-GlcNDaz Substrate Specificity—Here, we described the use of site-directed mutagenesis to improve the O-GlcNDaz photo-cross-linking method of discovering O-GlcNAc-mediated protein-protein interactions (19). Using an *in vitro* competition assay, we found that wtOGT prefers to modify the CKII peptide with O-GlcNAc, rather than O-GlcNDaz. This result was confirmed by kinetics analysis showing that UDP-GlcNAc is the preferred nucleotide sugar substrate for OGT. Indeed, inspection of a model of wtOGT complexed with UDP-GlcNDaz reveals a potential for steric conflict between the diazirine modification and the amino acid side chains in wtOGT (Fig. 4A). We speculate that wtOGT discrimination against UDP-GlcNDaz is an important factor limiting how much O-GlcNDaz-cross-linked material can be isolated for mass spectrometry analysis.

We screened OGT mutants to identify an enzyme capable of efficient O-GlcNDaz production. Although most mutants showed increased activity with UDP-GlcNDaz, the M501V mutant had very little activity with the modified nucleotide sugar. This result is consistent with a previous study in which the M501V mutation was introduced into sOGT (the short isoform of OGT). Ma *et al.* (27) prepared sOGT(M501V) with the goal of enhancing activity toward UDP-GlcNAc analogs with extended acyl chains but found that this mutant exhibited reduced activity toward the analogs. Although the M501V mutation did not appear to be beneficial for GlcNDaz transfer, we found that OGT(C917A) showed enhanced O-GlcNDaz production, which in turn enabled increased O-GlcNDaz-me-

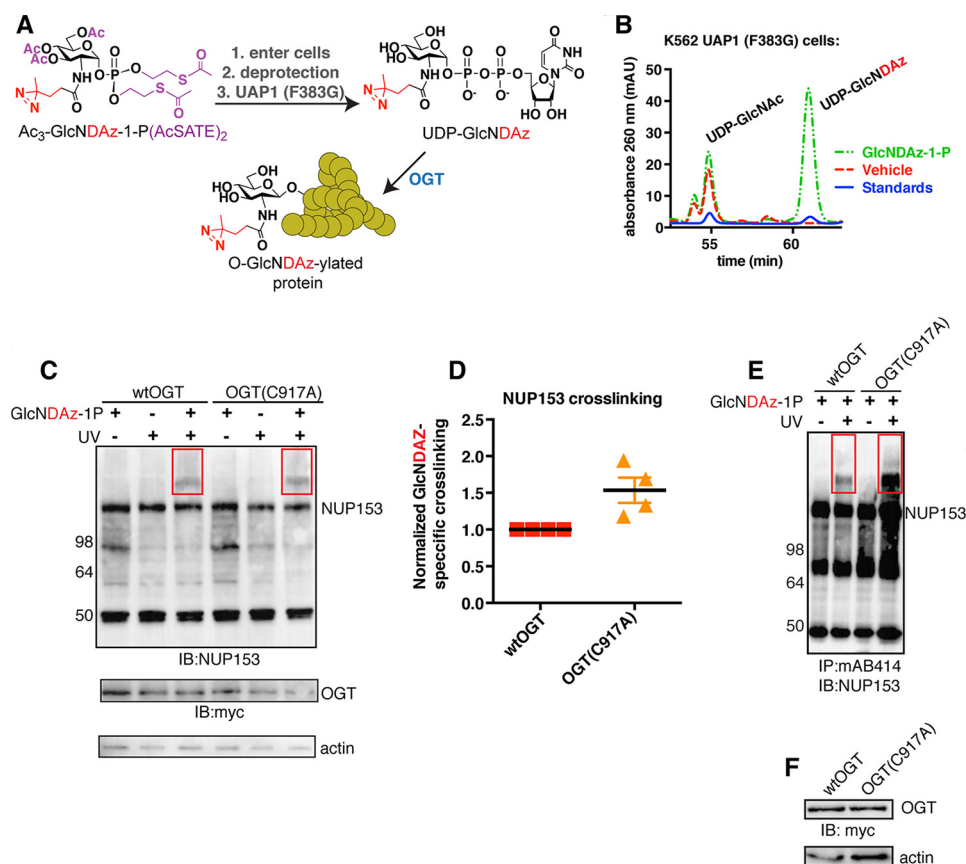


FIGURE 8. Expression of OGT(C917A) in cells results in enhanced O-GlcNDAz-dependent cross-linking. A, mammalian cells stably expressing human UAP1 with the F383G mutation are cultured with $\text{Ac}_3\text{GlcNDAz-1-P(AcSATE)}_2$. $\text{Ac}_3\text{GlcNDAz-1-P(AcSATE)}_2$ is deprotected inside of cells, yielding GlcNDAz-1-P, which is activated to UDP-GlcNDAz by UAP1(F383G). OGT transfers GlcNDAz from UDP-GlcNDAz to serine or threonine residues, producing O-GlcNDAz-modified proteins. B, K562 cells expressing UAP1(F383G) were cultured with $\text{Ac}_3\text{GlcNDAz-1-P(AcSATE)}_2$ or DMSO for 6 h. Metabolites were harvested and analyzed by HPAEC to detect production of UDP-GlcNDAz. C, K562 cells expressing UAP1(F383G) and nucleofected with plasmid encoding wtOGT or OGT(C917A) were cultured with or without $\text{Ac}_3\text{GlcNDAz-1-P(AcSATE)}_2$ for 7 h and then irradiated for 15 min. Immunoblot analysis (IB) was used to detect cross-linking of NUP153. Red boxes, putative cross-linked complexes. D, the extent of NUP153 cross-linking was analyzed in four independent experiments, as in C. The amount of cross-linking observed in cells nucleofected with wtOGT was arbitrarily set to 1, and the amount of cross-linking in cells nucleofected with OGT(C917A) was normalized relative to this control. Error bar, S.E. E, K562 cells expressing UAP1(F383G) were nucleofected with wtOGT or OGT(C917A). Cells were cultured with $\text{Ac}_3\text{GlcNDAz-1-P(AcSATE)}_2$ and were UV-irradiated for 15 min. After lysis, nucleoporins were purified using mAb414. The immunoprecipitate (IP) was probed by immunoblot using anti-NUP153. Red boxes highlight putative cross-linked complexes. Data presented are representative results from two independent trials. F, anti-Myc immunoblot reveals comparable expression of wtOGT and OGT(C917A) in nucleofected K562 cells used for the immunoprecipitation experiment shown in E. mAU, milliabsorbance units.

diated cross-linking both *in vitro* (Fig. 7) and in cell-based assays (Fig. 8, C and E). Although the OGT(C917A) mutant had previously been shown to be active (28), we were surprised and pleased to find that it transferred GlcNDAz better than GlcNAc. We speculate that the diazirine extension of UDP-GlcNDAz engages in productive interactions with OGT(C917A) that lead to one or both of the following: improved substrate binding and promotion of a catalytically active conformation.

OGT(C917A) may also aid in the transfer of other GlcNAc analogs. Indeed, a variety of unnatural UDP-GlcNAc analogs with extended *N*-acyl side chains have been prepared, but wild-type OGT tends to discriminate against those that add steric bulk close to the *N*-acyl group (25, 27, 29). The C917A mutation might facilitate transfer of these bulkier GlcNAc analogs. Variation in the *N*-acyl side chain of UDP-GlcNAc also occurs in nature; Vocadlo and co-workers (30) showed evidence of β -*N*-glycolylglucosamine production, suggesting that OGT accepts UDP- β -*N*-glycolylglucosamine. Similarly, small unnatural *N*-acyl substituents, such as the azide and alkyne, seem to be

tolerated by OGT (31–34), but it is possible that the C917A mutation could also facilitate discrimination between smaller analogs and the natural substrate. Overall, the observed utility of OGT(C917A) for O-GlcNDAz production adds to other studies showing that metabolic oligosaccharide engineering can be augmented through mutagenesis of carbohydrate-transforming enzymes to enhance metabolism of the unnatural sugar analogs (19, 22).

Our cell-based cross-linking data show that expression of OGT(C917A) results in enhanced O-GlcNDAz cross-linking, implying that this OGT mutant can increase production of O-GlcNDAz in cells. In cells, the naturally occurring O-GlcNAc modification is dynamic and can be removed by OGA. Previously, we showed that wild-type OGA is unable to remove O-GlcNDAz (19, 22). Thus, O-GlcNDAz probably persists in cells, and expression of OGT(C917A) will act to enhance its accumulation. It may be desirable to convert O-GlcNDAz from a static modification to a dynamic one to better mimic the natural O-GlcNAc modification. This could be accomplished by expressing an O-GlcNDAz-hydrolyzing mutant of OGA,

OGA(C215A) (22), along with the O-GlcNDaz-producing form of OGT, OGT(C917A). In this way, we envision creating cell lines that are optimized for O-GlcNDaz metabolism.

Author Contributions—A. C. R., S. H. Y., and J. J. K. conceived of and designed experiments. A. C. R. and S. H. Y. produced novel reagents, acquired data, and analyzed and interpreted data. B. L. and H. Z. supplied novel reagents and provided advice on experimental design. A. C. R., S. H. Y., and J. J. K. drafted the manuscript. All authors critically evaluated and approved the final version of the manuscript.

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References

- Torres, C. R., and Hart, G. W. (1984) Topography and polypeptide distribution of terminal *N*-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. *J. Biol. Chem.* **259**, 3308–3317
- Matsuura, A., Ito, M., Sakaidani, Y., Kondo, T., Murakami, K., Furukawa, K., Nadano, D., Matsuda, T., and Okajima, T. (2008) O-Linked *N*-acetylglucosamine is present on the extracellular domain of notch receptors. *J. Biol. Chem.* **283**, 35486–35495
- Bond, M. R., and Hanover, J. A. (2015) A little sugar goes a long way: the cell biology of O-GlcNAc. *J. Cell Biol.* **208**, 869–880
- Kreppel, L. K., Blomberg, M. A., and Hart, G. W. (1997) Dynamic glycosylation of nuclear and cytosolic proteins: cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. *J. Biol. Chem.* **272**, 9308–9315
- Lubas, W. A., Frank, D. W., Krause, M., and Hanover, J. A. (1997) O-Linked GlcNAc transferase is a conserved nucleocytoplasmic protein containing tetratricopeptide repeats. *J. Biol. Chem.* **272**, 9316–9324
- Gao, Y., Wells, L., Comer, F. I., Parker, G. J., and Hart, G. W. (2001) Dynamic O-glycosylation of nuclear and cytosolic proteins: cloning and characterization of a neutral, cytosolic β -*N*-acetylglucosaminidase from human brain. *J. Biol. Chem.* **276**, 9838–9845
- Taylor, R. P., Parker, G. J., Hazel, M. W., Soesanto, Y., Fuller, W., Yazzie, M. J., and McClain, D. A. (2008) Glucose deprivation stimulates O-GlcNAc modification of proteins through up-regulation of O-linked *N*-acetylglucosaminyltransferase. *J. Biol. Chem.* **283**, 6050–6057
- Bond, M. R., and Hanover, J. A. (2013) O-GlcNAc cycling: a link between metabolism and chronic disease. *Annu. Rev. Nutr.* **33**, 205–229
- Hart, G. W., Greis, K. D., Dong, L. Y. D., Blomberg, M. A., Chou, T.-Y., Jiang, M.-S., Roquemore, E. P., Snow, D. M., Kreppel, L. K., Cole, R. N., Comer, F. I., Arnold, C. S., and Hayes, B. K. (1995) O-Linked *N*-acetylglucosamine: the “Yin-Yang” of Ser/Thr phosphorylation? *Adv. Exp. Med. Biol.* **376**, 115–123
- Wang, Z., Udeshi, N. D., Slawson, C., Compton, P. D., Sakabe, K., Cheung, W. D., Shabanowitz, J., Hunt, D. F., and Hart, G. W. (2010) Extensive crosstalk between O-GlcNAcylation and phosphorylation regulates cytokinesis. *Sci. Signal.* **3**, ra2
- Rexach, J. E., Rogers, C. J., Yu, S.-H., Tao, J., Sun, Y. E., and Hsieh-Wilson, L. C. (2010) Quantification of O-glycosylation stoichiometry and dynamics using resolvable mass tags. *Nat. Chem. Biol.* **6**, 645–651
- Bauer, C., Göbel, K., Nagaraj, N., Colantuoni, C., Wang, M., Müller, U., Kremmer, E., Rottach, A., and Leonhardt, H. (2015) Phosphorylation of TET proteins is regulated via O-GlcNAcylation by the O-linked *N*-acetylglucosamine transferase (OGT). *J. Biol. Chem.* **290**, 4801–4812
- Zhu, Y., Liu, T.-W., Cecioni, S., Eskandari, R., Zandberg, W. F., and Vocadlo, D. J. (2015) O-GlcNAc occurs cotranslationally to stabilize nascent polypeptide chains. *Nat. Chem. Biol.* **11**, 319–325
- Fujiki, R., Hashiba, W., Sekine, H., Yokoyama, A., Chikanishi, T., Ito, S., Imai, Y., Kim, J., He, H. H., Igarashi, K., Kanno, J., Ohtake, F., Kitagawa, H., Roeder, R. G., Brown, M., and Kato, S. (2011) GlcNAcylation of histone H2B facilitates its monoubiquitination. *Nature* **480**, 557–560
- Dentin, R., Hedrick, S., Xie, J., Yates, J., 3rd, and Montminy, M. (2008) Hepatic glucose sensing via the CREB coactivator CRTC2. *Science* **319**, 1402–1405
- Lim, K., and Chang, H.-I. (2010) O-GlcNAc inhibits interaction between Sp1 and sterol regulatory element binding protein 2. *Biochem. Biophys. Res. Commun.* **393**, 314–318
- Gewinner, C., Hart, G., Zachara, N., Cole, R., Beisenherz-Huss, C., and Groner, B. (2004) The coactivator of transcription CREB-binding protein interacts preferentially with the glycosylated form of Stat5. *J. Biol. Chem.* **279**, 3563–3572
- Mizuguchi-Hata, C., Ogawa, Y., Oka, M., and Yoneda, Y. (2013) Quantitative regulation of nuclear pore complex proteins by O-GlcNAcylation. *Biochim. Biophys. Acta* **1833**, 2682–2689
- Yu, S.-H., Boyce, M., Wands, A. M., Bond, M. R., Bertozzi, C. R., and Kohler, J. J. (2012) Metabolic labeling enables selective photocrosslinking of O-GlcNAc-modified proteins to their binding partners. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 4834–4839
- Lazarus, M. B., Nam, Y., Jiang, J., Sliz, P., and Walker, S. (2011) Structure of human O-GlcNAc transferase and its complex with a peptide substrate. *Nature* **469**, 564–567
- Boyce, M., Carrico, I. S., Ganguli, A. S., Yu, S.-H., Hangauer, M. J., Hubbard, S. C., Kohler, J. J., and Bertozzi, C. R. (2011) Metabolic cross-talk allows labeling of O-linked β -*N*-acetylglucosamine-modified proteins via the *N*-acetylgalactosamine salvage pathway. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 3141–3146
- Rodriguez, A. C., and Kohler, J. J. (2014) Recognition of diazirine-modified O-GlcNAc by human O-GlcNAcase. *Medchemcomm* **5**, 1227–1234
- Kreppel, L. K., and Hart, G. W. (1999) Regulation of a cytosolic and nuclear O-GlcNAc transferase: role of the tetratricopeptide repeats. *J. Biol. Chem.* **274**, 32015–32022
- Lazarus, M. B., Jiang, J., Gloster, T. M., Zandberg, W. F., Whitworth, G. E., Vocadlo, D. J., and Walker, S. (2012) Structural snapshots of the reaction coordinate for O-GlcNAc transferase. *Nat. Chem. Biol.* **8**, 966–968
- Schimpl, M., Zheng, X., Borodkin, V. S., Blair, D. E., Ferenbach, A. T., Schüttelkopf, A. W., Navratilova, I., Aristotelous, T., Albarbarawi, O., Robinson, D. A., Macnaughtan, M. A., and van Aalten, D. M. F. (2012) O-GlcNAc transferase invokes nucleotide sugar pyrophosphate participation in catalysis. *Nat. Chem. Biol.* **8**, 969–974
- Lazarus, M. B., Jiang, J., Kapuria, V., Bhuiyan, T., Janetzko, J., Zandberg, W. F., Vocadlo, D. J., Herr, W., and Walker, S. (2013) HCF-1 is cleaved in the active site of O-GlcNAc transferase. *Science* **342**, 1235–1239
- Ma, X., Liu, P., Yan, H., Sun, H., Liu, X., Zhou, F., Li, L., Chen, Y., Muthana, M. M., Chen, X., Wang, P. G., and Zhang, L. (2013) Substrate specificity provides insights into the sugar donor recognition mechanism of O-GlcNAc transferase (OGT). *PLoS One* **8**, e63452
- Jiang, J., Lazarus, M. B., Pasquina, L., Sliz, P., and Walker, S. (2012) A neutral diphosphate mimic crosslinks the active site of human O-GlcNAc transferase. *Nat. Chem. Biol.* **8**, 72–77
- Gross, B. J., Kraybill, B. C., and Walker, S. (2005) Discovery of O-GlcNAc transferase inhibitors. *J. Am. Chem. Soc.* **127**, 14588–14589
- Macauley, M. S., Chan, J., Zandberg, W. F., He, Y., Whitworth, G. E., Stubbs, K. A., Yuzwa, S. A., Bennet, A. J., Varki, A., Davies, G. J., and Vocadlo, D. J. (2012) Metabolism of vertebrate amino sugars with *N*-glycolyl groups: intracellular β -O-linked *N*-glycolylglucosamine (GlcNGc), UDP-GlcNGc, and the biochemical and structural rationale for the substrate tolerance of β -O-linked β -*N*-acetylglucosaminidase. *J. Biol. Chem.* **287**, 28882–28897

Mutant OGT Prefers GlcNDAz

31. Vocadlo, D. J., Hang, H. C., Kim, E. J., Hanover, J. A., and Bertozzi, C. R. (2003) A chemical approach for identifying O-GlcNAc-modified proteins in cells. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 9116–9121
32. Leavy, T. M., and Bertozzi, C. R. (2007) A high-throughput assay for O-GlcNAc transferase detects primary sequence preferences in peptide substrates. *Bioorg. Med. Chem. Lett.* **17**, 3851–3854
33. Zaro, B. W., Yang, Y.-Y., Hang, H. C., and Pratt, M. R. (2011) Chemical reporters for fluorescent detection and identification of O-GlcNAc-modified proteins reveal glycosylation of the ubiquitin ligase NEDD4-1. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 8146–8151
34. Bateman, L. A., Zaro, B. W., Chuh, K. N., and Pratt, M. R. (2013) *N*-Propargyloxycarbamate monosaccharides as metabolic chemical reporters of carbohydrate salvage pathways and protein glycosylation. *Chem. Commun. (Camb.)* **49**, 4328–4330

Enhanced Transfer of a Photocross-linking *N*-Acetylglucosamine (GlcNAc) Analog by an *O*-GlcNAc Transferase Mutant with Converted Substrate Specificity

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