Insights into O-Linked N-Acetylglucosamine (O-GlcNAc) Processing and Dynamics through Kinetic Analysis of O-GlcNAc Transferase and O-GlcNAcase Activity on Protein Substrates

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Background: O-GlcNAc modification of several hundred proteins, with no apparent consensus, is modulated by just two enzymes. Results: Kinetic parameters for O-GlcNAcase do not fluctuate between protein substrates, whereas for O-GlcNAc transferase they are more variable.

Conclusion: O-GlcNAcase recognizes only the sugar moiety on proteins, whereas O-GlcNAc transferase probably plays a senior role in determining O-GlcNAc levels.

Significance: Indication of the interplay between O-GlcNAc transferase and O-GlcNAcase with different protein substrates.

Cellular O-linked N-acetylglucosamine (O-GlcNAc) levels are modulated by two enzymes: uridine diphosphate-N-acetyl-D-glucosamine:polypeptidyltransferase (OGT) and O-GlcNAcase (OGA). To quantitatively address the activity of these enzymes on protein substrates, we generated five structurally diverse proteins in both unmodified and O-GlcNAc-modified states. We found a remarkably invariant upper limit for $k_{cat}/K_m$ values for human OGA (hOGA)-catalyzed processing of these modified proteins, which suggests that hOGA processing is driven by the GlcNAc moiety and is independent of the protein. Human OGT (hOGT) activity ranged more widely, by up to 15-fold, suggesting that hOGT is the senior partner in fine tuning protein O-GlcNAc levels. This was supported by the observation that $K_{m, app}$ values for UDP-GlcNAc varied considerably (from 1 μM to over 20 μM), depending on the protein substrate, suggesting that some OGT substrates will be nutrient-responsive, whereas others are constitutively modified. The ratios of $k_{cat}/K_m$ values obtained from hOGT and hOGA kinetic studies enable a prediction of the dynamic equilibrium position of O-GlcNAc levels that can be recapitulated in vitro and suggest the relative O-GlcNAc stoichiometries of target proteins in the absence of other factors. We show that changes in the specific activities of hOGT and hOGA measured in vitro on calcium/calmodulin-dependent kinase IV (CaMKIV) and its pseudophosphorylated form can account for previously reported changes in CaMKIV O-GlcNAc levels observed in cells. These studies provide kinetic evidence for the interplay between O-GlcNAc and phosphorylation on proteins and indicate that these effects can be mediated by changes in hOGT and hOGA kinetic activity.

The attachment of 2-acetamido-2-deoxy-D-glucopyranose residues onto serine and threonine residues (O-GlcNAc) of intracellular proteins is a common post-translational modification within multicellular eukaryotes (1). O-GlcNAc is a dynamic modification, turning over more quickly than the polypeptide backbone of the proteins it modifies (2), and has been found in certain cases at or near residues known to be phosphorylated. O-GlcNAc can therefore probably influence phosphorylation and contribute to cellular signaling (2–5), which has stimulated interest in the field. In recent years, O-GlcNAc has been implicated in diverse cellular functions, including, for example, regulation of gene expression (6–8), trafficking (9–11), and signaling (12–14).

This increasing interest has prompted consideration of how O-GlcNAc levels on specific proteins are regulated. Remarkably, unlike phosphorylation, which is regulated by over 600 Ser/Thr kinases and phosphatases in humans (15), O-GlcNAc modification is regulated by only two carbohydrate-processing enzymes (16), although both exist in more than one isoform, which may provide some degree of substrate specificity (17). The enzyme catalyzing installation of O-GlcNAc onto protein residues from the substrate donor UDP-GlcNAc is a glucosyltransferase called uridine diphosphate-N-acetyl-D-glucosamine:polypeptidyltransferase (OGT) (18, 19). The enzyme...
responsible for catalyzing cleavage of O-GlcNAc from modified proteins and returning them to their unmodified state is a glycoside hydrolase known as O-GlcNAcase (OGA) (20, 21) (Fig. 1). These two enzymes appear able to regulate the O-GlcNAc modification of myriad targets that possess no apparent consensus sequence and direct which residues are modified. Various regulatory mechanisms that might govern OGA and OGT have been considered, including formation of an OGA-OGT complex that modulates their activities (22), accessory proteins that alter the activity of these enzymes on specific substrates (23), oxidative pathways leading to different enzyme activity (24), and tyrosine phosphorylation of OGT that might influence activity (18). One central mechanism that has been little explored is whether regulation of O-GlcNAc levels on various proteins depends on OGT and OGA showing quantitatively different activities toward different target proteins. We reasoned that differences in OGA and OGT activities, and moreover the ratios of OGA and OGT activity, toward different protein substrates are probably a contributing factor in the maintenance of protein-specific O-GlcNAc levels. This consideration motivated us to examine, using enzyme kinetic assays, the detailed activities of OGT and OGA on a series of different protein substrates in vitro.

Previous kinetic studies on the enzymatic activity of OGT have generally been limited to studying its activity on peptide substrates (25–27), which limits the ability to discern differences in its activity on protein substrates. Indeed, quantitative kinetic studies of the activity of OGT on protein substrates have been limited to investigating the modification of only one protein, Rattus norvegicus nucleoporin 62 (Nup62) (18, 28, 29). With respect to OGA, there is a greater paucity of kinetic studies. To our knowledge, there have been no detailed kinetic studies evaluating OGA activity on O-GlcNAc-modified proteins. Because these two enzymes are central players in modulating O-GlcNAc levels, an appreciation of their quantitative kinetic behavior toward genuine protein substrates would be beneficial. Indeed, such insights should improve our understanding of how O-GlcNAc levels are regulated on various protein substrates as well as provide insight into why certain proteins are more heavily modified than others.

To gain improved insight into the processing of protein substrates by these two enzymes, we performed a series of kinetic studies examining both human OGT (hOGT; the nucleocytoplasmic isoform) and human OGA (hOGA; the full-length isoform) activity on five structurally diverse proteins that can be recombinantly expressed at high levels within Escherichia coli both in an O-GlcNAc-modified form and in their unmodified state. We find that the upper limit for the activity of hOGA is surprisingly similar for a series of protein substrates and a synthetically modified peptide, whereas the activity of hOGT differs more widely. These kinetic studies suggest that hOGA activity depends primarily on the presence of the sugar residue and is independent of protein substrate or sequence. We also find that $K_{m}$ values for UDP-GlcNAc with hOGT vary depending on the protein substrate being studied, suggesting that some proteins will show more nutrient sensitivity than others with respect to their O-GlcNAc levels. Finally, we observe that when hOGA and hOGT are both present in reactions, hOGT acts in a manner predicted by assays performed using hOGT alone, indicating that the presence of hOGA does not influence its activity. These data provide a fundamental framework for understanding the kinetic relationship between OGA and OGT and should facilitate quantitative in vitro analysis of cellular factors that might influence relative OGA and OGT activities.

**Experimental Procedures**

**Cloning and Site-directed Mutagenesis**—cDNA encoding human TAB1 (TAK-binding protein 1) and CaMKIV were obtained from Origene. CDNA encoding CARM1 (coactivator-associated arginine N-methyltransferase 1) was purchased from the ATCC (Manassas, VA). Each gene was amplified by PCR using Pfu (Fermentas) and the primers listed in supplemental Table 1. The amplified DNA products were digested using the appropriate restriction enzymes and ligated into a pET28a vector previously described (30). The pET28a vector allows protein expression with a polyhistidine binding tag (His tag) for protein purification. The plasmid encoding *R. norvegicus* Nup62 in a PET3 vector was a kind gift from J. Hanover (National Institutes of Health, Bethesda, MD). To facilitate higher protein expression levels, the gene encoding Nup62 was amplified and cloned into a pMAL-c2X vector (New England Biolabs), which enables fusion of a maltose-binding protein (MBP) tag to the protein to aid soluble protein expression. The gene encoding Nup62 and the region of the pMAL-c2X vector encoding the MBP tag (Nup62-MBP) were then subcloned into a pET28a vector to give higher levels of Nup62 expression with the desired antibacterial resistance for the co-expression system (see below). The plasmid encoding hOGT, which has been described previously (28), was subcloned into the pMAL-c2X vector using the primers listed in supplemental Table 1. The plasmids encoding hOGA (and mutants) and *Bacteroides thetaiotaomicron* OGA (BgGH84) have been described previously (31, 32). The CaMKIV T200E mutant was generated using the QuikChange® site-directed mutagenesis kit (Stratagene) with the primers listed in supplemental Table 1.

**Protein Expression and Purification**—Plasmids harboring the genes for TAB1, CaMKIV, CARM1, Tau, Nup62, BgGH84, hOGA, hOGA D174A mutant, and hOGT (all in a pET28a vector with kanamycin antibiotic resistance and encoding a polyhistidine tag in frame with the protein product) were trans-
formed into Tuner (αDE3) cells (Novagen) and selected on an LB-agar plate containing 50 mg/liter kanamycin (Bioshop). A colony was selected for a starter culture, which was grown in Luria-Bertani (LB) medium containing antibiotic at 37 °C overnight. 10 ml of the starter culture was used to inoculate 1 liter of LB containing the antibiotic, and the culture was allowed to grow to an A700 of ~0.6. Recombinant protein expression was induced by supplementing each culture with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (Bio-background) at 25 °C for 4 h. Cells were harvested by centrifugation, treated with 1 mg/ml lysozyme (Bio-background), and sonicated, and finally centrifugation was used to remove insoluble cellular debris. The recombinant proteins were purified on a 5-ml HisTrap nickel column (GE Healthcare) according to the manufacturer’s protocol. Proteins were eluted using 50 mM sodium phosphate, 100 mM NaCl, 250 mM imidazole, pH 7.4, and then the buffer was exchanged to PBS (Lonza) using a PD-10 desalting column (GE Healthcare). The MBP tag was liberated from Nup62 by the addition of 50 μg/ml thrombin (Roche Applied Science) overnight at 4 °C and Nup62 was repurified using an MBPTrap column (GE Healthcare). Protein was washed off using PBS with 10 mM EDTA. The protein concentration was determined by Bradford assay (Bio-Rad), nanodrop (ND-1000, NanoDrop Technologies), or DC assay (Bio-Rad). Proteins were concentrated using centrifugation concentrating devices and stored at either 4 or −20 °C until they were used in the assays.

Co-expression System—The hOGT co-expression system was implemented as described previously (33). Plasmids harboring TAB1, CaMKIV, CARM1, Tau, Nup62, and CaMKIV T200E (each in a pET28a vector) and hOGT (in a pMAL-c2X vector) were co-transformed into Tuner cells and selected on an LB-agar plate containing 50 mg/liter kanamycin and 100 mg/liter ampicillin (Bio-background). One colony was picked and subjected to the same expression procedure as described above. The optimal induction time for protein expression and glycosylation level were examined at 4 and 20 h by SDS-PAGE and immunoblot analysis with the anti-O-GlcNAc CTD110.6 antibody. For subsequent experiments, Tau and Nup62 overexpression was induced for 20 h, and TAB1, CaMKIV, CaMKIV T200E, and CARM1 overexpression was induced for 4 h. Protein purification was carried out as described above using a HisTrap nickel column for the protein substrates; hOGT was subjected to the same expression procedure as described above. Protein purification was carried out as described above using a HisTrap nickel column for the protein substrates; hOGT was subjected to the same expression procedure as described above.

OGT and OGA Kinetic Analysis on Protein Substrates

O-GlcNAc Transferase Assays—hOGT assays were performed using radiolabeled [3H]UDP-GlcNAc (American Radiolabel) as the substrate donor and TAB1, CaMKIV, CARM1, Tau, or Nup62 as the protein acceptor. A suitable hOGT concentration for use in the assay was determined individually for each protein substrate using a range of concentrations of His-tagged hOGT between 0.1 and 1 μM, 20 μM each protein acceptor, and 20 μM [3H]UDP-GlcNAc (constant specific activity of 0.14 Ci/mmol). The K\textsubscript{m(app)}(UDP-GlcNAc) was determined through assays using a range of [3H]UDP-GlcNAc concentrations (constant specific activity of 0.092 Ci/mmol) in the presence of each of the protein acceptors (20 μM) and His-tagged hOGT (250 nM). The hOGT assay to determine the kinetic parameters on different acceptor proteins consisted of acceptor proteins ranging in concentration (1–40 μM), 20 μM (for CaMKIV and CARM1 protein substrates) or 100 μM (for Nup62, TAB1, and Tau protein substrates) [3H]UDP-GlcNAc and 200 nM His-tagged hOGT. The specific activity of the UDP-GlcNAc was 0.14 Ci/mmol when using 20 μM UDP-GlcNAc and 0.092 Ci/mmol when using 100 μM. Reactions were initiated by the addition of enzyme and were incubated at 37 °C for 1 h, a time over which linear rates were verified. Reaction mixtures were placed on ice after this incubation and immediately applied to 1.5 × 3-cm pieces of nitrocellulose membrane (Bio-Rad) that were then allowed to air-dry. The quantity of protein loaded onto each piece of nitrocellulose membrane was at least 10 times less than the binding capacity of the membrane (as detailed in the manufacturer’s protocol). The membranes were then washed with four consecutive large volumes (100 ml) of PBS and air-dried. The pieces of membrane were loaded into scintillation vials, 4 ml of scintillation fluid (Amersham Biosciences) was added, and the levels of tritium were quantified using a liquid scintillation counter (Beckman LS6000). All assays were done in at least triplicate. The counts in disintegrations/min from the scintillation counting were converted into rates with units of Ci/mmol of GlcNAc/min/mg of hOGT, by taking into account the length of time for the assay and the enzyme concentration. The background rate in the absence of enzyme was subtracted, and the resulting rates were plotted against the substrate concentration (which was determined as described below) using GRAFIT (34) to establish the apparent k\textsubscript{cat} and K\textsubscript{m} values for each substrate, which for simplicity are referred to below as k\textsubscript{cat} and K\textsubscript{m} values.

O-GlcNACase Assays—hOGA-catalyzed hydrolysis reactions of O-GlcNAc-modified TAB1, CaMKIV, CARM1, Tau, and Nup62 were carried out in PBS with a total reaction volume of 250 μl. Reactions contained different concentrations of substrates ranging from 1 to 3000 μM and were initiated by the addition of hOGA (1 μM). Assays were incubated at 37 °C for 1 h (a time over which linear rates were obtained using these assay conditions). Reactions were terminated by the addition of 750 μl of 95% cold ethanol and stored at −20 °C for 30 min in order to ensure complete quenching of the radioactivity.
OGT and OGA Kinetic Analysis on Protein Substrates

to precipitate the proteins. Upon termination of the reaction, 5 μl of a 1 mM solution of fucose was added to each reaction as an internal standard. Following protein precipitation, the suspension was centrifuged, and the supernatant was collected and dried by vacuum centrifugation. The residue was dissolved in 150 μl double-distilled H2O, vortexed, and centrifuged to remove any residual insoluble debris. Carbohydrates were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (ASI 100 automated sample injector, Carbopack PA20 column, ICS 3000, and ED detector, Dionex). An isocratic elution profile composed of 20 mM NaOH was used, which afforded clear separation of fucose (retention time = 3 min) and the GlcNAc product of the enzymatic reaction (retention time = 8 min). The GlcNAc/fucose peak integration ratio was calculated to determine the amount of product from the hydrolysis reaction relative to the standard. The absolute amount of GlcNAc produced was determined using a standard curve constructed from GlcNAc at concentrations between 1 and 250 μM. The rate of hOGA hydrolysis was calculated after taking into account the length of time for the assay and the enzyme concentration. The background rate in the absence of enzyme was subtracted, and the resulting rates were plotted against the substrate concentration and the apparent pseudo-second order rate constant values (kcat/Km) were determined using GRAFIT (34). All reactions were carried out in quadruplicate.

Synthesis and Purification of O-GlcNAc-modified (at Ser-208) Tau Peptide—Fmoc-protected and pentafluorophenyl-acetylated peracetylated β-O-GlcNAc-modified serine (Fmoc-Ser(Ac3-B-β-O-GlcNAc)-pentafluorophenyl) was synthesized as described previously (35, 36). 1H NMR chemical shifts were identical to the published values. Glycopeptide synthesis was carried out at BioBasic Inc. (Markham, Canada) by solid phase peptide synthesis using standard Fmoc chemistry with O-benzotriazolone-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate/N,N-diisopropylethylamine couplings to generate the peptide Ac-CGYSSPSGSPGTPgSRSH2 (Ser-208 OG-Tau peptide, where g5 represents the site of glycosylation) with an acetylated N terminus on Rink amide resin. Upon receipt of the fully protected Ser-208 OG-Tau peptide coupled to the Rink amide resin, the O-acetyl groups on the GlcNAc moiety were deacetylated by resuspending the resin (0.4 g) in 5 ml of dry methanol. The solution was adjusted to pH >9 (judged using pH paper) using a solution of 0.5 M sodium methoxide in dry methanol. The solution was shaken gently for 24 h using an orbital shaker at room temperature. The resin was then washed with methanol three times and resuspended in a solution containing trifluoroacetic acid (TFA), water, 1,2-ethanedithiol, and triethylsilane in a ratio of 85:2.5:2.5:10 and allowed to shake for 2 h at room temperature. The resin was filtered off and washed three times with fresh TFA. The filtrate was concentrated in vacuo using a high vacuum rotary evaporator. Cold diethyl ether was added to the filtrate until a white precipitate formed in the flask. The precipitate was centrifuged at 800 rpm in a Sorvall Legend RT centrifuge in a disposable 50-ml conical centrifuge tube using a swinging bucket TTH-750 rotor. The resulting pellet was washed three times with cold diethyl ether followed by centrifugation. Finally, the diethyl ether was removed, and the crude peptide pellet was dried under a stream of nitrogen gas, resuspended in water, lyophilized to dryness, and stored at −20 °C until required. To purify the crude Ser-208 OG-Tau peptide by high performance liquid chromatography (HPLC), 10 mg of material was loaded per run onto an Agilent Zorbax 300 SB-C8 (9.4 × 250 mm) semi-preparative HPLC column housed in an 1100 series Agilent HPLC. The peptide was purified using a linear gradient of 5% acetonitrile to 70% acetonitrile over 40 min operating at 2 ml/min. The major peak eluted at ~16.3 min, and the corresponding fractions were pooled and high resolution mass spectrometry was carried out to ensure the correct identity of the deprotected peptide. High resolution mass spectrometry predicted: 992.9466 Da [M + 2H]2+; found: 992.9305 Da [M + 2H]2+.

hOGA Kinetic Assays with Ser-208 OG-Tau Peptide—Ser-208 OG-Tau peptide, ranging from 25 to 125 μM (in PBS, pH 7.4), was mixed with 1 μM hOGA in 500-μl reactions and allowed to proceed for 1 h. Following the reaction, fucose was added as an internal standard, and the hOGA was inactivated by heating at 100 °C for 5 min. Immediately after, the reactions were cooled and then passed through 1-ml Bond Elut-C18 columns (Agilent) pre-equilibrated with water. The water was removed by vacuum centrifugation, and the samples were resuspended in 150 μl of water. The quantity of liberated GlcNAc was determined using HPAEC-PAD as described above.

hOGT and hOGA Competition Assay on TAB1 and Nup62—The assay consisted of 5 μM TAB1 or 10 μM Nup62, 20 μM [3H]UDP-GlcNAc (constant specific activity of 0.14 Ci/mmol), 0.25 μM His-tagged hOGT, and 0.25 μM hOGA. The catalytically inactive hOGA D174A mutant was used, at the same concentration, in place of hOGA in the negative control reactions. Reactions were initiated by the addition of the enzymes and incubated at 37 °C. Time points were taken at 0, 30, 60, 90, 120, 150, 180, and 240 min for TAB1 and 0, 20, 40, 80, 120, 160, and 240 min for Nup62 by applying the reaction mixtures to 1.5 × 3-cm pieces of nitrocellulose membrane, which were then allowed to air-dry. The levels of tritium present were determined by scintillation counting after processing as described above.

Hydrolysis with hOGA and BtGH84—For total O-GlcNAc cleavage, 10 μM modified TAB1, CaMKIV, CARM1, Tau, or Nup62 was treated with 25 μM BtGH84 for 3 h at 37 °C in a volume of 200 μl. 20 μl of the sample was used for immunoblot analysis, and the rest of the sample was subjected to HPAEC-PAD analysis as described above. For time-dependent O-GlcNAc cleavage, 10 μM modified TAB1, CaMKIV, CARM1, Tau, or Nup62 was treated with 1 μM hOGA for 80 min at 37 °C in a volume of 200 μl, and samples were removed at various time points. 20 μl of the sample was used for immunoblot analysis, and the rest of the sample was subjected to HPAEC-PAD analysis as described above. The modified T200E CaMKIV mutant (12 μM) and wild type (WT) CaMKIV (4 μM) proteins were subjected to O-GlcNAc hydrolysis using 2 μM hOGA or BtGH84 at 37 °C for 2 h. The reaction was stopped by the addition of Laemmli sample buffer and subjected to immunoblot analysis as described above.
Mass Determination by Mass Spectrometry (MS)—Samples were desalted using a PD-10 column (GE Healthcare) into MS grade water (Mallinckrodt Baker) and further concentrated to the desired concentration using a centrifugal device (Millipore). Samples were centrifuged prior to analysis to remove any debris. Samples were analyzed using an Agilent 6210 LC/MS (Agilent Technology) equipped with an electrospray ionization source and a ZORBAX 300SB-C8 column (Agilent Technology, 2.1 × 50 mm, 3.5 μm). Proteins were separated on the column using HPLC grade water with 0.1% formic acid as the mobile phase and eluted into the MS using a gradient containing HPLC grade acetonitrile with 0.1% formic acid. An ion spray voltage of 3500 V was used, and the data acquisition was done at a scan rate of 1000 atomic mass units/s over a mass range of 300–3000 atomic mass units. The raw averaged data were selected by comparison with the peak retention times on the LC chromatogram and subjected to deconvolution using MassHunter workstation software (Agilent Technology, version B.02.00) from 700 to 1850 atomic mass units.

O-GlcNAc Stoichiometry Estimation by Acid Hydrolysis—To estimate the stoichiometry of O-GlcNAc modification on Tau, 26 nmol of O-GlcNAc-modified Tau was treated with 6 M HCl at 110 °C in a 5-ml Reacti-Vial reaction vial (Thermo). After 4 h, half of the reaction mixture was removed and concentrated to dryness in vacuo using a high vacuum rotary evaporator. After a further 2 h (6 h total), the other half of the reaction mixture was concentrated to dryness as described above. The released glucosamine (GlcN) was labeled with 2-aminoacridone (2-AMAC), and levels were determined as described previously with minor modifications as follows (37). Briefly, the GlcN was dissolved in 10 μl of 0.1 M 2-AMAC in 3:17 (v/v) glacial acetic acid/DMSO and vortexed for 30 s. 10 μl of 1 M NaCNBH3 in water was added, and the reaction was heated at 90 °C for 30 min. The reaction was diluted to 150 μl in water and analyzed by reversed phase HPLC. Chromatography was conducted using a Waters C-18 Symmetry column (4.6 × 250 mm) housed in an Agilent 1100 series HPLC connected to a diode array detector monitoring a wavelength of 440 nm. The mobile phase was 100 mM ammonium acetate, pH 6.8 (Solvent A), and acetonitrile (Solvent B). Separation of the free 2-AMAC and the 2-AMAC GlcN was achieved using a gradient of 10–50% acetonitrile over 45 min at a flow rate of 1 ml/min. A 2-AMAC-labeled GlcN standard was used to establish the elution time of the 2-AMAC-labeled GlcN from the acid hydrolysis reaction of O-GlcNAc-modified Tau (14.7 min). A standard curve using free 2-AMAC was prepared and used to calculate an extinction coefficient (1600 M⁻¹ cm⁻¹) of 2-AMAC at 440 nm. This extinction coefficient was used to calculate the concentration of labeled GlcN in the samples, using the peak absorbance. The stoichiometry of O-GlcNAc modification was determined by dividing the concentration of GlcN liberated by the concentration of O-GlcNAc modified Tau in the reaction. The stoichiometry estimation was made for both the 4 and 6 h time points, and the values were not found to differ significantly (indicating that the reaction had gone to completion, as is consistent with the literature (37)) and were thus averaged and the appropriate S.D. value reported.

RESULTS

Generation of Diverse Substrate Proteins as Substrates for hOGA and hOGT—Assays of OGT and OGA processing of protein substrates are complicated by the limited number of O-GlcNAc-modified proteins identified that are also readily available in recombinant form at the high levels required for detailed kinetic studies. For OGA, the difficulty is compounded by the need to obtain such proteins in a form having significant levels of O-GlcNAc modification. To address these issues, we looked to previous reports that revealed a number of hOGT target proteins (23, 38) as well as studies showing that co-expression of substrate proteins with hOGT in E. coli results in high levels of O-GlcNAc modification (33, 39). Based on these reports, we selected the genes encoding TAB1, CaMKIV, CARM1, microtubule-associated protein Tau, and Nup62 for cloning and co-expression with hOGT to yield O-GlcNAcylated forms of these proteins. Immunoblot analysis using an anti-O-GlcNAc antibody CTD110.6 revealed that O-GlcNAcylated forms were obtained for TAB1, CaMKIV, CARM1, Tau, and Nup62 after treatment with 6 M HCl (Fig. 1, bottom). The results showed that hOGT and hOGA processing of protein substrates from the co-expression system are O-GlcNAc-modified (Fig. 2A, bottom).

We determined the O-GlcNAcylation levels on each protein using two independent methods whenever possible. We first used electrospray ionization mass spectrometry (supplemental Fig. 1) and observed that proteins were glycosylated with varying numbers of O-GlcNAc residues on each protein molecule; TAB1 showed up to five O-GlcNAc sites, whereas both CaMKIV and Tau showed up to two sites. By taking into account the relative abundance of each species as observed by integration from the reconstructed mass spectra, we were able to calculate the average stoichiometry for TAB1 to be 2.1, for CaMKIV to be 0.96, and for Tau to be 0.61 O-GlcNAc residues per protein.
molecule (supplemental Table 2). Unfortunately, CARM1 and Nup62 failed to give readily interpretable spectra. We therefore used an alternative approach to determine the stoichiometry of O-GlcNAc residues on all five modified proteins. This approach involved measuring the amount of GlcNAc liberated during prolonged enzymatic digestion of O-GlcNAc from the protein substrates. To accomplish this aim, we used a bacterial homologue of OGA (BtGH84), which can remove O-GlcNAc from diverse protein substrates (32), and evaluated its ability to completely digest O-GlcNAc from these protein substrates. As judged using the CTD110.6 antibody, we found that, except for CaMKIV, O-GlcNAc could be essentially entirely removed from all proteins (Fig. 2B). The amount of enzymatically liberated GlcNAc was quantified using HPAEC-PAD analysis in conjunction with an internal standard and a standard curve. We found the average number of O-GlcNAc residues per molecule of protein substrate determined using this approach was 2.7 ± 0.1 for TAB1, 0.10 ± 0.03 for CaMKIV, 1.0 ± 0.1 for CARM1, 1.4 ± 0.1 for Nup62, and 0.38 ± 0.15 for Tau. Although there are various sources of error inherent to both the mass spectrometry and BtGH84 total O-GlcNAc hydrolysis approaches, the consistency of the results obtained for both Tau and TAB1 supports the use of these methods (Table 1). A further analytic method was used to verify these approaches to determining the O-GlcNAc stoichiometry. We used Tau as the substrate protein sample to analyze using this method because this allowed testing of both the MS and total enzymatic digestion approaches, the consistency of the results obtained for both approaches, the accuracy of the methods, and therefore allowed direct comparison of the results of these three independent approaches. Acid hydrolysis was used to remove the O-GlcNAc modification from modified Tau to liberate glucosamine. The glucosamine was labeled with 2-aminoacridone and quantified by reversed phase HPLC. This approach gave a stoichiometry of 0.40 ± 0.06 O-GlcNAc residues per molecule of Tau, which was consistent with the values obtained using the other two methods.

One important exception, however, is that O-GlcNAcylated CaMKIV showed markedly lower stoichiometry when estimated by enzymatic digestion as compared with mass spectrometry, almost certainly due to the incomplete BtGH84-catalyzed hydrolysis of O-GlcNAc from CaMKIV (Fig. 2B). Therefore, although enzymatic digestion is a useful approach, and digestion can be validated qualitatively by immunoblotting, mass spectrometric analysis is probably a more reliable method for proteins for which spectra can be obtained. The total acid-catalyzed hydrolysis method used with Tau supports the use of this enzyme digestion approach in cases when complete digestion can be verified. In cases where enzymatic digestion is found to be incomplete, using either MS or total acid-catalyzed hydrolysis methods is advised.

Human OGA Kinetics on TAB1, CaMKIV, CARM1, Nup62, Tau, and Ser-208 OG-Tau Peptide—Using the O-GlcNAc-modified protein substrates described above, hOGA kinetics were carried out at 37 °C and pH 7.4 over a period of 60 min using 1 μM hOGA. To ensure substrate depletion did not complicate the kinetic analysis, we analyzed the progress of the reactions at several time points; both immunoblot and HPAEC-PAD analyses showed that O-GlcNAc hydrolysis remained linear over the entire assay period for each of the protein substrates (supplemental Fig. 2).

The limited solubility of most of these protein substrates precluded using them at saturating concentrations. For this reason, we were limited to using concentrations of less than 60 μM for most of the proteins studied. We find that the initial velocity of the reactions increased linearly with respect to O-GlcNAc-modified protein substrate concentration. Under the conditions of the assay, this linear region can be described by a second order rate equation in which the pseudo-second order rate constant is given by $k_{cat}/K_m$ (Fig. 3, A–E, and Table 1). Only O-GlcNAc modified Tau is highly soluble, which enabled us to use Tau protein concentrations of up to 3 mM. In this way, we obtained initial velocities over a wide range of O-GlcNAc-modified Tau concentrations, observing traditional saturation kinetics and enabling the determination of the Michaelis-Menten parameters ($k_{cat} = 1.50 \times 10^{-3} \pm 0.07 \times 10^{-3} \mu M$ GlcNAc s$^{-1}$ μM hOAG$^{-1}$, $K_m = 0.8 \pm 0.1$ mm modified Tau, and $k_{cat}/K_m = 1.8 \pm 0.2 \mu M$ GlcNAc s$^{-1}$ μM hOGA$^{-1}$ μM modified Tau$^{-1}$; Fig. 3E). The $k_{cat}/K_m$ value established in this way agrees quite well with the value measured for Tau obtained by using substrate concentrations well below the $K_m$ value and extracting the pseudo-second order rate constant from the slope of reaction velocity versus protein substrate concentration ($k_{cat}/K_m = 1.34 \pm 0.07 \mu M$ GlcNAc s$^{-1}$ μM hOGA$^{-1}$ μM modified Tau$^{-1}$; Fig. 3E, inset). The agreement of these two values supports our approach of using a series of low concentrations of protein substrates to obtain the pseudo-second order rate constants for hOGA. Because a determinant of the substrate concentration in these hOGA assays is not only the protein concentration but also the stoichiometry of O-GlcNAc modification on each of the protein substrates, the $k_{cat}/K_m$ values for each substrate can be considered to be more meaningful.

**Table 1**

| O-GlcNAc-modified protein | $k_{cat}/K_m$ (μM GlcNAc s$^{-1}$ μM hOAG$^{-1}$) | Modification ratio (GlcNAc to protein) | $k_{cat}/K_m$ (μM GlcNAc s$^{-1}$ μM hOAG$^{-1}$) |
|---------------------------|-----------------------------------------------|----------------------------------------|-----------------------------------------------|
| TAB1                      | 31.9 ± 0.8                                    | 2.7 ± 0.1 (MS = 2.1)                   | 11.8 ± 0.5                                    |
| CaMKIV                    | 0.40 ± 0.2                                    | 0.10 ± 0.03 (MS = 0.96)                | 0.42 ± 0.2                                    |
| CARM1                     | 8.5 ± 0.5                                     | 1.0 ± 0.06                             | 8.5 ± 0.7                                     |
| Nup62                     | 1.4 ± 0.1                                     | 0.4 ± 0.1 (MS = 0.61)                  | 4 ± 1                                         |
| Tau                       | 1.34 ± 0.07                                   | 1                                       | 68 ± 4                                        |

*The O-GlcNAc modification levels determined by BtGH84 complete hydrolysis of the modified substrate were used in the standardization by O-GlcNAc level except for CaMKIV. A more accurate estimation of O-GlcNAc level on CaMKIV was determined by MS analysis due to the inability of BtGH84 to remove O-GlcNAc.

*This value is an underestimate because digestion of O-GlcNAc from CaMKIV by BtGH84 was incomplete.
when the extent of O-GlcNAcylation of each target protein is taken into account using the O-GlcNAc/protein ratio estimated from HPAEC-PAD analysis (Table 1). Interestingly, the $k_{\text{cat}}/K_m$ values of hOGA obtained in this way for each of the target proteins, with the exception of CaMKIV, are strikingly similar. It is interesting in this regard that CaMKIV was a poor substrate for both hOGA and BtGH84 as compared with the other protein substrates we tested; the significance of the similarity of $k_{\text{cat}}/K_m$ values and the rationale for the difference observed with CaMKIV will be discussed below and under “Discussion.” hOGA kinetics were similarly measured on the synthetic Tau-derived peptide, which was stoichiometrically O-GlcNAc-modified at serine 208 (Ser-208 OG-Tau peptide) (Fig. 3E and Table 1). The $k_{\text{cat}}/K_m$ value was determined to be $68 \pm 4 \mu\text{M GlcNAc} \cdot \text{s}^{-1} \cdot \mu\text{M hOGA}^{-1} \cdot \text{M O-GlcNAc on the modified peptide}^{-1}$, a value which is $\sim$20-fold higher than on the full-length Tau protein.

**Human OGT Kinetics on TAB1, CaMKIV, CARM1, Nup62, and Tau**—We next performed hOGT kinetics on the protein substrates we had examined with hOGA using an established radioactive hOGT assay (28). First, an appropriate hOGT concentration to use and an assay period over which hOGT activity remained linear needed to be established; we found that linear rates were observed when using hOGT concentrations of between 100 and 500 nM over an assay time of 60 min (supplemental Fig. 3). hOGT itself is observed to become O-GlcNAc-modified when co-expressed with the protein substrates in *E. coli*. Therefore, these assays could be complicated by background rates arising from OGT-catalyzed modification of hOGT. For this reason, we performed control assays containing hOGT alone. The amount of O-GlcNAc transferred to hOGT in the absence of acceptor protein was found in all cases to be less than 10% of total O-GlcNAc modification in the presence of the protein substrate. These control values were in all
cases subtracted from the rates obtained in the presence of the protein substrates.

We also measured the kinetic parameters for UDP-GlcNAc as a substrate when using each of the protein acceptor substrates. Surprisingly, we find that the $K_{m,\text{app}}(\text{UDP-GlcNAc})$ values for UDP-GlcNAc varied, depending on the protein substrate used: 8.6 ± 0.7 μM for TAB1, 25 ± 3 μM for CaMKIV, 26 ± 3 μM for CARM1, and 1.3 ± 0.1 μM for Nup62 (supplemental Fig. 4A and Table 3). Interestingly, under these conditions, recombinant Tau protein was not O-GlcNAc-modified by hOGT at a detectable level. We also used immunoblotting, by probing with CTD110.6, as an independent method to qualitatively evaluate the results from the radioactive assays. The immunoblot results were in accord with the radioactivity assays; they also showed that O-GlcNAc modification of Tau does not occur to a measurable level even at a concentration of 100 μM UDP-GlcNAc (supplemental Fig. 4B). Because the CTD110.6 antibody was shown to detect O-GlcNAc-modified Tau generated from the co-expression system (Fig. 2A), these observations suggest that full-length hOGT has limited activity against Tau, at least in vitro, and the findings are consistent with previous qualitative data showing that Tau is a relatively poor substrate for the full-length hOGT isoform used here as well as the other isoforms (41).

To assess the kinetic parameters governing the action of hOGT on protein substrates, including TAB1, CaMKIV, CARM1, and Nup62, we carried out radioactive hOGT assays using fixed concentrations of UDP-GlcNAc that were at least 2-fold above the measured $K_{m,\text{app}}(\text{UDP-GlcNAc})$ value for each protein substrate: 20 μM (specific activity of 0.14 Ci/mmol) for TAB1 and Nup62 and 100 μM (specific activity 0.092 Ci/mmol) for CaMKIV and CARM1. A range of protein acceptor concentrations extending to the limit of solubility of each protein was used, and in all cases, we observed traditional saturation Michaelis-Menten kinetics (Fig. 4). Once again, however, even at high concentrations of Tau, it was not possible to detect any full-length hOGT activity against Tau. The $K_{m,\text{app}}(\text{protein})$ value obtained for Nup62 using this assay is similar to a previously reported value (1.2 μM) (26). More generally, it is notable that the $K_{m,\text{app}}(\text{protein})$ values determined for each substrate are remarkably similar (2–7 μM), whereas greater variation is observed in the $k_{\text{cat}}$ values (Table 2). The potential significance of this observation will be discussed below.

**In Vitro O-GlcNAc Modification Dynamics for Protein Substrates**—Obtaining hOGT and hOGA kinetic parameters for processing of the same protein substrates allows some insight regarding the dynamics of O-GlcNAc modification. Indeed, the dynamic regulation of O-GlcNAc levels on a specific protein will be governed in vitro by the velocity of the installation of O-GlcNAc catalyzed by OGT ($v_{\text{ON}}$) and the velocity of the removal of O-GlcNAc catalyzed by OGA ($v_{\text{OFF}}$), both of which are directly proportional to second-order rate constants established for each enzyme-substrate pair. To this end, we summarize (Table 3) the results obtained using hOGA acting on O-GlcNAc-modified TAB1, CaMKIV, CARM1, and Nup62 as well as the unmodified proteins acted upon by hOGT. The ratio of $k_{\text{cat}}/K_{m}$ for the hOGT-catalyzed reaction ($k_{\text{cat}}/K_{m,\text{OGT}}$) which is proportional to $v_{\text{ON}}$ and $k_{\text{cat}}/K_{m}$ for the hOGA-catalyzed reaction ($k_{\text{cat}}/K_{m,\text{OGA}}$, which is proportional to $v_{\text{OFF}}$) therefore provides an indication of the relative extent...
to which these proteins are likely to be modified in the presence of specified concentrations of hOGA, hOGT, and UDP-GlcNAc, in the absence of other factors. Thus, a high 
\((k_{\text{cat}}/K_m)_{\text{OGT}}/(k_{\text{cat}}/K_m)_{\text{OGA}}\) ratio obtained for one protein suggests that the stoichiometry of O-GlcNAc will be higher than for a protein having a low 
\((k_{\text{cat}}/K_m)_{\text{OGT}}/(k_{\text{cat}}/K_m)_{\text{OGA}}\) ratio. The values we observe for CaMKIV are notable in that hOGA acts poorly on this protein; the rationale and potential significance of this observation will be discussed below. The 
\((k_{\text{cat}}/K_m)_{\text{OGT}}/(k_{\text{cat}}/K_m)_{\text{OGA}}\) ratio for the rest of the protein substrates ranged from 2 to 14, with Nup62 showing the largest value (Table 3).

To test these predictions regarding expected relative stoichiometries on protein substrates in vitro and to evaluate the proposed regulatory interaction between OGA and OGT (22), we incubated TAB1 or Nup62 with equal concentrations of hOGA and hOGT as well as with radiolabeled UDP-GlcNAc. As a control experiment, we performed the same experiment but used the D174A mutant of hOGA in place of the wild type (WT) enzyme, which is known to be catalytically inactive (42). In this way, we were able to judge whether hOGA protein itself influences hOGT activity. We find that O-GlcNAc levels on both TAB1 and Nup62 in the presence of both hOGT and hOGA as well as with radiolabeled UDP-GlcNAc. As a control experiment, we performed the same experiment but used the D174A mutant of hOGA in place of the wild type (WT) enzyme, which is known to be catalytically inactive (42). In this way, we were able to judge whether hOGA protein itself influences hOGT activity. We find that O-GlcNAc levels on both TAB1 and Nup62 in the presence of both hOGT and hOGA as well as with radiolabeled UDP-GlcNAc.

### TABLE 2

Summary of hOGT kinetic parameters on TAB1, CaMKIV, CARM1, Nup62, and Tau with respect to protein acceptor concentration

| Protein substrate | \(K_m\) (\(\mu\)M GlcNAc s\(^{-1}\) \(\mu\)M hOGT\(^{-1}\)) | \(k_{\text{cat}}/K_m\) ratio | \(k_{\text{cat}}/K_m\) (\(\mu\)M GlcNAc s\(^{-1}\) \(\mu\)M hOGT\(^{-1}\) M protein substrate\(^{-1}\)) |
|-------------------|---------------------------------|----------------|--------------------------------------------------|
| TAB1              | 0.57 ± 0.07                     | 7 ± 2          | 80 ± 20                                          |
| CaMKIV            | 0.077 ± 0.002                   | 2.2 ± 0.3      | 35 ± 5                                           |
| CARM1             | 0.073 ± 0.003                   | 4.7 ± 0.7      | 16 ± 3                                           |
| Nup62             | 0.93 ± 0.05                     | 5.8 ± 0.7      | 160 ± 40                                        |
| Tau               | <0.001                          | Not measurable | Not measurable                                    |

### TABLE 3

Summary of the apparent pseudo-second order rate constants for O-GlcNAc modification on TAB1, CaMKIV, CARM1, Nup62, and Tau by hOGT and hOGA and the ON/OFF ratio

| Protein substrate | ON rate constant (\(\mu\)M GlcNAc s\(^{-1}\) \(\mu\)M hOGT\(^{-1}\) M protein substrate\(^{-1}\)) | OFF rate constant (\(\mu\)M GlcNAc s\(^{-1}\) \(\mu\)M hOGA\(^{-1}\) O-GlcNAc on modified protein\(^{-1}\)) | “ON” to “OFF” ratio |
|-------------------|-------------------------------------------------|-------------------------------------------------|-------------------|
| TAB1              | 80 ± 20                                         | 11.8 ± 0.5                                      | 7 ± 2             |
| CaMKIV            | 35 ± 5                                          | 0.42 ± 0.2                                      | 80 ± 20           |
| CARM1             | 16 ± 3                                          | 8.5 ± 0.7                                       | 2.0 ± 0.4         |
| Nup62             | 160 ± 40                                        | 11.4 ± 0.9                                      | 14 ± 6            |
| Tau               | Not measurable                                  | 4 ± 1                                           | Not measurable    |

### OGT and OGA Kinetic Analysis on Protein Substrates

**Protein Modification**—We found that O-GlcNAc modified CaMKIV was a remarkably poor substrate for both hOGA and BtGH84, as demonstrated using both immunoblot (Fig. 2B and supplemental Fig. 2, A and B) and HPAEC-PAD analysis (Fig. 3B and supplemental Fig. 2C). The observation that it is a poor OGA substrate appears inconsistent with previous *in vivo* data, wherein it was shown that O-GlcNAc levels of CaMKIV decreased in a highly dynamic manner upon stimulation (4). In that study, Dias et al. (4) showed that ionomycin induced both phosphorylation of residue Thr-200 and decreased CaMKIV O-GlcNAc levels. Thr-200 itself is not an O-GlcNAc site, indicating that direct competition is not the explanation. Further, consistent with phosphorylation playing a key role, a pseudophosphorylation mimic at Thr-200 (T200E) resulted in decreased mutant CaMKIV O-GlcNAc levels within cells. We felt that our kinetic results could contribute to understanding the molecular basis by which Thr-200 phosphorylation and the T200E pseudophosphorylation mutation act to decrease O-GlcNAc levels. We also speculated that the results could be attributable to increased hOGA and/or decreased hOGT activity toward phosphorylated or pseudophosphorylated CaMKIV as compared with WT CaMKIV. To address this question, we used the CaMKIV T200E (CaMKIV\(_{T200E}\)) mutant as a Thr-200
pseudophosphorylation mimic, as reported previously (4). We expressed the CaMKIV_{T200E} mutant protein and its O-GlcNAc-modified form using the co-expression system and found that the mutant has less than one-third the O-GlcNAc levels of the WT protein (CaMKIV_{WT}) expressed under the same conditions (supplemental Fig. 1D and Table 2). O-GlcNAc-modified CaMKIV_{T200E} and CaMKIV_{WT} were assayed as substrates for hOGA. Notably, we found that hOGA showed increased activity on the T200E mutant as compared with the WT protein (following normalization of O-GlcNAc levels between WT and mutant CaMKIV) (Fig. 6A). The bacterial OGA homologue, BtgH84, however, demonstrated negligible activity against both CaMKIV_{T200E} and CaMKIV_{WT} (as determined by densitometry analysis on the immunoblot shown in Fig. 6A) (data not shown), suggesting that the pseudophosphorylation mimic exerts an hOGA-specific effect. Taking into account the stoichiometry of O-GlcNAc on CaMKIV_{T200E} (0.27 residues of O-GlcNAc/molecule of CaMKIV_{T200E}), we found that the pseudo-second order rate constant governing hOGA action on CaMKIV_{T200E} is 14 ± 2 μM O-GlcNAc  s⁻¹ μM hOGA⁻¹ M O-GlcNAc on CaMKIV_{T200E}. This value is 35-fold higher than the value measured for CaMKIV_{WT}, 0.41 ± 0.03 μM O-GlcNAc  s⁻¹ μM hOGA⁻¹ M O-GlcNAc on CaMKIV_{WT} (Fig. 6B and Table 4). hOGT showed k_{cat} values toward CaMKIV_{T200E} and CaMKIV_{WT} that were similar, but the K_{m} for CaMKIV_{T200E} was measured to be 12.0 ± 0.4 μM, which is 5-fold higher than for the WT protein (2.4 ± 0.4 μM) (Fig. 6C and Table 4). Taking into account the effect on both OGA and hOGT activity as reflected in the (k_{cat}/K_{m})_{OGT}/(k_{cat}/K_{m})_{OGA} ratio, we predict that the O-GlcNAc modification of CaMKIV_{WT} is favored 150-fold over CaMKIV_{T200E} (Table 4). These in vitro kinetics using CaMKIV_{WT} and CaMKIV_{T200E} indicate that phosphorylation at Thr-200 is expected to significantly decrease O-GlcNAc modification of CaMKIV, a finding that is consistent with the changes seen using ionomycin and the CaMKIV_{T200E} pseudophosphorylation mutant within cells (4). Thus, the effects of Thr-200 phosphorylation on CaMKIV O-GlcNAc levels seen within cells can probably be accounted for by differences in the kinetic parameters of OGA and OGT toward CaMKIV_{T200E} as compared with CaMKIV_{WT}.  

**DISCUSSION**

Despite the large numbers of nucleocytoplasmic proteins now known to be modified with O-GlcNAc and the increasing number of modified residues that have been mapped, there is no obvious consensus sequence that governs which residues are modified. Indeed, knowledge regarding the molecular basis by which OGT and OGA recognize their protein substrates is limited. Structures of a bacterial homologue of hOGT (28, 43), in conjunction with a structure of the tetratricopeptide repeats of hOGT (44), and more recently of hOGT itself (45), have suggested that substrates bind within a superhelical groove formed from a series of tetratricopeptide repeats that extends right into the glycosyltransferase active site. There is little understanding, however, of the elements within proteins that are recognized. Furthermore, only one protein (Nup62) has been examined as a substrate of OGT using enzyme kinetics (26, 28, 45). For OGA, our knowledge of how this enzyme recognizes substrates is still less understood. Structures of bacterial homologues of hOGA have been determined (32, 46, 47), and a conserved peptide substrate binding groove has been proposed (47); however, no detailed kinetic studies evaluating hOGA activity on O-GlcNAc-modified proteins have been performed. To gain insight into the processing of protein substrates by these two enzymes, we performed a series of kinetic studies examining both hOGT and hOGA activity on five structurally diverse proteins that can be recombinantly expressed at high levels within *E. coli* both in an O-GlcNAc-modified form and in their unmodified state.

One limitation stemming from the recombinant process used to generate these O-GlcNAc-modified proteins is that there are heterogeneous populations with O-GlcNAc present at one or more different sites. The generation of entirely homogeneous O-GlcNAc modified protein populations using this expression system is not readily achievable. Nevertheless, some insight into the molecular determinants driving hOGA-catalyzed hydrolysis can be gained by considering the relative values of the kinetic parameters obtained for the structurally diverse series of protein substrates studied here. Indeed, the measured k_{cat}/K_{m} values for hOGA on CARM1, TAB1, Tau, Nup62, and CaMKIV_{T200E} differ only by 4-fold, which suggests that the heterogeneous extent of O-GlcNAc modification does not signifi-

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**FIGURE 5.** The dynamic equilibrium established by simultaneous treatment of either TAB1 or Nup62 with hOGA and hOGT. TAB1 (A) and Nup62 (B) were incubated with equal concentrations of hOGT and hOGA and [³H]UDP-GlcNAc. Assays containing catalytically inactive hOGA (D174A) showed linear rates over the entire assay period. Steady state O-GlcNAc levels were observed after 120 min with TAB1 and 160 min with Nup62. The data are displayed as an average of triplicate determinations with associated S.D. values shown as error bars.
cantly complicate analysis. Furthermore, in the case of Tau, for which we were able to measure the $K_m$ value, we find that the data are well fitted by the Michaelis-Menten equation. Given that different populations of Tau are produced using this recombinant co-expression system, the data further support the view that some heterogeneity in O-GlcNAc levels on these proteins does not notably complicate the kinetic analysis of hOGA activity.

The second order rate constant, $k_{cat}/K_m$, governs the rate of the reaction from free enzyme and substrate to the transition state of the first irreversible step of enzyme catalyzed reactions. This value therefore provides an indication as to how the structures of different substrates contribute to making them better or worse substrates. The similarity in $k_{cat}/K_m$ values for hOGA action on these protein substrates suggests that they are all similarly good substrates despite the only clear commonality, in terms of substrate structure, being the O-GlcNAc residue and peptide backbone. Similarly, hOGA-catalyzed hydrolysis of O-GlcNAc from a synthetically modified peptide derived from Tau, Ser-208 OG-Tau peptide, has a $k_{cat}/K_m$ value that is only around 20-fold higher than for the full-length Tau protein. In this regard, it is worth noting that previous kinetic studies using methyl 2-acetamido-2-deoxy-$\beta$-D-glucopyranoside (MeO-GlcNAc) revealed a $k_{cat}/K_m$ value of 440 ± 50 $\mu\text{M GlcNAc}^{-1} \cdot \mu\text{M hOGA}^{-1} \cdot \text{M MeO-GlcNAc}^{-1}$ for hOGA-catalyzed hydrolysis (48), a value that is 40–100-fold higher than the values we determined here for the protein substrates (3.6–11.8 $\mu\text{M GlcNAc}^{-1} \cdot \mu\text{M hOGA}^{-1} \cdot \text{M modified protein}^{-1}$) and around 6.5-fold higher than for the peptide substrate (68 $\mu\text{M GlcNAc}^{-1} \cdot \mu\text{M hOGA}^{-1} \cdot \text{M peptide substrate}^{-1}$) (Table 1). The $O$-GlcNAc-modified protein substrates studied here are therefore actually worse substrates than the peptide substrate, which is itself worse than the simple substrate MeO-GlcNAc. This result suggests that neither the protein component of O-GlcNAc-modified proteins nor the peptide backbone of proteins or peptides contains molecular features that are exploited by hOGA to facilitate its processing. Rather, the data indicate that only the sugar moiety plays a critical role in determining

### TABLE 4

Summary of the apparent second and pseudo-second order rate constants for O-GlcNAc modification on CaMKIV WT and T200E by hOGA and hOGT and the ON/OFF ratio

| Protein substrate | OGT assay (ON rate constant) | OGA assay (OFF rate constant) |
|-------------------|-----------------------------|-------------------------------|
|                   | $k_{cat}$ ($10^{-3} \mu\text{M GlcNAc} \cdot \mu\text{M hOGA}^{-1}$) | $K_m$ (M GlcNAc) | $k_{cat}/K_m$ ($\mu\text{M GlcNAc} \cdot \mu\text{M hOGA}^{-1} \cdot \text{M modified protein}^{-1}$) | $k_{cat}/K_m$ ($\mu\text{M GlcNAc}^{-1} \cdot \mu\text{M hOGA}^{-1} \cdot \text{M O-GlcNAc on modified protein}^{-1}$) | ON/OFF ratio |
| CaMKIV WT         | 0.073 ± 0.003                | 2.4 ± 0.4                     | 0.39 ± 0.03 | 0.41 ± 0.03 | 78 ± 5 |
| CaMKIV T200E      | 0.080 ± 0.005                | 12 ± 2                        | 3.9 ± 0.5  | 14 ± 2      | 2 ± 0.2 |

The pseudophosphorylation CaMKIV T200E mutant affects the activity of both hOGA and hOGT in favor of producing overall lower O-GlcNAc levels. A, top, processing of O-GlcNAc-modified CaMKIV T200E mutant (T200E) by hOGA is more rapid than processing of CaMKIV WT (WT). Bottom, BtGH84 processing of CaMKIV WT and CaMKIV T200E occurs at the same negligible rate. B, HPAEC-PAD analysis of hOGA kinetics on O-GlcNAc-modified CaMKIV T200E and CaMKIV WT shows a 10-fold difference in the pseudo-second order rate constant. C, measurement of hOGT activity on CaMKIV T200E and CaMKIV WT shows hOGT transfers O-GlcNAc with a similar $k_{cat}$ value, but CaMKIV T200E has a 5-fold higher $K_m$ value than CaMKIV WT. The data in B and C are displayed as an average of triplicate determinations with associated S.D. values shown as error bars.

FIGURE 6.
how good a substrate is for hOGA. The faster processing of the small molecule substrate and peptide as compared with the protein substrates probably stems from their difference in size, which inversely influences their collisional frequency. The data thus support the O-GlcNAc residue being the key determinant for hOGA-catalyzed processing of protein substrates. Notably, the observation that CaMKIV shows a lower \( k_{cat}/K_m \) value for processing by hOGA is not inconsistent with this view. Rather, it suggests that some structural feature within CaMKIV impairs hOGA-catalyzed removal of O-GlcNAc from this protein. Indeed, the pseudophosphorylation of Thr-200 relieves this effect, and CaMKIV\( _{T200E} \) shows a \( k_{cat}/K_m \) value comparable with that seen for the other O-GlcNAc-modified protein substrates. Following on from these observations, the absence of saturation kinetics for most of the O-GlcNAc-modified protein substrates indicates that their \( K_m \) values must be at least in the high micromolar range. The \( K_m \) value for Tau was estimated to be 0.8 ± 0.1 mM, which differs only 3-fold when compared with the \( K_i \) value measured for MeO-GlcNAc with hOGA (\( K_i = 2.2 ± 0.3 \) mM; data not shown). Although \( K_m \) is a composite of kinetic parameters, the similarity of these values is consistent with hOGA using the sugar as the key structural determinant to mediate both substrate binding and catalysis.

hOGT is a bisubstrate enzyme, most likely operating by an ordered sequential mechanism (45), which therefore encouraged us to evaluate the effects of independently varying both UDP-GlcNAc and protein substrate concentrations. We find that the \( K_{m,app}(UDP-GlcNAc) \) values vary, depending on which protein substrate is used. Nup62 has a \( K_{m,app}(UDP-GlcNAc) \) value of 1.3 µM, whereas CARM1 and CaMKIV have \( K_m \) values of over 20 µM. Because the UDP-GlcNAc concentration in cells typically ranges from single to low double digit micromolar (49, 50), the modification levels of some protein substrates, such as CaMKIV and CARM1, could vary significantly in response to UDP-GlcNAc availability, whereas others such as Nup62 are likely to vary to a lesser extent. When varying the protein substrate concentration and keeping UDP-GlcNAc constant, clear saturation kinetics were observed for all proteins except Tau, which was not modified at a detectable level. hOGT assays show \( K_{m,app}(Protein) \) values for the protein substrates that are quite similar, ranging between 2 and 7 µM, but \( k_{cat} \) values that vary by up to 20-fold for different protein substrates. These \( K_{m,app}(Protein) \) values are much lower than the \( K_m \) values found for peptide substrates (27, 45), which is consistent with previous observations showing that the tetratricopeptide repeat domains of hOGT are directly involved in binding protein substrates (26, 51). This observation suggests that hOGT may have a similar binding affinity for the different protein substrates examined here, except Tau, yet differences in protein substrate structure probably alter the ability of hOGT to transfer the GlcNAc residue to the protein once bound. That Tau can be modified in the co-expression system but not under the assay conditions may perhaps be explained by a need for much higher UDP-GlcNAc concentrations. Unfortunately, it is not feasible to test these conditions due to the relatively low specific activity and concentration of the commercially available radioactive material. Other reasons for the lack of modification on Tau in vitro might include, for example, the need for high Tau concentrations or the absence of certain cellular machinery that is present in the prokaryotic system and/or in human cells. In any event, hOGT shows a wider range of activities toward the protein substrates examined here than does hOGA, indicating that OGT is probably the senior partner in the dynamic cycle and may generally dictate which proteins are more heavily modified with O-GlcNAc.

Comparison of the hOGT and hOGA kinetics data shows that hOGT has a higher apparent second order rate constant than the hOGA pseudo-second order rate constant for the protein substrates assayed here, with the exception of Tau. This result suggests that, if hOGT and hOGA are present at equivalent concentrations within cells, protein substrates are more likely to be O-GlcNAc-modified, which is consistent with our observations. However, there is no study, to our knowledge, that measures the relative concentration or activity of hOGT and hOGA in any cell line or tissue, so these values provide only a prediction of the relative cellular O-GlcNAc levels on these proteins. Recently, Rexach et al. (52) developed a method to quantify the extent of O-GlcNAc modification of proteins using a resolvable polyethylene glycol mass tag. Their results demonstrated that Nup62 from adult rat brain is heavily O-GlcNAcylated, yielding a range of O-GlcNAc-modified species, an observation that is consistent with previous findings showing that Nup62 is heavily modified in cells (53). The results of the hOGT and hOGA kinetics also enable us to estimate the relative O-GlcNAc levels expected on different protein substrates at fixed concentrations of UDP-GlcNAc and both of the enzymes, assuming that no other factors are operative. To investigate this idea further, we added equivalent concentrations of hOGT and hOGA to an assay containing TAB1 or Nup62 and \([\text{H}]\text{UDP-GlcNAc}\) and monitored the extent of radioactive O-GlcNAc incorporation over time. We find, as expected, that the reaction reached a dynamic equilibrium as the rate of hOGA processing of the O-GlcNAc-modified protein gradually increased to reach a balance with the rate of hOGT processing. At this steady state, the rate of hOGT activity matches the rate obtained in the assays using hOGT alone with either TAB1 or Nup62 as the protein substrate. Unfortunately, the same comparison cannot be made for hOGA because the level of O-GlcNAc modification on the protein substrates during the course of the experiment does not exceed the hOGA concentration, so Michaelis-Menten kinetics do not hold. The dynamic action of both hOGA and hOGT being present together with TAB1 or Nup62 resulted in a 2-fold higher O-GlcNAc level on Nup62 as compared with TAB1, results that match the prediction on the basis of kinetic measurements made when using either hOGT or hOGA independently.

There has been significant attention focused on the dynamic interplay between O-GlcNAc and O-phosphate. Interactions between these modifications may arise from the following mechanisms: 1) direct competition for the same serine or threonine residue; 2) modification at one site causing steric hindrance for a modifying enzyme at a proximal site; 3) the activities of OGT and OGA being altered upon phosphorylation; or 4) the activities of kinases or phosphatases being regulated by O-GlcNAc modification. Phosphorylation and O-GlcNAc have been proposed to synergistically regulate various processes (4,
O-GlcNAc-modified recombinant CaMKIV is highly resistant to hOGA and BtOGA hydrolysis, and we find that the pseudophosphorylation mutation CaMKIV<sub>T200E</sub> has a clear effect on the kinetic activity of both O-GlcNAc-regulating enzymes. hOGA showed a 10-fold increase in activity toward the CaMKIV<sub>T200E</sub> mutant compared with the WT CaMKIV, an observation in accord with a previous study showing that hOGA associated better with phosphorylated CaMKIV (4). The observation that the activity of BtGH84 remains unaffected by the mutation suggests that the effect of pseudophosphorylation, and by extension phosphorylation, of CaMKIV is a hOGA-specific effect. Although the experiments we performed cannot clarify the basis for this difference in susceptibility to hOGA processing, one possibility is that the pseudophosphorylation mutation may induce a conformational change making this region more susceptible to O-GlcNAc cleavage. Further studies will be needed to clarify the molecular basis for this difference in the susceptibility of CaMKIV to processing by hOGA. Although the effect is less pronounced, we also found that hOGT has decreased activity against the CaMKIV<sub>T200E</sub> mutant as compared with CaMKIV<sub>WT</sub>. The difference stems from a 5-fold increased value that suggests an alteration in the substrate affinity for hOGT. The effects of pseudophosphorylation of CaMKIV on hOGA and hOGT activity therefore cause a decrease in the overall level of O-GlcNAc modification, which can account for earlier observations made in cells showing that phosphorylation of CaMKIV leads to decreased CaMKIV O-GlcNAc levels (4). These observations offer a simple kinetic explanation as to how phosphorylation on protein substrates could act to regulate O-GlcNAc levels.

In summary, these in vitro experiments give insights into the dynamics of hOGA and hOGT activity on a series of diverse protein substrates. The data suggest that the O-GlcNAc residues themselves are the key structural element driving hOGA catalysis but that molecular features of proteins can impair their processing by hOGA. The variability of $K_{m,app}$ (UDP-GlcNAc) values observed with different protein substrates suggest that some proteins may have O-GlcNAc levels that are responsive to changes in cellular UDP-GlcNAc levels, whereas others may not. These studies also suggest that interaction of hOGA and hOGT in vitro does not alter the enzymatic activity of hOGT. These data also provide quantitative evidence that phosphorylation of protein substrates can influence their processing by both hOGA and hOGT. Finally, these collective results form a fundamental framework for understanding the kinetic relationship between OGA and OGT. The ability to manipulate this system should facilitate the design of well controlled in vitro experiments that will enable quantitative testing of cellular factors that could contribute to regulating the kinetic behavior of OGA and OGT, including, for example, accessory proteins and post-translational modifications, including phosphorylation.

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