Regulation of a Cytosolic and Nuclear O-GlcNAc Transferase

ROLE OF THE TETRATRICOPÉPTIDE REPEATS

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The O-GlcNAc transferase (OGT) is a unique nuclear and cytosolic glycosyltransferase that contains multiple tetratricopeptide repeats. We have begun to characterize the mechanisms regulating OGT using a combination of deletion analysis and kinetic studies. Here we show that the p110 subunit of the enzyme forms both homo- and heterotrimeric forms that appear to have different binding affinities for UDP-GlcNAc. The multimerization domain of OGT lies within the tetratricopeptide repeat domain and is not necessary for activity. Kinetic analyses of the full-length trimer and the truncated monomer forms of OGT suggest that both forms function through a random bi-bi kinetic mechanism. Both the monomer and trimer have similar specific activities and similar \( K_m \) values for peptide substrates. However, they differ in their binding affinities for UDP-GlcNAc, indicating that subunit interactions affect enzyme activity. The findings that recombinant OGT has three distinct \( K_m \) values for UDP-GlcNAc and that UDP-GlcNAc concentrations modulates the affinity of OGT for peptides suggest that OGT is exquisitely regulated by the levels of UDP-GlcNAc within the nucleus and cytoplasm.

O-GlcNAc is an abundant intracellular posttranslational modification consisting of a single N-acetylglucosamine O-linked to serine/threonine residues. Unlike other carbohydrate modifications, O-GlcNAc is not further modified and is found almost exclusively in the nucleus and cytoplasm. Since it was first described in lymphocytes (1) O-GlcNAc has been found on an ever increasing number of proteins, including RNA polymerase II and its transcription factors, nuclear pore proteins, tumor suppressor proteins, intermediate filaments, viral proteins, and oncoproteins (reviewed in Refs. 2–5). O-GlcNAc is an abundant and dynamic modification exhibiting properties more like phosphorylation than typical N- and O-linked glycosylation (2, 3). The O-GlcNAc modification (termed O-GlcNAcylation) has been suggested to play a direct role in regulating a number of cellular functions including protein synthesis (6, 7), neurofilament assembly (8), and transcription (9–11). In our laboratory, we have purified and characterized both a UDP-N-acetylglucosamine:peptide N-acetylglucosaminyl-transferase (O-GlcNAc transferase) (12) specific for the attachment of O-GlcNAc to proteins and a soluble N-acetyl-\( \beta \)-d-glucosaminidase (O-GlcNAcase) (13) specific for the removal of O-GlcNAc from proteins. These enzymes may work together to regulate the attachment and removal of O-GlcNAc in response to cellular signals in much the same way that kinases and phosphatases regulate protein phosphorylation.

Although significant progress has been made in our understanding of the distribution of O-GlcNAc in the cell, little is known about how the attachment of O-GlcNAc to proteins is regulated. The problem is significant because numerous proteins are O-GlcNAcylated, many at more than one site. A further complexity is added by the lack of a canonical consensus site for the attachment of O-GlcNAc to proteins (4, 5). Additionally, the O-GlcNAc modification turns over more rapidly then the protein backbone (14, 15) and is responsive to cellular stimuli (16, 17). Thus the cell must regulate not only which proteins to modify with O-GlcNAc but also which site(s) on a protein to modify in response to specific cellular signals.

The gene encoding the catalytic subunit of an O-GlcNAc transferase (OGT) was recently cloned and characterized from rat (18), human, and nematode (19). The protein (p110) encoded by this gene represents a novel glycosyltransferase that has a unique cellular distribution and shares no sequence or structural similarity to any previously described secretory glycosyltransferase (20, 21). The gene encoding OGT is found in all higher eukaryotes examined and has been highly conserved throughout evolution. It is modified by O-GlcNAc and tyrosine phosphorylation, which may play a role in the regulation of the OGT. Further examination of the protein sequence indicate that p110 can be divided into two distinct domains, the amino-terminal half of the protein containing multiple tetratricopeptide repeats (TPRs) and the carboxyl-terminal half of the protein representing a novel polypeptide believed to contain the catalytic portion of the enzyme. TPRs are found in a large number of proteins of diverse function and play a role in modulating a variety of cellular processes, including cell cycle (22–24), transcription regulation (25–27), and protein transport (28) by mediating specific protein-protein interactions (reviewed in Refs. 29 and 30). Thus, the TPR domain of p110 may represent a protein interaction domain that facilitates protein-protein interactions that regulate enzymatic activity.

In this paper, we begin to address the question of how the cell regulates the attachment of O-GlcNAc by further characterizing the p110 subunit of the OGT cloned from rat liver. Using the insect cell baculovirus system to overexpress p110, we show that it is the catalytic subunit of the enzyme and that different portions of the TPR domain are required for multimerization of the OGT and for enzymatic activity. We also find that recombinant p110 contains the same posttranslational modifications.
as p110 purified from rat. Kinetic studies suggest that OTG functions through a random bi-bi kinetic mechanism and that the protein has three distinct binding constants for the UDP-GlcNAc sugar donor. In addition we find that binding of UDP-GlcNAc is regulated by multimerization of the enzyme. These data suggest that multimerization and the intracellular concentration as well as binding of UDP-GlcNAc are among the mechanisms regulating OTG activity in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—Peptides were synthesized on a Perseptive Biosystems (Framingham, MA) automated peptide synthesizer and purified by reverse phase high performance liquid chromatography. Prepared Superoxide 12 PC 3.2/30, and Hi-Trap Chelating columns were from Amersham Pharmacia Biotech. Uridine diphospho-N-acetyl[6-3H]glucosamine (50 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Uridine diphospho-N-acetylglucosamine was from Oxford Glycosystems (Oxford, UK). Ultrafree Centrifugal Filter Devices were from Millipore Inc. All other reagents were of the highest quality available.

**General Recombinant DNA Techniques**—Restriction endonuclease digestions and ligations were carried out as described (31). Plasmids were isolated using Wizard Prep Kits (Promega) according to the manufacturer’s directions.

**DNA Sequencing**—Sequence information of the various fusion clones was obtained by automated DNA sequencing on an Applied Biosystems (Foster City, CA) model 373A automated DNA sequencer.

**Cloning of Full-length p110 into Baculovirus for Expression in Insect Cells**—The 5′-untranslated region of the previously cloned full-length cDNA fragment (18) was removed by digestion with BsaHI and replaced with a linker adaptor (CGCGATCGCGATGG), which adds a BamHI linker upstream of the restored ATG. The full coding region was excised with a linker adaptor (CGCGATCGCGATGG), which adds a BamHI linker upstream of the restored ATG. The full coding region was excised by digestion with BamHI and HindIII and ligated into pBlueBacHis A (Invitrogen). The resulting plasmid (pLK59) was recombined in vivo into baculovirus using the Bac-N-Blue Transfection kit (Invitrogen) as per the manufacturer’s protocol resulting in the viral strain VLK59.

**Creation of the TPR Deletion Mutants by Polymerase Chain Reaction**—Five oligonucleotides were synthesized (GGATCCGCAATTGAG, 5′-GACCCACGCGGAAATG-3′, 5′-ATGGGCGGCGGAAATG-3′, 5′-CGCGATCGCGATGG-3′) and PCR-amplified using the CKII peptide at 3 mM final concentration. The UDP-

**Concentration of UDP-GlcNAc**—UDP-GlcNAc was synthesized using the CKII peptide at 3 mM final concentration. The UDP-

**Purification of Recombinant p110 Constructs**—Insect cell pellets were resuspended in Buffer A (20 mM Tris, pH 7.9, 0.5 mM NaCl) and lysed by sonication (2 × 30 s pulses powered with a 550 Sonic Dismembrator; Fisher Scientific). Lysates were spun for 30 min at 30,000 × g, and the supernatant was filtered through a 0.45-μm filter and loaded at a flow rate of 1 ml/min onto a 1-ml Hi-Trap chelating column precharged with NiSO₄ as per the manufacturer’s instructions and pre-equilibrated with buffer A. The column was washed with 20 column volumes buffer A, followed by a 10-ml wash at 5% buffer B and then eluted with a 25-min linear gradient 5-100% buffer B (20 mM Tris, pH 7.9, 0.5 mM NaCl, 100 mM imidazole) at 1 ml/min. Fractions (1 ml) were collected throughout the separation were assayed for activity as described below. The fractions were also examined by SDS-PAGE followed by visualization with Coomassie G-250 staining (33) or by Western blot analysis (see below). The most pure active fractions were pooled and further purified using an ultrafree-15 concentrator per manufacturer’s directions. Buffer was exchanged by resuspending the concentrate in 12 ml of buffer B (20 mM Tris, pH 7.5, 20% glycerol, 1 mM dithiothreitol) and concentrating again. The concentrate was removed, and the concentrator was rinsed with 0.5 ml of buffer. The pooled concentrate (~600 μl) was brought to 40% glycerol, 1 mM dithiothreitol and annealed at −20 °C.

**O-GlcNAc Transferase Activity Assay**—Activity assays were performed as described previously (34) with minor modifications. Because the nickel affinity purified enzyme is concentrated in a salt-free buffer, the enzyme is assayed directly without desalting. For the assays described here the enzyme was diluted 1:25 with cold desalt buffer (20 mM Tris, pH 8, 20% glycerol, 0.02% NaN₃) just prior to use, and the reactions were started with the addition of 25 μl of the diluted enzyme mix. In addition to the previously described peptide substrate YSDDSPTSTST (9-mer peptide), an additional peptide PGGSTPVSANNM (CKII peptide) was used for kinetic analysis. A number of other peptides were also used (see Fig. 6A). All peptides were used at a final concentration of 3 mM unless otherwise noted. The peptides were eluted from the SP-Sepharose 6 Fast Flow (Amersham Pharmacia Biotech) and were cleaned up after the reaction on a Sep-pak C18 cartridge (Waters Corp.) as follows: cartridge was washed with 10 ml of methanol, 10 ml of 50 mM formic acid, the reaction was then loaded onto the cartridge, and the cartridge was washed with 10 ml of 50 mM formic acid, 10 ml of 50 mM formic acid containing 0.5 mM NaCl, and 10 ml of distilled H₂O. The peptides were eluted from the cartridge directly into scintillation vials with 2 × 1.5 ml of methanol. Units are defined as micromoles of GlcNAc transferred per min.

**Western Blot Analysis**—The proteins were separated by SDS-PAGE and transferred to polyvinylidine difluoride membrane (Millipore). Purified rabbit polyclonal IgG AL-25 (1:5000) (18) or monoclonal anti-phosphotyrosine (1:500) (Transduction Laboratories) was used as a primary antibody with anti-rabbit or anti-mouse IgG coupled to horse-radish peroxidase (Amersham Pharmacia Biotech) as the secondary antibody (1:20,000 dilution). Detection of the horseradish peroxidase activity was enhanced chemiluminescence and fluorography as described by the manufacturer (Amersham Pharmacia Biotech).

**Galactosyltransferase Labeling**—Nickel affinity purified recombinant enzyme (described above) was probed for terminal GlcNAc using 0.5 mM UDP-[3H]galactose (Amersham Pharmacia Biotech) as the secondary antibody (2.5 μCi/μl) (35). The labeled protein was brought to 1 ml with RIPA buffer (10 mM Tris, pH 8.15, 50 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS, 0.5% NaDOC) and incubated with AL-25 IgG (18) on ice for 3 h. The IgGs were then precipitated with protein A-Sepharose CL-4 B (Amersham Pharmacia Biotech). The resin was washed extensively in RIPA buffer followed by a wash in 50 mM Tris, pH 6.5. The resin was then resuspended in equivalent volumes of either SDS-PAGE sample buffer or in 0.5 M urea and resolved on a 7.5% SDS-acrylamide gel, and proteins tagged with [3H]galactose were detected by fluorography of the gel treated with 1 μM sodium salicylate.

**Gel Filtration Chromatography of Recombinant Proteins**—A portion (50 μl) of the nickel affinity purified recombinant enzyme was further concentrated by centrifugation in an Ultrafree-0.5 concentrator and exchanged into buffer F containing either 150 mM NaCl or 1 mM NaCl. The concentrate was then loaded onto a Superose 12 column using a Amersham Pharmacia Biotech Smart fast protein liquid chromatography system equilibrated in buffer F containing both 150 mM NaCl or 1 mM NaCl using a Amersham Pharmacia Biotech Smart fast protein liquid chromatography system, and the protein was eluted at 15 μl/min. The absorbance at 280 nm was followed, and 25-μl fractions were collected that were assayed for activity, and analyzed by SDS-PAGE followed by visualization with Coomassie G-250 staining (33) or by Western blot analysis (see above). In separate runs standards were loaded onto the column to calculate the apparent molecular weight values.

**Calculation of Michaelis-Menten Constants (Kₘ)**—The Kₘ values for both the CKII and 9-mer peptides were performed at 2.5 μM UDP-GlcNAc, and concentrations of peptide were varied from 5 μM to 15 μM, and the Kₘ values were determined by Eadie-Scatchard analysis (data not shown). Further kinetic studies were performed using only the CKII peptide. To determine the Kₘ for UDP-GlcNAc across the full spectrum of concentrations from 0.05 μM to 4.8 μM, similar analysis were performed using the CKII peptide at 3 mM final concentration. The UDP-
The p110 Subunit Is the Catalytic Subunit of the OGT—Photolabeling studies on the OGT purified from rat liver cytosol (34) suggested that the p110 subunit was the catalytic subunit of the enzyme. To confirm this hypothesis a His-tagged protein was expressed and purified from insect cells. These data indicate that the His-p110 subunit expressed and purified from insect cells is a good model for studying the mechanism(s) regulating OGT activity.

[6-3H]GlcNAc was cold diluted from 50 Ci/mmol to either 0.2 Ci/mmol for the lower concentration of UDP-GlcNAc or to 0.62 Ci/mmol at the higher concentrations of UDP-GlcNAc. Addition kinetic studies were performed on each fraction analyzed in the activity assays per fraction. All assays were performed in duplicate, and each experiment was performed at least twice.

RESULTS

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The specific activity (1.23 nmol/min/mg) of the purified His-p110 is comparable with OGT purified from liver (1.1 nmol/min/mg), and the Km for the YSDSPSTST (9-mer) peptide is the same (10 mM) for both enzymes. In addition the recombinant p110, like the liver OGT, exists as a trimer (described below) and bears the same posttranslational modifications present on liver purified OGT (described below). These data indicate that the His-p110 subunit expressed and purified from insect cells is a good model for studying the mechanism(s) regulating OGT activity.

His-p110 Is a Tightly Associated Trimer—OGT purified from liver is a heterotrimer composed of two p110 subunits and one highly related p78 subunit with an apparent molecular weight of 240 kDa (34). The apparent molecular weight of His-p110 was determined by gel filtration chromatography at both physiological salt concentrations (150 mM) and high salt concentrations (1 M). At both salt concentrations the apparent molecular weight was ~360 kDa, indicating that the His-p110 is a homotrimer and that the subunits are tightly associated. The absorbance at 280 nm and activity profiles at 1 M NaCl are shown in Fig. 2A. Although the homotrimer is not found in liver it may
represents the subunit composition of native OGT in tissues that do not contain the p78 subunit (18). Note that the contaminating 70-kDa band separates from the His-p110 under these conditions and that no other protein co-migrates with the His-p110 protein by G-250 Coomassie staining (data not shown).

**TPRs Are Required for Subunit-Subunit Interactions and Enzymatic Activity**—The amino-terminal half of the p110 subunit contains 11 tandem TPRs (18). TPRs are involved in modulating specific protein-protein interactions, suggesting that the TPR domain of p110 is responsible for the subunit interactions. To address this possibility and to determine whether the TPR domain is required for OGT activity, a series of amino-terminal deletion constructs removing 3, 6, 9, or all 11 TPRs were made. These constructs (referred to as pD3 pD6, pD9, and pD11 respectively) were expressed in insect cells and purified as described for the His-p110 construct (data not shown). The specific activity and size of each construct was determined as described above (summarized in Fig. 3).

The specific activities of pD3 and pD6 were similar (1.17 and 1.06 nmol/min/mg, respectively) to His-p110 (1.23 nmol/min/mg), indicating that only five TPRs are required for activity. pD9 and pD11 had no detectable activity (<0.002 nmol/min/mg); however, neither of the inactive constructs was expressed well in our system, and the large amounts of degraded protein present indicate that these truncated proteins are unstable and may not be folded correctly. Further studies were done only on the pD9 protein as it was slightly more stable than the pD11 protein.

pD3 runs as a trimer (apparent molecular weight, ~290 kDa) at physiological salt concentrations (Fig. 2B). However, at high salt concentrations (1 M NaCl) pD3 runs as a dimer (apparent molecular weight, ~175) (indicated by the asterisk in Fig. 2B), indicating that the full complement of TPRs play a role in stabilizing subunit interactions. Interestingly both the active pD6 and the inactive pD9 are monomers (apparent molecular weights, ~90 and ~70 kDa, respectively) at both high salt (data not shown) and at physiological salt concentrations (Fig. 2C and D). Thus, trimerization is not necessary for OGT activity in our standard assay.

**The Posttranslational Modifications of Recombinant p110**—The purified p110 is modified by both tyrosine phosphorylation and by O-GlcNAc. We wanted to examine the posttranslational modification state of the His-p110 as well as several of the deletion constructs. Western blot analysis of the nickel affinity purified proteins using an anti-phosphotyrosine antibody shows that both the His-p110 and pD6 but not the inactive pD9 are immunoreactive. This reactivity is blocked by the addition of 10 mM phosphotyrosine (Fig. 4A) but not 10 mM tyrosine (data not shown). Similar experiments using antibodies against phosphoserine and phosphothreonine showed no immunoreactivity (data not shown). Densitometry analysis indicates that His-p110 and pD6 are phosphorylated to a similar degree (data not shown); however, exact quantitation of the modification is not possible using this methodology.

We also probed these proteins with galactosyltransferase (see “Experimental Procedures”). Galactosyltransferase is a specific probe for terminal GlcNAc resides (35, 36) that is commonly used to detect O-GlcNAc by covalently labeling the GlcNAc with UDP-[^3H]galactose. Following the galactosyltransferase labeling the proteins were further purified by immuno precipitation with the AL-25 antibody. All three recombinant proteins are labeled with[^3H]galactose (Fig. 4B, lanes 1–3), indicating that they are modified by GlcNAc. The level of O-GlcNAc modification detected here is substoichiometric with approximately 0.1 O-GlcNAc residue/protein molecule for each construct examined. However, exact quantitation of the O-GlcNAc modification is complicated by many factors including the ability of the modification during purification and accessibility to the galactosyltransferase (35). Thus, it is important to keep in mind that this value reflects a lower limit and the exact level of O-GlcNAcylation may be somewhat higher.

**Binding Constants for UDP-GlcNAc**—It was previously reported that the $K_m$ for UDP-GlcNAc of the purified liver enzyme was 0.5 μM. However, these studies were conducted using UDP-GlcNAc concentrations only in the low micromolar range (0.05 μM to 10 μM) and used the 9-mer acceptor peptide, which does not contain a known in vivo O-GlcNAc attachment site. We wanted to examine the binding affinity for the purified p110 subunit over a much broader range of UDP-GlcNAc concentrations that better reflected the wide range of UDP-GlcNAc concentration seen in tissues (37). In addition, we used the peptide PGGSTPVSSANM (CKII), which represents an endogenous O-GlcNAc acceptor site. Three apparent $K_m$ values, 6, 35, and 217 μM, were calculated from the linear regions of the reciprocal plot from 0.05 μM to 4.8 mM UDP-GlcNAc (Fig. 5A). The OGT activity in rabbit reticulocyte lysate shows similar kinetics for the binding of UDP-GlcNAc. We performed similar studies on the pD6 protein and found that only two apparent N-terminal halves of the protein believed to contain the catalytic portion of the enzyme. The predicted molecular weight of each construct is based on the amino acid sequence, whereas the apparent molecular mass was determined by gel filtration chromatography at 150 mM NaCl. Each number represents the average molecular mass determined by three separate runs.

$^{1}$T. Matsuoka, D. Dellamanna, L. K. Kreppel, and G. W. Hart, manuscript in preparation.

$^{2}$R. S. Haltiwanger, personal communication.

[Table 1: Deletion Construct Specific Activity Predicted Apparent Complex Size](#)

| Deletion Construct | Specific Activity | Predicted MW (kDa) | Apparent MW (kDa) | Complex Size |
|--------------------|------------------|--------------------|------------------|-------------|
| His-p110           | 1.23             | 117                | 360              | trimer      |
| pD6                | 1.17             | 100                | 100              | trimer      |
| pD9                | 1.06             | <89                | 90               | monomer     |
| pD11               | <0.002           | 77                 | 70               | monomer     |

[Fig. 3: Separate portions of the TPR domain are required for multimerization and for activity. The name of each protein construct is next to the stick figure representation. The numbers in the open boxes indicate the number of TPRs remaining, the crosshatched boxes represent the carboxyl-terminal halves of the protein believed to contain the catalytic portion of the enzyme. The predicted molecular weight of each construct is based on the amino acid sequence, whereas the apparent molecular mass was determined by gel filtration chromatography at 150 mM NaCl. Each number represents the average molecular mass determined by three separate runs.]

[Fig. 4: Posttranslational modifications on insect cell expressed p110 and TPR deletion constructs. A, nickel affinity purified His-p110 (lane 1), pD6 (lane 2), and pD9 (lane 3) was electrophoresed on a 7.5% polyacrylamide gel and immunoblotted with AL-25, the anti-phosphotyrosine antibody PY-20 alone, or PY-20 in the presence of 10 μM free phosphotyrosine (P-Tyr). B, the same protein preparations were labeled with[^3H]galactose using galactosyltransferase as a probe for terminal GlcNAc residues, further purified by immunoprecipitated with AL-25. Immunopurified proteins were electrophoresed on a 7.5% polyacrylamide gel and visualized by fluorography. The positions of each protein are indicated by the arrows.]
K_m values, 6 and 60 μM, are present. Although these numbers are quite similar to the first two found for His-p110, there is clearly no apparent K_m in the 200 μM range for this protein.

Activity of OGT on Acceptor Substrates Is Dependent on UDP-GlcNAc Concentrations—Fig. 6 lists several peptides representing known in vivo sites of O-GlcNAcylation (bold type in Fig. 6A). However, only the CKII peptide is a substrate in our standard assay where the concentration of UDP-GlcNAc is 0.2–0.6 μM (data not shown). The observation that OGT has three separate binding constants for UDP-GlcNAc suggested that UDP-GlcNAc concentration could directly affect OGT activity. Thus, we would predict that some peptides that are poor acceptors substrates at low concentrations of UDP-GlcNAc would be better acceptors at higher UDP-GlcNAc concentrations. To test this hypothesis we examined the ability of the peptides listed in Fig. 6A to serve as acceptor substrates at four different UDP-GlcNAc concentrations (20, 50, 100, and 500 μM). Nearly all of the peptides became better acceptors as the concentration of UDP-GlcNAc increased with the exception of the CTD-2 peptide, which was a poor substrate under all conditions examined (Fig. 6B). The CKII peptide was the best acceptor at all UDP-GlcNAc concentrations (Fig. 6B, inset). The cMyc peptide showed the most dramatic change, as it was not an acceptor substrate at 20 μM UDP-GlcNAc and remained a poor acceptor until UDP-GlcNAc concentrations reached 500 μM. These data suggest that UDP-GlcNAc levels modulate the affinity of OGT for individual glycosylation sites differentially.

The Kinetic Mechanism of the O-GlcNAc Transferase—The kinetic behavior of the active His-p110 and pΔ6 proteins was examined by varying both the peptide (CKII) and UDP-GlcNAc concentrations and measuring the initial reaction velocities (panels A in Figs. 7–10). We conducted these studies at UDP-GlcNAc concentrations flanking the lowest K_m of UDP-GlcNAc. Five kinetic parameters can be determined by these studies as defined in Scheme 1. Figs. 7 and 9 show the dependence of v on UDP-GlcNAc concentrations from 0.3 to 18.75 μM for His-p110 and pΔ6, respectively. Figs. 8 and 10 show the dependence of v on CKII peptide concentrations.
on CKII peptide concentrations from 50 to 500 \(\mu M\). The data are fit to the equation 
\[ y = \frac{V_{\text{max}} \cdot x}{K_m + x}, \]
where \(y\) is the measured velocity and \(x\) is the substrate concentration, using least squares analysis.

The extrapolation of a linear plot of \(1/K_m(\text{app})\) of UDP-GlcNAc versus [CKII] (Figs. 7B and 9B) reveals a \(K_d\) UDP-GlcNAc of 10.5 \(\mu M\) for His-p110 and 7.5 \(\mu M\) for p\&6. Plotting \(1/K_m(\text{app})\) of the CKII peptide versus [UDP-GlcNAc] gives a \(K_d\) CKII of 198 \(\mu M\) for His-p110 and 540 \(\mu M\) for p\&6 (Figs. 8B and 10B).

The \(K_m\) of the CKII peptide was determined to be 107 \(\mu M\) for His-p110 and 215 \(\mu M\) for p\&6 from a secondary plot of \(1/V_{\text{max}}\) versus \(1/[\text{UDP-GlcNAc}]\) (Figs. 7C and 9C). Secondary plots of \(1/V_{\text{max}}\) versus \(1/[\text{UDP-GlcNAc}]\) (Figs. 8C and 10C) give \(K_m\) UDP-GlcNAc values of 5.25 \(\mu M\) for His-p110 and 3 \(\mu M\) for p\&6. These \(K_m\) values agree well with the values obtained above; the small differences may be due to slight variations in the purity and activity of the enzyme preparations used for each experiment. The \(V_{\text{max}}\) values determined from these double reciprocal plots are 0.0040 and 0.0033 \(\mu mol/\text{min/mg}\) for His-p110 and p\&6, respectively. Double reciprocal plots of \(1/V\) versus \(1/\text{substrate}\) yielded a series of converging lines (data not shown) that are consistent with a random bi-bi enzymatic mechanism for both His-p110 and p\&6. The results of these studies are summarized in Table I.

**DISCUSSION**

We have found that the p110 subunit of the OGT displays several features that appear to regulate its enzymatic activity and provide insights into how the cell ultimately regulates O-GlcNAcylation: 1) The p110 subunit is active and forms a homotrimer in the absence of the p78 subunit. 2) The TPR domain is a protein-protein interaction domain which facilitates subunit interactions. 3) Multimerization of the enzyme is not necessary for basal enzymatic activity, but the affinity for the UDP-GlcNAc sugar donor is dramatically affected by the multimerization state of the enzyme. 4) The enzyme has three
though these studies have helped to narrow down the regions of
the protein that are modified, further studies are necessary to
determine the functional consequences of these modifications.

These deletion mutants have also been used to begin to define the functional role of the TPR domain.

Numerous studies have demonstrated that TPRs modulate a variety of cellular processes via protein-protein interactions. This raises the possibility that OGT activity could be regulated by its TPR domain in a similar manner. We have found that five TPRs are sufficient for basal activity but not for multimORIZATION. Eight TPRs are needed for formation of trimers, and the trimerization is further stabilized by the presence of the full complement of 11 TPRs. Previous work has demonstrated that individual TPRs are responsible for mediating specific protein-protein interactions (26, 27), so it is possible that additional studies will delineate the TPR requirements into individual TPRs. Although a portion of the TPR domain is clearly responsible for OGT subunit interactions, it may also serve as a protein interaction domain for effector proteins that regulate the activity or substrate specificity of the enzyme.

Both the p110 trimer and the pΔ6 monomer are active at levels that are virtually indistinguishable in our standard assay. They have similar Km values for each of the peptide substrates examined and have nearly identical specific activity and V_max values. Also, both enzymes appear to function via a random bi-bi reaction sequence. In contrast the p110 trimer has three distinct binding constants for UDP-GlcNAc, whereas only two are observed for the pΔ6 monomer. The finding that the binding affinities for UDP-GlcNAc are strikingly different for the trimer and monomer whereas their posttranslational states are similar supports the idea that subunit composition may play a role in regulating OGT activity. This also suggests that other proteins may interact with the TPR domain and may affect OGT activity in vivo.

The lowest K_m,UDP-GlcNAc for p110 (5.25 μM) is 10-fold higher than that reported for the liver enzyme (0.545 μM). This difference may reflect the different subunit composition of these two enzyme sources or differences in the posttranslational modification states of the proteins. It was not possible to make a direct comparison between our data on the levels of tyrosine phosphorylation and O-GlcNAcylation on the recombinant OGT to the published data for the liver OGT. Previous K_m studies of the liver OGT were not done over a sufficient range of UDP-GlcNAc concentrations to detect K_m values in the higher micromolar range. However, the OGT present in rabbit reticulocyte lysate also appears to have three K_m values. The presence of multiple K_m values suggests the following: 1) There are two or more forms of the enzyme present, perhaps representing different posttranslational modification states. In support of this hypothesis our data indicate that the O-GlcNAc modification is stoichiometric on the purified OGT constructs so only a subpopulation is modified by O-GlcNAc. 2) There are two or more UDP-GlcNAc binding sites that regulate the activity of the enzyme. 3) There may be negative cooperativity between the subunits, although this seems unlikely in view of the fact that the pΔ6 protein is a monomer and still has two K_m values. Additional studies will be necessary to fully address each of these possibilities.

The studies presented here demonstrate that the activity of the enzyme can be directly regulated by UDP-GlcNAc concentrations, suggesting that in vivo OGT activity would be sensitive to the UDP-GlcNAc levels in the cytosol and nucleus. Cellular levels of UDP-GlcNAc are known to vary dramatically between tissues (37), during differentiation (38), during inflammation (39), and in response to stimuli such as glucocorticoids (40). However, the exact distribution of UDP-GlcNAc between the cytoplasm, nucleus and lumenal compartments is unknown. The levels of UDP-GlcNAc in the nucleus and cytosol could be regulated by the UDP-GlcNAc transporters in the

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### TABLE 1

| Kinetic constants | His-p110 | pΔ6 |
|------------------|----------|-----|
| K_m (μM)         | 6.0      | 6.0 |
| K_m (μM)         | 35       | 60  |
| V_max (μmol/min/mg) | 217     | none |
| K_m (μM)         | 0.0040   | 0.0033 |
| K_m (μM)         | 198      | 540 |
| K_m (9-mer (mM)) | 10       | 6   |

*a* From Fig. 5.  
*b* From Figs. 6–9.  
* From data not shown (see "Experimental Procedures").
endoplasmic reticulum and Golgi apparatus, which rapidly move UDP-GlcNAc from the cytosol to the luminal compartments (41). In this manner the cell could rapidly alter the cytoplasmic levels of UDP-GlcNAc, thereby regulating OGT activity. Alternatively, OGT could be sequestered away from UDP-GlcNAc in the cell by association with other proteins.

The model for OGT regulation emerging from these studies combines several different mechanisms including protein-protein interactions and changes in substrate binding affinities. OGT can be regulated by its multimerization state, which appears to alter its binding affinities for UDP-GlcNAc. The identification of both p110 homotrimers and p110/p78 heterotrimers suggests that other combinations may exist in tissues that express additional isoforms of the p110 or other interacting proteins. Another means of regulation may include the posttranslational modifications found on OGT. Additionally, the presence of three separate $K_m$ values for UDP-GlcNAc strongly suggests that OGT activity is modulated by the UDP-GlcNAc levels in the nucleus and cytoplasm. Taken together these observations provide a working model for how OGT could be regulated to modulate O-GlcNAcylation in the cell.

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