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Turnover and characterization of UDP-*N*-acetylglucosaminyl transferase in a stably transfected HeLa cell line ☆

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Abstract

To estimate the turnover of UDP-*N*-acetylglucosaminyl transferase (OGT), we exposed stably transfected HeLa cells to tetracycline for 16 h to induce OGT gene expression and increase cytosolic enzyme levels. Removal of tetracycline led to a progressive decrease in OGT activity (after a 6 h lag period), yielding an estimated OGT half-life of 13 h. A similar half-life (12 h) was obtained by measuring the loss of biosynthetically labeled OGT ([^{35}S]methionine pulse-chase experiments). Since OGT turnover was relatively slow, it is unlikely that changes in OGT gene expression or protein expression play a role in the short-term regulatory actions mediated by the hexosamine signaling pathway. We also found that the overexpressed 110 kDa murine OGT subunit (recombinant enzyme) was enzymatically similar to the endogenous holoenzyme derived from rat brain tissue. Thus, stably transfected HeLa cells provide an abundant source of enzyme that can be used to study the structure, function, and regulation of OGT.
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Keywords: UDP-*N*-acetylglucosaminyl transferase; Hexosamine signaling pathway; Transfected HeLa cells; Enzyme turnover; Hyperglycemia; Signal transduction

The hexosamine signaling pathway (HSP) was first identified in isolated rat adipocytes in 1991 [1] and found to function as an intracellular glucose-sensor (or nutrient-sensor) coupled to a complex signal transduction system that regulates glucose uptake and storage [2,3]. Under hyperglycemic conditions, increased flux through the HSP culminates in desensitization of the insulin-responsive glucose transport system (GTS) and up-regulation of lipogenic mRNA levels that encode fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and glycerol-3-P dehydrogenase [1,4]. These adaptive responses are functionally linked in that glucose uptake is reduced (by development of insulin resistance) and excess incoming glucose is diverted into a lipogenic pathway where it can be stored as triglyceride.

Two enzymes play a major role in the glucose sensing/transductional capabilities of the HSP. The first is glutamine:fructose-6-phosphate amidotransferase (GFAT), the first and rate-limiting enzyme of the hexosamine pathway. In isolated adipocytes, pharmacological inactivation of GFAT has been shown to block glucose-induced desensitization [1]. Conversely, transgenic mice studies have demonstrated that overexpression of GFAT in muscle and fat leads to the progressive

☆ *Abbreviations:* HSP, hexosamine signaling pathway; OGT, UDP-*N*-acetylglucosaminyl transferase; GlcN, glucosamine; UDP-GlcNAc, UDP-*N*-acetylglucosamine; GlcN-6-P, glucosamine-6-phosphate; GFAT, glutamine:fructose-6-phosphatemidotransferase; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hepes-buffered balanced saline solution.

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development of insulin resistance [5]. Thus, one functional consequence of increased hexosamine flux is induction of insulin resistance. In this case, GFAT exerts regulatory control by influencing the rate of glucose entry into the HSP. The second crucial enzyme of the HSP is UDP-*N*-acetylglucosaminyl transferase (OGT). This enzyme uses UDP-GlcNAc to attach a single monosaccharide (GlcNAc) onto the serine/threonine backbone of various proteins found in the cytosol and nucleus [6–10]. Since this unique covalent modification has been shown to alter protein function, it is apparent that OGT functions as a transduction element that can convert changes in metabolite levels into regulatory signaling events. Based on the identification, subcellular localization, and tissue distribution of various O-linked glycoproteins, it has been postulated that excessive glycosylation underlies the pathogenesis of several human diseases including diabetes, cancer, and Alzheimer's disease [11–14].

Many of the regulatory actions of the HSP occur over relatively short times spanning several minutes to about 4 h. These actions include activation of glycogen synthase and stimulation of glycogen formation [15], desensitization of the insulin responsive GTS [1], up-regulation of pyruvate kinase activity [16], and down-regulation of GFAT activity [17]. To gain a better understanding of the mechanisms underlying these short-term regulatory actions, it was of interest to determine the turnover (half-life) of OGT. To this end, we assessed turnover using a stable, tetracycline-inducible HeLa cell line that overexpresses murine OGT [18]. We also enzymatically characterized recombinant OGT to ascertain whether recombinant enzyme (the p110 kDa subunit) exhibited the same characteristics as the endogenous holoenzyme derived from brain tissue [19]. If this were the case, then recombinant OGT could provide an abundant source of enzyme for future studies on the structure, function, and regulation of OGT.

Materials and methods

Materials. Talon metal affinity resin was from Clontech Labs, Palo Alto, CA. Mouse monoclonal anti-myc tag antibody (1 μg/μl) was from Stressgen Biotechnologies, Victoria, BC, Canada. [^{3}H]UDP-*N*-acetyl-D-glucosamine ([^{3}H]UDP-GlcNAc) and ^{35}S express tag label were from New England Nuclear, Boston, MA. NP-40 was from CalBiochem, La Jolla; all other chemical reagents were from Sigma, St. Louis, MO, or Fisher, Santa Clara, CA, unless otherwise indicated.

Generation of a stable, tetracycline-inducible HeLa cell line using an OGT expression vector. The murine OGT sequence was put under the inducible control of the T-RexTM tetracycline operator as previously described [18], and then transfected into T-RexTM-HeLa human cervical adenocarcinoma cells (HeLa-OGT) where it was expressed with C-terminal tandem myc-tag and 6×-histidine fusion partners. HeLa cells were induced to overexpress OGT by treatment with 3 mM sodium butyrate and 1 μg/ml tetracycline for 16–36 h. Analysis of cytosolic OGT activity typically revealed a 15- to 50-fold increase in OGT activity compared to uninduced cells [18].

Preparation of HeLa cytosol. Cells were detached from 60 mm plates using trypsin–EDTA, and cytosol was harvested as previously described [18]. Fractions were desalted by PEG precipitation to remove salts, cellular UDP, and sugar nucleotides, which interfere with the OGT assay. Protein concentrations were determined using a protein assay kit (Pierce, Rockford, IL). PEG-desalted crude cytosol is referred to as cytosol unless otherwise specified.

OGT assay. The OGT assay was performed as previously described [18,20] by adding the following reagents to a 0.5 ml microcentrifuge tube: 5 μl of 10× Hepes buffer, 0.1 μCi [^{3}H]UDP-GlcNAc, 10 μl p62ST (~200 ng), cellular protein, and water (50 μl final volume). The mixture was vortexed and incubated for 2 h at room temperature to allow enzymatic glycosylation of a p62ST acceptor protein. The reaction was stopped by adding trichloroacetic acid (TCA) and ^{3}H-glycosylated p62ST was separated from free [^{3}H]UDP-GlcNAc by pelleting precipitated protein. The pellet was resuspended, transferred to a scintillation vial, and counted in a liquid scintillation counter. All OGT measurements were performed in duplicate and averaged unless otherwise indicated.

Assay of immunopurified OGT (OGT-IP assay). In an earlier study, we used an anti-OGT antibody to immunopurify endogenous OGT [19]. In the current study, we used a commercially available anti-myc antibody to immunopurify recombinant OGT (through the myc tag epitope). Briefly, HeLa cytosol and 0.1 μl of anti-myc antibody were added to a 0.5 ml microfuge tube (50 μl final vol.). The antibody was allowed to bind OGT for 40 min at room temperature, after which time 10 μl of prewashed protein A–agarose beads was added. The tubes were then rotated for 20 min (at room temp.) to allow binding of the OGT–antibody complex to agarose beads. The OGT–antibody complex was separated from other cellular proteins by washing the agarose beads four times with 400 μl buffer. The final agarose bead pellet was resuspended in 43 μl buffer. The OGT–antibody complexes (attached to the agarose beads) were assayed for OGT activity by adding 5 μl p62ST (~200 ng) and 0.1 μCi [^{3}H]UDP-GlcNAc (50 μl final vol.) and rotating the mixture for 2 h at room temperature. The assay reaction was terminated by pelleting the agarose beads by centrifugation (20 s/15,800*g*). The supernatant (containing free isotope and radiolabeled P62ST) was transferred to a new 0.5 ml tube to which prewashed Talon (70 μl) was added for 10 min to bind recombinant p62ST (through the myc tag). The Talon was then washed and counted in scintillation vials as in the OGT assay.

Metabolic labeling of OGT and assessment of OGT protein turnover. HeLa-OGT cells were seeded at 1.2 × 106/cells 60 mm dish and incubated in medium containing methionine and cysteine-free DMEM, 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 3 mM butyrate, 1 μg/ml tetracycline, and 55 μCi/ml ^{35}S express tag label. Cells were incubated at 37 °C with 5% CO_2. After 14 h, cells the plates were washed twice with 2 ml complete media containing DMEM (Biowhithaker, Walkersville, MD), 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. After washing, the cells were chased with 2 ml complete media supplemented with 3 mM butyrate and additional unlabeled amino acids (1 mM methionine, 0.5 mM cysteine) and incubated at 37 °C for the indicated time. Cytosol was prepared as above without PEG precipitation.

Immunomagnetic purification of radiolabeled OGT and radiometric quantitation. We originally tried to purify radiolabeled OGT by traditional immunoprecipitation techniques, but found that radiolabeled cellular proteins bound non-specifically to the agarose beads and raised background levels to unacceptable levels. Therefore, we used immunomagnetic separation of OGT to reduce background radioactivity and vastly improve assay sensitivity. In this immunopurification technique, we first biotinylated anti-myc tag antibody with biotin reagent from Sigma (biotinamidocaproic acid 3-sulfo-*N*-hydroxy-

succinimide ester) according to the vendor's instructions using a 3:1 molar ratio of biotin reagent to antibody. The mixture was incubated at room temperature for 30 min, after which time free biotin was removed by adsorbing the antibody to protein A–agarose beads (Invitrogen) for 15 min and then washing beads four times with PBS containing 0.05% Triton-X 100 (PBST). The biotinylated antibody was eluted from the protein A–agarose beads with 0.1 M glycine/0.15 M NaCl, pH 2.4 (15 min incubation), and the eluted solution was neutralized by the addition of a 2 M Tris, pH 8.0, solution. Streptavidin beads (Dynal, Brown Deer, WI) were washed twice with PBST. After each wash, the supernatant was removed using a Dynal particle concentrator. We then combined 100 μg of biotinylated antibody and 500 μl of streptavidin beads and incubated the mixture at room temperature for 15 min with occasional vortexing. After incubation, beads were washed three times with PBST and then resuspended in the original volume with the same buffer. For immunomagnetic separation of radiolabeled OGT, 20 μg of cytosol was incubated with 8 μl of anti-myc tag dynabeads in 100 μl PBST for 1 h at room temperature with constant mixing. Beads were then washed three times with PBST and OGT was eluted with 2× Tris–glycine SDS sample buffer (Invitrogen). The eluted protein was resolved on a 7.5% SDS–polyacrylamide gel and then transferred onto a nitrocellulose membrane as previously described [18]. The radiolabeled OGT on the membrane was then quantified using ImageQuant and the Molecular Dynamics Storm 840 phosphorimaging system.

Results

Estimate of OGT turnover in HeLa cells

As shown in Fig. 1A, cytosolic OGT activity progressively increased over 24 h when stably transfected HeLa cells were incubated in the presence of tetracycline (and butyrate). Western blot analysis using an antibody to the myc-tag epitope of recombinant OGT (myc) or an anti-OGT antibody (HxP4) showed that increased enzyme activity was due to elevated levels of OGT protein. Inclusion of a protein synthesis inhibitor (cycloheximide) during the induction period completely blocked both OGT protein expression (inset) and the rise in OGT enzyme activity.

When tetracycline was removed (Fig. 1B), the enhanced levels of OGT activity gradually diminished after an initial 6 h lag period. Based on the rapidity of loss, we calculated an OGT half-life of about 13 h. To assess the half-life of OGT protein, we used a pulse-chase protocol (Fig. 1C) in which OGT expression in HeLa cells was induced in the presence of [^{35}S]methionine and [^{35}S]cysteine. After 14 h, the radiolabeled amino acids and tetracycline were removed by washing, and the cells were then incubated in tetracycline-free media containing excess unlabeled amino acids. At various times after tetracycline withdrawal, cytosol was harvested and radiolabeled recombinant OGT was purified by immunomagnetic separation using biotinylated anti-myc-tag antibody coupled to streptavidin-coated dynabeads. The immunopurified OGT was than eluted, resolved by SDS–PAGE, and transferred to a nitrocellulose membrane. The OGT signal was quantified using

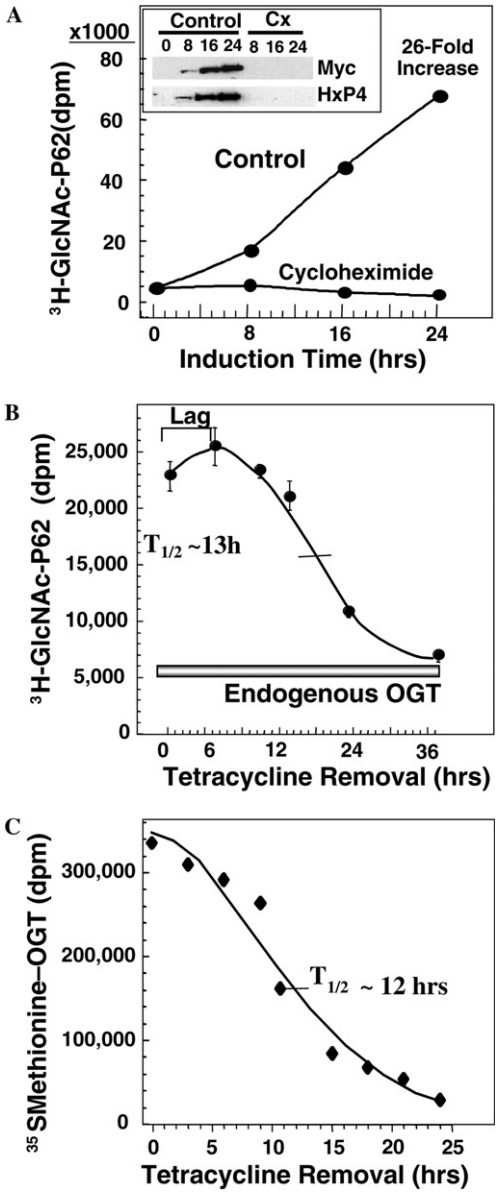

Fig. 1. Estimate of OGT turnover in HeLa Cells. (A) Stably transfected HeLa cells were incubated with 10 mM butyrate and 1 μg/μl tetracycline for various times to induce OGT overexpression. Time-dependent increases in OGT activity were determined as described under Materials and methods and increases in OGT protein levels were assessed by Western blotting using either anti-OGT antibodies (HxP4) or anti-myc antibodies specific for the OGT myc-tag epitope. (B) After an 18 h induction period, butyrate and tetracycline were removed (by cell washing) and HeLa cells were incubated under tetracycline-free conditions for various times up to 36 h. The return of elevated OGT activity towards endogenous levels was assessed and an estimated half-life for OGT ($T_{1/2}$ value) was derived. (C) Pulse-chase experiment in which recombinant OGT was biosynthetically labeled during a 14 h induction period. After removing labeled amino acids and tetracycline, loss of immunopurified, radiolabeled OGT was measured over 24 h to derive an estimated half-life for OGT protein.

ImageQuant and a Molecular Dynamics phosphorimaging system. Under these conditions, we estimated the half-life of OGT protein at about 12 h.

Enzymatic characterization of the recombinant p110 subunit of OGT

Fig. 2 depicts several dose–response studies examining the effects UDP-sugars, nucleotides, and salts on recombinant OGT activity. As can be seen, recombinant OGT derived from HeLa cytosol exhibited a high affinity for UDP-GlcNAc (IC_{50} of 0.14 μM) and a much lower affinity for UDP-glucose (IC_{50} of 3.7 μM) and UDP-galactose (IC_{50} of 11 μM). Cytosolic OGT also had a high affinity for UDP (IC_{50} of 0.47 μM) and UTP (IC_{50} of 3.8 μM), but a much lower affinity for ATP ($IC_{50} > 3$ mM). Salts, such as NaCl and NaH_2PO_4, had a profound inhibitory effect on activity with an IC_{50} of 50 mM for NaCl and 5 mM for NaH_2PO_4.

Since cytosol contains numerous proteins that could potentially alter OGT activity, it was important to confirm the aforementioned results using immunopurified enzyme. To facilitate the rapid purification of recombinant OGT, we used an immunoprecipitation method that employs an anti-myc-tag monoclonal antibody that binds recombinant OGT through a myc-tag epitope on the enzyme. Fig. 3A depicts an experiment in which we determined the optimal concentration of myc-tag antibody necessary for immunopurification. As can be seen, only 0.05 μg of antibody was required to harvest maximal amounts of enzymatically active OGT. In the first phase of this method, anti-myc antibody was incubated with cytosol to form a soluble enzyme–antibody complex. To separate this complex from other cytosolic proteins, we used agarose beads containing covalently attached protein A. The inset (Fig. 3B) indicates that 10 μl of protein A–agarose beads is more than sufficient to precipitate the OGT–antibody complex. Fig. 3C de-

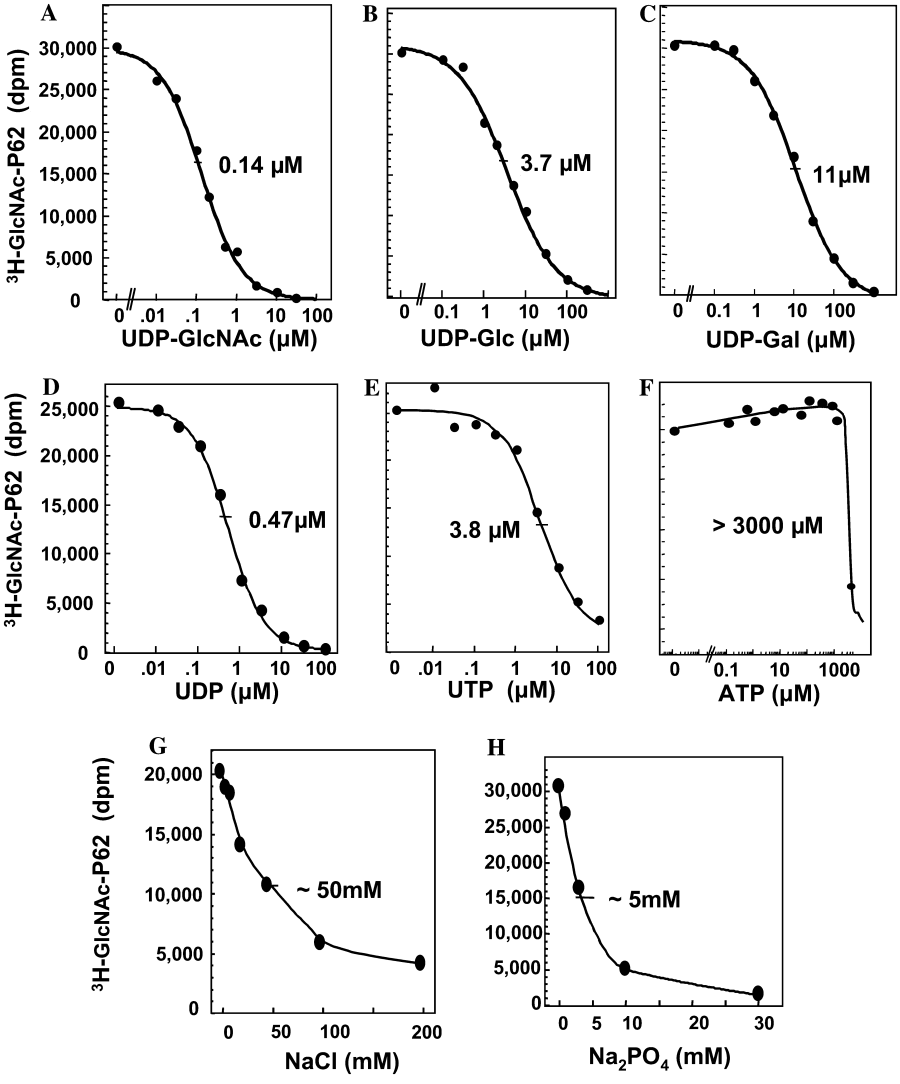

Fig. 2. Effect of nucleotide-sugars, nucleotides, and salts on recombinant OGT activity. Cytosol was harvested from HeLa cells after a 36 h induction period with 10 mM butyrate and 1 μg/μl tetracycline. The effects of UDP-sugars (A–C), nucleotides (D–F), and salts (G–H) on OGT activity were assessed after adding the indicated concentration of agents to the assay mixture. ID_{50} values were calculated and are shown in each panel.

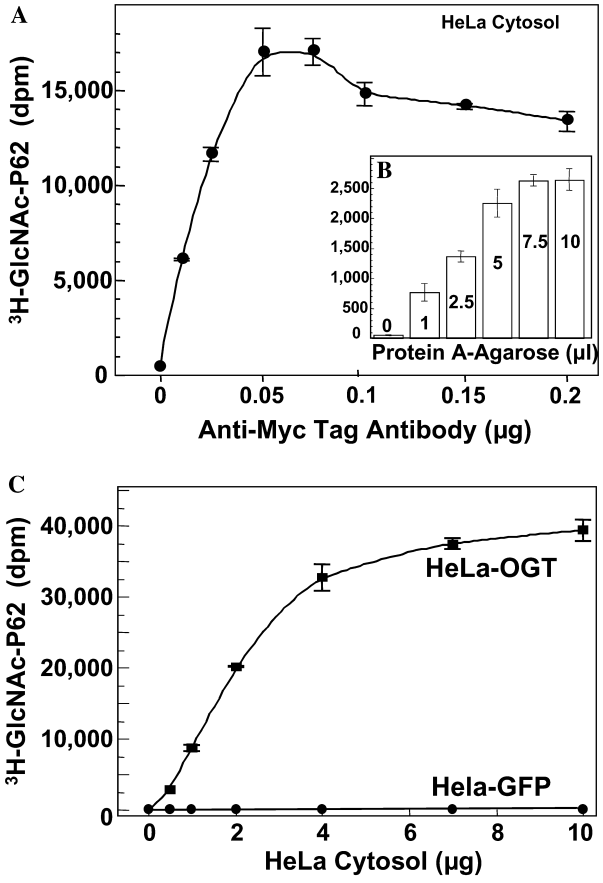

Fig. 3. Immunopurification of recombinant OGT using anti-myc tag antibody. (A) Enzymatic activity of immunopurified OGT was determined in a two-step procedure. In the purification step, recombinant enzyme in HeLa-OGT cytosol (2 µl) was incubated for 40 min with various amounts of monoclonal anti myc-tag antibody. OGT–antibody complexes were then harvested using protein A–agarose. In the second step (the OGT-IP assay), immunopurified OGT bound to agarose beads was assayed by mixing beads with 0.1 µCi [^{3}H]UDP-GlcNAc and 200 ng of p62ST (50 µl total vol.) for 2 h at room temperature. The OGT assay was stopped by pelleting the agarose beads (by centrifugation) and harvesting the supernatant. Talon metal affinity resin was used to rapidly purify p62ST and remove free [^{3}H]UDP-GlcNAc as described under Materials and methods. After extensive washing, Talon was transferred to a scintillation vial and counted. Each point represents the mean ± SEM of duplicate determinations. (B) HeLa cytosol was incubated with 0.05 µg of anti-myc antibody after which time recombinant OGT was harvested using various amounts of protein A–agarose beads. OGT activity was assessed as described above. (C) OGT was immunopurified from the indicated amounts of HeLa-OGT or HeLa-GFP cytosol using 0.05 µg of anti-myc-tag antibody and assayed for OGT activity as described in (A). Each point represents the mean ± SEM of duplicate determinations.

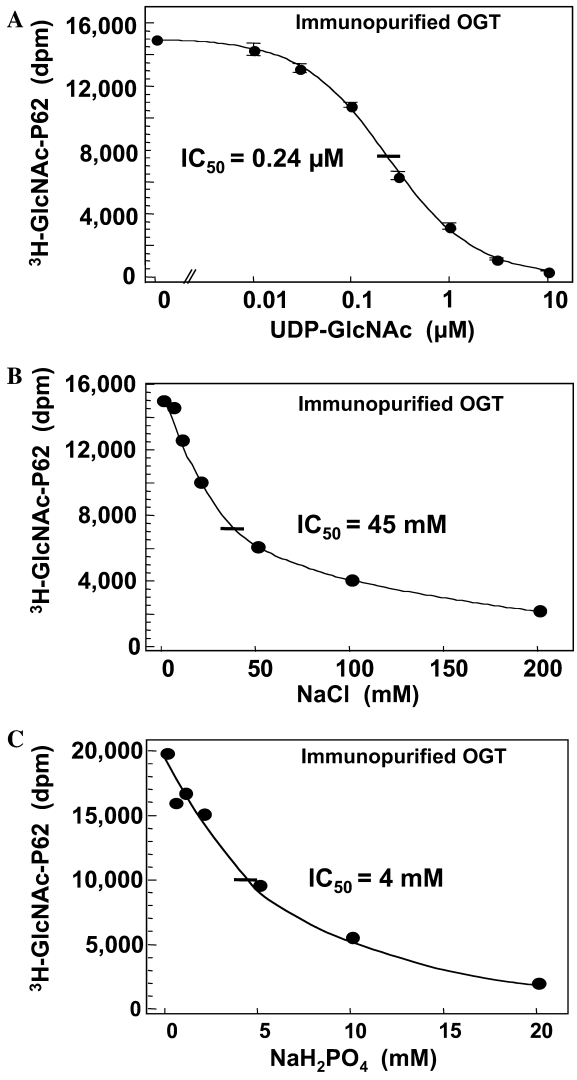

Fig. 4. Characterization of immunopurified OGT. OGT was immunopurified using anti-myc tag antibodies as described in Fig. 3. Activity of purified OGT was assessed in the presence of various concentrations of UDP-GlcNAc (A), NaCl (B) or NaH_2PO_4 (C). ID_{50} values were calculated and are shown in each panel.

picts an experiment in which we used 0.1 µg of antibody and 10 µl of protein A–agarose to immunopurify OGT from various amounts of cytosol. As can be seen, OGT activity of the precipitated complex markedly increased as we added increasing amounts of cytosol harvested from transfected HeLa cells (up to about 4 µg cytosol). No OGT activity was detected in cytosol in the absence of antibody (0 µg data point) or when using cytosol from HeLa cells containing the construct for green fluorescent protein (GFP). This indicates that anti-myc-tag antibody specifically recognizes recombinant OGT but not the endogenous enzyme Fig. 4.

Using this method, we found that that immunopurified OGT retained a high affinity for UDP-GlcNAc (IC_{50} of 0.24 µM) and a unique sensitivity to salts (IC_{50} of 45 mM for NaCl, 4 mM for NaH_2PO_4) that was comparable to unpurified cytosolic OGT (Fig. 2). We interpret this to mean that other cytosolic proteins do not alter OGT affinity for its preferred substrate (UDP-GlcNAc) and that salts can act directly on the enzyme. In a previous study, we examined the characteristics and subcellular distribution of brain OGT protein and activity [19]. The salient findings from this study

Table 1
Comparison of recombinant OGT from HeLa cells with endogenous OGT harvested from rat brain tissue

	ID_{50} values (mM)	
	Recombinant HeLa cell OGT	Endogenous brain OGT
Nucleotide sugars		
UDP-GlcNAc	0.14	0.25
UDP-Glc	3.7	4.2
UDP-Gal	11.5	13
Nucleotides		
UDP	0.47	0.6
UTP	3.8	0.5
ATP	>3000	>900
Salts		
NaCl	50	26
Na_2PO_4	5	2.5

regarding the effects of nucleotide sugars, nucleotides, and salts on OGT activity are summarized in Table 1. As can be seen, similar data were obtained using recombinant OGT, leading to the conclusion that the p110 subunit of OGT is enzymatically similar, if not identical, to the endogenous holoenzyme derived from rat brain.

Discussion

In the early 1980s, Hart and co-workers [21,22] discovered a novel form of protein-saccharide linkage consisting of a single *N*-acetylglucosamine (GlcNAc) attached in O-linkage directly to the serine/threonine backbone of proteins. The soluble enzyme catalyzing O-linked glycosylation [6–8,10] was originally believed to be a heterotrimer consisting of two 110 kDa subunits and one 78 subunit [23]; however, subsequent studies revealed that the 110 kDa subunit was immunologically related to the 78 kDa subunit. The OGT holoenzyme is now believed to be a homotrimer of three 110 kDa subunits each containing a C-terminal catalytic domain and a variable number of tetratricopeptide repeats (TPRs) on the N-terminus [24,25]. Although OGT had been cloned and partially characterized [23–27], progress in this glycobiology area was hampered by the lack of a rapid, sensitive, and economical OGT assay. In 2003, we developed such an assay [20] and found that OGT activity and protein expression were 10-fold higher in brain tissue compared to OGT activity in muscle, adipose, heart, liver, and brain tissue [19].

With the ability to readily measure OGT activity, we next created a stable, tetracycline-inducible HeLa-OGT cell line using an expression construct that encoded a recombinant protein containing murine OGT, a myc-tag epitope, and a 6× histidine tag [18]. The myc-tag serves primarily as a recognition site for a commercially available monoclonal anti-myc-tag antibody, whereas the 6×-his epitope on the recombinant protein allows for the rapid purification of OGT through a commercially available immobilized metal affinity chromatography resin (Talon) designed for purifying recombinant polyhistidine-tagged proteins.

Although our stably transfected HeLa cell line provides an abundant source of OGT, it was important to show that the 110 kDa recombinant enzyme was enzymatically similar to the endogenous holoenzyme. As previously reported [19], endogenous OGT from brain tissue has a high affinity for UDP-GlcNAc and a much lower affinity for UDP-glucose and UDP-galactose. Uridine nucleotides (UDP and UTP) are also effective inhibitors of OGT with apparent affinities similar to UDP-GlcNAc. Interestingly, salts such as NaCl markedly decrease OGT activity [19,23] and a recent study has shown that Na_2PO_4 is 10-fold more potent than NaCl in reducing OGT activity [19]. These data characterizing OGT harvested from brain tissue are summarized in Table 1 and are compared with the ID_{50} values derived from dose–response curves using recombinant OGT (from Fig. 2). As can be seen, recombinant OGT exhibited traits remarkably similar to those of endogenous OGT. We also found that immunopurified recombinant OGT retained a high affinity for UDP-GlcNAc (ID_{50} of 0.24 μM) and a sensitivity to salt (ID_{50} of 45 mM for NaCl and 4 mM for NaH_2PO_4). This indicates that other proteins in transfected HeLa cytosol do not alter the basic characteristics of OGT.

To gain new insights into the cellular mechanism(s) by which hyperglycemic conditions induce insulin resistance, we assessed the turnover (half-life) of OGT using stably transfected HeLa cells. The rationale for addressing this question is based on the idea that the amount of OGT protein may influence the extent of O-linked glycosylation under conditions of increased hexosamine flux. Turnover of OGT was examined by exposing HeLa cells to tetracycline for 16 h to induce OGT overexpression (by >10-fold) and then assessing the loss of enzyme activity at various times after the removal of tetracycline. Under these conditions, OGT progressively decreased to endogenous levels after an initial 6 h lag time, yielding an OGT half-life of 13 h. To estimate the turnover of OGT protein, we used a similar protocol in which recombinant protein was biosynthetically labeled with [^{35}S]methionine/cysteine and loss of radiolabeled OGT was followed during a 24 h chase period. These studies yielded an OGT half-life of 12 h based on the loss of overexpressed, recombinant OGT. Since both methods indicated that OGT turnover is relatively slow, we conclude that physiological or pharmacological changes in OGT gene expression do not play a significant role in the short-term regulatory actions of the HSP.

A more likely explanation for the rapid adaptive changes after glucose or glucosamine treatment is that enhanced hexosamine flux elevates intracellular levels

of UDP-GlcNAc, which in turn facilitates O-linked glycosylation [28,29]. Other regulatory mechanisms could also potentially affect the extent of glycosylation including altered OGT activity through covalent modification of the enzyme, allosteric regulation of OGT, or regulatory changes in the enzyme mediating de-glycosylation of O-linked glycoproteins. It is important to note that mechanisms other than O-linked glycosylation appear to be operative within the HSP. Specifically, elevated levels of GlcN-6-P have been shown to allosterically regulate various enzymes, such as glycogen synthase and hexokinase [15,29]. Thus, it appears that multiple mechanisms underlie the rapid glucose sensing/transductional capabilities of the HSP. Although short-term control of OGT gene expression appears not be involved in acute regulatory actions, future studies should assess whether long-term adaptation to hyperglycemic conditions includes changes in OGT protein levels mediated by altered gene expression.

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