Potential Regulation of Nuclear UDP-*N*-Acetylglucosaminyl Transferase (OGT) by Substrate Availability: Ability of Chromatin Protein to Bind UDP-*N*-Acetylglucosamine and Reduce OGT-Mediated *O*-Linked Glycosylation

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UDP-*N*-acetylglucosaminyl transferase (OGT) resides in both cytosolic and nuclear compartments and catalyzes *O*-linked glycosylation of various proteins. In the current study, we have extracted protein from nuclear DNA (chromatin protein) using 0.2% NP-40 detergent. Addition of chromatin protein to either cytosolic or nuclear preparations (containing abundant OGT) resulted in a dose-dependent loss of OGT activity. Since chromatin-mediated loss of OGT activity could be restored by immunopurification of OGT, we conclude that loss of enzyme activity is not due to direct inactivation of OGT. Addition of UDP-galactose (to saturate potential UDP binding proteins) effectively restored OGT activity in cytosol containing chromatin protein. This indicates that chromatin protein inhibits OGT activity by binding UDP-GlcNAc. These studies suggest that nuclear substrate availability may comprise one of the *in vivo* mechanisms regulating OGT activity and *O*-linked glycosylation of nuclear proteins. This is potentially significant, since most transcription factors are *O*-linked glycosylated and such post-translational modifications can alter gene expression.

Key words UDP-*N*-acetylglucosaminyl transferase; chromatin; subcellular fractionation; enzyme; substrate availability

In the early 1980's, Hart and coworkers in a well-designed series of studies 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.^{1,2)} The soluble enzyme catalyzing *O*-linked glycosylation of cytosolic and nuclear proteins is UDP-*N*-acetylglucosaminyl transferase (OGT).^{3–6)} The holoenzyme is believed to be a heterotrimer consisting of two p110 subunits and one p78 subunit⁷⁾ and has been shown to be covalently modified by phosphorylation and glycosylation.^{7–11)} In 1997, the p110 OGT subunit was cloned^{8,9)} and shown to contain a catalytic domain, a nuclear localization site, and a variable number of tetratricopeptide repeats (TPR's).

Although OGT was originally thought to be a cytosolic enzyme, recent immunolocalization evidence has shown that OGT also resides in the nucleus.^{12,13)} Functional studies in brain tissue also revealed that nuclear OGT protein was enzymatically active and had an affinity for UDP-GlcNAc and a salt sensitivity that was similar to the cytosolic enzyme.^{12,14)} The presence of OGT in two distinct compartments raises questions regarding the mechanism(s) regulating OGT in each compartment.

A serendipitous finding led us to postulate that substrate availability may play a role in regulating nuclear OGT activity. From previous studies,¹²⁾ we knew that a low concentration of detergent (NP-40) was required to lyse brain nuclei and allow for the harvesting of nuclear OGT.¹²⁾ In experiments designed to optimize detergent solubilization, we recently found that lysis of nuclei with NP-40 concentrations greater than 0.07% markedly reduced nuclear OGT activity without affecting extraction of OGT protein. The current report provides a mechanistic explanation for this observation and discusses the practical and physiological implications of this discovery.

MATERIALS AND METHODS

Preparation of Brain Cytosol and Nucleosol and Extraction of Chromatin Protein As previously described,^{12,14)} rat brain tissue was minced, homogenized and filtered through cheesecloth. The filtered homogenate was then diluted to 5 volumes (v/w) of sucrose buffer and centrifuged at 600×*g* for 10 min to remove nuclei and cellular debris. The supernatant was transferred to new tubes and centrifuged at 20000×*g* for 10 min to obtain crude cytosol. Protein concentrations were determined using a protein assay kit (Pierce, Rockford, IL). Cytosol was desalted by PEG precipitation to remove salts, cellular UDP, and sugar nucleotides. PEG-desalted cytosol is referred to as cytosol unless otherwise specified. Brain nucleosol was obtained by resuspending the low speed nuclear pellet in a high sucrose buffer (1.5 M sucrose, 10 mM Tris, pH 7.4, 1 mM MgCl₂) and centrifuging the mixture at 14000×*g* for 30 min. Purified nuclei were resuspended in a nuclear lysis buffer (NLB) containing 20 mM Hepes, pH 7.5, 600 mM NaCl, 0.5 mM EDTA and 0.05% Nonidet P-40 (NP-40) at a volume of 1 ml per 1 g initial brain tissue. After 20 min on ice, lysed nuclei were centrifuged at 20000×*g* for 10 min and the gelatinous DNA pellet was removed by syringe. Desalting of nucleosol was accomplished as described for cytosol. PEG-desalted nucleosol is referred to as nucleosol unless otherwise specified.

To extract chromatin-associated proteins, we lysed nuclei with 0.05% NP-40 as described above and centrifuged the mixture to pellet the gelatinous chromosomal fraction. The supernatant was discarded and the chromatin was resuspended and washed by adding 1 ml of NLB without NP-40. Chromatin was reharvested by centrifugation at 20000×*g* for 10 min. The chromatin pellet was transferred to a new tube and resuspended in NLB containing 0.2% NP-40 (at a volume of 0.6 ml per 1 g of initial brain tissue). The mixture was centrifuged and the gelatinous chromatin pellet was removed

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by syringe and discarded. The supernatant containing chromatin protein was desalted with PEG and assayed for protein as described above.

OGT Assay The OGT assay was performed as previously described^{14,15} by adding the following reagents to a 0.5 ml microcentrifuge tube: 5 μ l of 10 $\times$ Hepes buffer, 0.1 μ Ci (2.2–5 pmol) of ³H-UDP-GlcNAc, 10 μ l of p62ST (ca. 200 ng), cellular protein, and water (50 μ l final volume). The mixture was vortexed and incubated for 2 h at room temperature to allow enzymatic glycosylation of the p62ST acceptor protein. The reaction was stopped by adding trichloroacetic acid (TCA) and ³H-glycosylated p62ST was separated from free ³H-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.

Immunopurification of OGT and Measurement of OGT Activity (OGT-IP Assay) OGT was immunopurified as previously described¹⁴ by adding nucleosol and 1 μ l of 1897k anti-OGT antiserum to a 0.5 ml microfuge tube (50 μ l final vol.). The antibody was allowed to bind OGT for 40 min at room temperature, after which time 40 μ l of prewashed protein A-agarose beads were added. The microtubes 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 4 times with 400 μ l transferase assay buffer (TAB) containing 25 mM Hepes, 10 mM MgCl₂ and 1 mM EDTA, pH 7.0. The final agarose bead pellet was resuspended in 43 μ l TAB. The OGT-antibody complexes attached to the agarose beads were assayed for OGT activity by adding 5 μ l of p62ST (ca. 200 ng) and 0.1 μ Ci of ³H-UDP-GlcNAc (50 μ l final vol.) and then rotating the mixture for 2 h at room temperature to allow enzymatic glycosylation of p62ST. The reaction was terminated by pelleting the agarose beads by centrifugation (20 s/15800 g). The supernatant (containing free isotope and radiolabelled p62ST) was transferred to a new 0.5 ml tube to which prewashed Talon (70 μ l) was added for 10 min to bind recombinant p62ST (through 6 $\times$ polyhistidine tag). The Talon was then washed and counted in scintillation vials as in the OGT assay.

RESULTS

To determine the effect of detergent on extraction of nuclear OGT, we made aliquots with identical amounts of brain nuclei and added nuclear lysis buffer containing various concentrations of NP-40 (from 0 to 0.2%). As shown in Fig. 1A, minimal OGT activity was extracted in the absence of NP-40. Addition of increasing amounts of detergent (from 0.02 to 0.07%) resulted in a dose-dependent increase in OGT activity. At higher NP-40 concentrations, OGT activity was reduced by >80%. We believe that the initial rise in OGT activity (from 0.02 to 0.07% NP-40) is due to detergent-mediated lysis of nuclei based on microscopic examination of nuclei preparations (data not shown). It is also important to note that nuclear proteins were desalted with PEG, which we have previously shown to effectively remove >98% of detergent.¹⁶ Thus, residual detergent in the assay cannot account for differences in OGT activity. In addition, direct addition of

Fig. 1. Extraction of Nuclear OGT Activity by NP-40 and Inhibition of OGT Activity by Nucleosol Extracted with 0.2% NP-40

(A) Isolated nuclei were harvested from homogenized brain tissue and incubated with various concentrations of NP-40. The OGT assay was performed using the indicated volume of nuclear protein. Each point represents the mean of duplicate determinations. (B) Various amounts of nucleosol (0.2% NP-40) were mixed with brain cytosol and then assayed for OGT activity. Each point represents the mean of duplicate determinations.

Fig. 2. OGT Activity in Nucleosol after Immunopurification

Enzymatic activity of immunopurified OGT was determined in OGT-IP assay described in Materials and Methods. Each point represents the mean of duplicate determinations.

NP-40 to OGT assay did not affect the enzyme activity up to 0.2% (data not shown), eliminating the possibility that remained NP-40 inhibits the OGT activity. To determine whether 0.2% nucleosol contains an OGT inhibitor, we added various amounts of 0.2% nucleosol to a fixed volume of brain cytosol (containing an abundance of OGT activity). As can be seen in Fig. 1B, addition of increasing amounts of 0.2% nucleosol progressively inhibited cytosolic OGT activity (by >80% at 2 μ l of nucleosol).

Shown in Fig. 2 is an experiment in which OGT was immunopurified from equal volumes of either 0.05% nucleosol or 0.2% nucleosol and then assayed for OGT activity. It is clear from the results that OGT purification and the concomitant removal of nuclear proteins restores OGT activity in 0.2% nucleosol. Collectively, the data depicted in Figs. 1 and 2 indicate that the low OGT activity in the 0.2% nucleosol is not due to loss or inactivation of OGT protein, but rather is mediated by the presence of an inhibitory protein.

Chromosomal DNA is known to contain numerous proteins and the DNA-protein complex is known as chromatin. To test the hypothesis that OGT inhibitory proteins are derived from chromatin, we harvested chromatin protein by lysing nuclei with 0.05% NP-40 buffer and collecting the gelatinous DNA pellet. Chromatin proteins were then extracted with high detergent buffer (0.2% NP-40) and the residual DNA was pelleted and discarded. Figure 3 shows that addition of chromatin protein to cytosol (containing abundant OGT activity) results in reduced OGT activity. Only 1 μ g of chromatin protein was able to inhibit OGT activity by about 70%; almost complete inhibition was seen at 2 μ g. To test the hypothesis that chromatin protein was reducing OGT activity by binding ³H-UDP-GlcNAc, we used

Fig. 3. Ability of Chromatin Protein to Inhibit Cytosolic OGT Activity

OGT activity of brain cytosol in the absence or presence of the indicated concentrations of chromatin protein. Each point represents the mean of duplicate determinations.

Fig. 4. Ability of UDP-Galactose to Restore OGT Activity in Cytosol Containing Chromatin Protein

(A) OGT activity was determined in cytosol containing the indicated concentrations of UDP-galactose. Each point represents the mean of duplicate determinations. (B) OGT activity in cytosol containing 3 μ g of chromatin protein and the indicated concentrations of UDP-galactose. Each point represents the mean of duplicate determinations. (C) OGT activity depicted in panel B is corrected for the competitive inhibition of UDP-galactose. Data are expressed as a percent of control (OGT activity in cytosol alone).

UDP-galactose. The rationale behind this protocol is that UDP-galactose is a low affinity substrate for OGT, so it could be used as substrate to saturate any potential UDP binding proteins. In other words, saturation of the inhibitory binding protein by UDP-galactose would prevent the binding of 3 H-UDP-GlcNAc, thus increasing substrate availability in the OGT assay. This indeed appeared to be the case as shown by the results in Fig. 4B. The addition of chromatin protein to cytosol markedly reduced OGT activity; however, inclusion of increasing amounts of UDP-galactose (up to 10 μ M) increased OGT activity. The increase in OGT activity is really an underestimate, since UDP-galactose itself is a competitive inhibitor of 3 H-UDP-GlcNAc (Fig. 4A). When the OGT data in Fig. 4B are corrected for UDP-galactose competition in the OGT assay (Fig. 4A), it can be seen that OGT activity is restored to almost full activity by the inclusion of UDP-galactose (Fig. 4C).

DISCUSSION

In the current study, we have obtained evidence that protein(s) extracted from DNA (chromatin protein) can markedly inhibit the ability of OGT to glycosylate protein. When 0.2% NP-40 was used to lyse nuclei, OGT activity in the nucleosol was minimal despite an abundance of OGT protein (as assessed by Western analysis). Since addition of 0.2% nucleosol to cytosol (containing abundant OGT activity) reduced OGT activity by >90%, we concluded that loss of activity was due to solubilization of inhibitory proteins (rather than inactivation of OGT). This was substantiated by the finding that removal of chromatin and other nuclear proteins from 0.2% nucleosol (by immunopurification of OGT) restored OGT activity. The amount of detergent used to lyse nuclei was a key variable. Low detergent (0.05% NP-40), could effectively lyse nuclei and extract enzymatically active OGT. In contrast, higher detergent concentrations (>0.07%) yielded OGT with greatly diminished OGT activity (>80% loss at 0.2% NP-40).

To test the possibility that loss of OGT activity was mediated by extraction of chromatin protein, we lysed nuclei with 0.05% NP-40, harvested the gelatinous DNA pellet (chromatin fraction), and then extracted chromatin protein using 0.2% NP-40. When chromatin protein was added to cytosol, OGT activity was reduced by >90% (with only 2 μ g of protein). These studies indicate that when high detergent is used to lyse nuclei, chromatin protein is extracted (together with OGT) and this protein inhibits OGT activity.

Chromatin protein appears to inhibit OGT activity by binding 3 H-UDP-GlcNAc and reducing substrate availability (rather than acting directly on the OGT enzyme). This was demonstrated by adding unlabeled UDP-galactose to an OGT assay containing cytosol (source of OGT), chromatin protein, and 3 H-UDP-GlcNAc. Under these conditions, OGT activity was restored. The rationale for using UDP-galactose is that it has a much lower affinity for OGT compared to UDP-GlcNAc (only 2%). Therefore, it would saturate uridine-sugar binding sites on chromatin protein without altering the ability of OGT to use 3 H-UDP-GlcNAc as a substrate. A direct binding of chromatin protein to UDP-GlcNAc must be further confirmed. Also, a molecular substance of this UDP-sugar binding protein should be elucidated.

From a physiological perspective, our studies suggest that nuclear substrate availability may comprise one of the *in vivo* mechanisms regulating OGT activity and O-linked glycosylation of nuclear proteins. This is potentially significant, since most transcription factors are O-linked glycosylated and such post-translational modification can alter gene expression. Since no evidence was found for the binding and sequestration of UDP-GlcNAc in cytosol, this suggests that the levels of this key substrate are determined by synthesis (through the hexosamine biosynthesis pathway) and utilization. On the other hand, free UDP-GlcNAc levels in the nucleus would appear to involve, cytosolic levels, transport of substrate into the nucleus, and sequestration of UDP-GlcNAc by chromatin proteins. This would provide for differential regulation of OGT activity in the cytosol and nucleus. Additional studies will be required to test this hypothesis and identify other mechanism(s) mediating regulation of OGT activity in cytosolic and nuclear subcellular compartments.

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