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Characterization of the Specificity of O-GlcNAc Reactive Antibodies Under Conditions of Starvation and Stress

Russell A. Reeves¹, Albert Lee¹, Roger Henry¹, and Natasha E. Zachara^{1,2,3}

¹The Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205-2185, USA

Abstract

The dynamic modification of nuclear, cytoplasmic, and mitochondrial proteins by *O*-linked β -*N*-acetyl-D-Glucosamine (O-GlcNAc) has been shown to regulate over 3,000 proteins in a manner analogous to protein phosphorylation. O-GlcNAcylation regulates the cellular stress response, the cell cycle, and is implicated in the etiology of neurodegeneration, type II diabetes, and cancer. The antibody CTD110.6 is often used to detect changes in the O-GlcNAc modification. Recently, it has been demonstrated that CTD110.6 recognizes *N*-linked *N,N'*-diacetylchitobiose, which is thought to accumulate in cells experiencing severe glucose deprivation. In this study, we have addressed two questions: 1) Which other antibodies used to detect O-GlcNAc cross-react with *N*-linked *N,N'*-diacetylchitobiose? 2) Does *N*-linked *N,N'*-diacetylchitobiose accumulate in response to other cellular stressors? To delineate between O-GlcNAc and *N*-linked *N,N'*-diacetylchitobiose, we developed a workflow that has been used to confirm the specificity of a variety of O-GlcNAc specific antibodies. Using this workflow we demonstrated that heat shock, osmotic stress, endoplasmic reticulum stress, oxidative stress, DNA damage, proteasomal inhibition, and ATP depletion induce O-GlcNAcylation but not *N*-linked *N,N'*-diacetylchitobiose. Moreover, we demonstrated that while glucose deprivation results in an induction in both O-GlcNAc and *N*-linked *N,N'*-diacetylchitobiose, the induction of *N*-linked *N,N'*-diacetylchitobiose is exacerbated by the removal of fetal bovine serum.

Keywords

CTD110.6; O-GlcNAc; nutrient deprivation; specificity; stress-response

Introduction

In recent years, the covalent modification of nuclear, mitochondrial and cytoplasmic proteins by *O*-linked β -*N*-acetyl-D-glucosamine (O-GlcNAc) has been implicated in the

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²To whom correspondence should be addressed: Tel: 1-410-955-7049; Fax: 1-410-955-5759; nzachara@jhmi.edu.

³Author responsible for proofs and reprints.

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regulation of signal transduction, nutrient sensing, transcription, the cell cycle, and the cellular stress response [1]. The addition of O-GlcNAc to serine and threonine residues is catalyzed by the O-GlcNAc transferase (OGT) [2] and this enzyme utilizes the high energy sugar nucleotide uridinediphosphate-GlcNAc (UDP-GlcNAc) [3]. Removal of O-GlcNAc from proteins is catalyzed by a neutral β -N-acetylhexosaminidase (O-GlcNAcase), initially characterized as hexosaminidase-C [4].

UDP-GlcNAc levels are maintained by flux through the hexosamine biosynthetic pathway (HBP). In adipocytes, between 2-5% of the glucose that enters the cell is converted by the HBP to UDP-GlcNAc and other metabolites [5]. As a result of this, acute hyperglycemia causes UDP-GlcNAc levels to increase and this is reflected by an increase in the O-GlcNAc modification [3]. Paradoxically, it has been shown that glucose starvation also causes an increase in O-GlcNAcylation [6-9] suggesting a key role of O-GlcNAc in the regulation of cellular metabolism.

A growing body of data suggests that O-GlcNAc is a key regulator of the cellular stress response. Diverse forms of cellular stress lead to elevated O-GlcNAc levels on numerous intracellular proteins in both cell culture and animal models [10]. Suggesting that this is a pro-survival response of cells, elevating O-GlcNAc levels before or after injury is protective in both *in vivo* and *in vitro* models of heat stress, oxidative stress, hypoxia, ischemia/reperfusion injury and trauma hemorrhage [11].

Recently, it was demonstrated that under conditions of glucose deprivation the commonly used O-GlcNAc-specific antibody, CTD110.6, cross-reacts with *N*-linked GlcNAc β (1-4)GlcNAc herein referred to as *N,N,N'*-diacetylchitobiose [12]. Notably, *N,N,N'*-diacetylchitobiose is thought to arise as the synthesis of the dolichol-P-P-GlcNAc₂Man₉Glc₃ is truncated at dolichol-P-P-GlcNAc₂ [12]. Collectively, these data have led to two questions: 1) Do other forms of cellular stress induce *N,N,N'*-diacetylchitobiose? 2) Which other O-GlcNAc-specific antibodies cross-react with *N,N,N'*-diacetylchitobiose?

Results and Discussion

A combination of PNGase F and mild β -elimination can be used to delineate between O-GlcNAc and *N,N,N'*-diacetylchitobiose

In order to determine which O-GlcNAc-specific antibodies cross-react with *N,N,N'*-diacetylchitobiose, we developed an experimental workflow that is capable of distinguishing between two different glycan classes (*N*-linked glycans and *O*-linked glycans) (Figure 1A). This workflow employs a combination of peptide *N*-glycosidase F (PNGase F) digestion to remove *N*-linked glycans and/or mild on-blot β -elimination to remove *O*-glycans (Figure 1).

We used total cell lysates from human osteosarcoma (U2OS) cells that were either fed with complete media (Dulbecco's modified eagle medium (DMEM), 5g/L glucose, 10% (v/v) fetal bovine serum (FBS)) or starvation media (STV; DMEM, 0g/L glucose, 10% (v/v) dialyzed FBS) as reported by Cheung and Hart [8]. The cell extracts were digested with PNGase F to remove *N*-linked oligosaccharides, separated by SDS PAGE, and duplicate gels were blotted onto polyvinylfluoride (PVDF) membrane. The membranes were

subsequently exposed to water, mild (40°C, 55mM NaOH) or harsh (60°C, 100mM NaOH) β -elimination conditions (Figure 1B). The *O*-glycosidic bond is more labile than the *N*-glycosidic bond, and as such can be β -eliminated under mild conditions (40°C, 55mM NaOH) without compromising the integrity to *N*-linked oligosaccharides or the protein backbone [13, 14].

Consistent with previous results [8, 12], and the appearance of *N,N,N'*-diacetylchitobiose, a significant increase in the reactivity of CTD110.6 was observed in cells grown in the starvation media when compared to cells grown in the control media. As expected, there was no reduction of CTD110.6 reactivity in the control cells (Fed) treated with PNGase F (Figure 1B). However, the increase in CTD110.6 reactivity was attenuated in lysates from starved cells digested with PNGase F (Figure 1B). Upon mild β -elimination, CTD110.6 reactivity was maintained in the starved samples and was diminished upon treatment with PNGase F. Together, these data suggest that the starvation conditions used induce *N,N,N'*-diacetylchitobiose, but not *O*-GlcNAc. Under harsh β -elimination conditions CTD110.6 reactivity was diminished across all sample treatments, suggesting that both *O*-linked and *N*-linked glycans were removed. Consistent with these observations, antibody reactivity to both OGT and Actin were diminished, suggesting that the antibody-recognizing epitopes were destroyed under harsh β -elimination conditions. These data underscore the importance of the mild- β -elimination conditions when differentiating between *O*-linked and *N*-linked glycans.

We confirmed that the PNGase F digestion worked effectively by probing for α -mannose, a common constituent of *N*-linked glycans, using Concanavalin A (ConA) lectin blotting (Figure 1C- Control). Consistent with the presence of abbreviated *N*-glycans, a mild reduction of ConA reactivity was observed upon starvation. Treatment with PNGase F further reduced ConA reactivity, suggesting that treatment with PNGase F removes *N*-linked glycans. Residual ConA reactivity upon both starvation and PNGase F treatment may be due to *O*-linked α -mannose structures. Although, as the ConA signal was reduced in all treatments upon mild β -elimination (Figure 1C- β -Elim. Mild), the β -elimination conditions may also induce a change in the *N*-glycan structure that affects ConA reactivity.

Together, our data suggest that we can differentiate between *N,N,N'*-diacetylchitobiose and *O*-GlcNAc. We next evaluated whether other commercially available *O*-GlcNAc antibodies cross-reacted with *N,N,N'*-diacetylchitobiose. Notably, we did not evaluate galactosyltransferase based methods, as these techniques have previously been reported to modify both complex *N*-linked glycans [15] and attenuated *N*-linked glycans bearing a single *N*-linked GlcNAc residue that are presumably produced by the endoplasmic reticulum associated degradation (ERAD) pathway [16].

Various antibodies used to detect GlcNAc also react with *N,N,N'*-diacetylchitobiose

We examined the specificity of the following *O*-GlcNAc and GlcNAc recognizing antibodies: CTD110.6 [17], RL2 [18], 18B10.C7(3), 1F5.D6(14), 9D1.E4(10) [19], and HGACs 39, 49, and 85 [20]. We utilized the same strategy described in Figure 1 using total cell lysates of U2OS cells exposed to either control or nutrient deprived conditions. We found that similar to CTD110.6, the antibody 9D1.E4(10) also had increased reactivity upon

starvation treatment (Figure 2). This signal was sensitive to PNGase F, but not mild β -elimination, suggesting that 9D1.E4(10) also cross-reacts with *N,N,N'*-diacetylchitobiose.

The O-GlcNAc specific antibodies 18B10.C7(3), 1F5.D6(14) and RL2 did not react with *N,N,N'*-diacetylchitobiose. Several of these antibodies have been reported to only react with O-GlcNAc in a specific 3-dimensional context defined by the underlying peptide and this presumably explains the improved specificity [18, 21, 22]. Interestingly, HGACs 39, 49 and 85, did not appear to react with *N,N,N'*-diacetylchitobiose, although they were raised against bacterial streptococcal group A glycoconjugate that is composed of a polyrhmannose backbone with GlcNAc side chains. These HGAC antibodies are commonly used for cytologic analysis and assessment of the distribution of O-GlcNAc residues on proteins associated with the nuclear periphery, nucleoplasm and cytoplasm of mammalian cells [20].

Severe nutrient deprivation, not glucose starvation, induces elevation of *N,N,N'*-diacetylchitobiose on N-linked glycoproteins

To study the specificity of CTD110.6 we sought to recapitulate earlier reports that suggested that O-GlcNAc and *N,N,N'*-diacetylchitobiose levels increase upon glucose deprivation [8, 12, 23]. We were able to detect *N,N,N'*-diacetylchitobiose in extracts of cells grown in DMEM with 0g/L glucose supplemented with either 1% (v/v) FBS (Supplemental Figure 1) or 10% (v/v) dialyzed FBS (treatment 4, Figure 3). However, when cells were grown in glucose free DMEM supplemented with 10% (v/v) FBS, for up to 48h, *N,N,N'*-diacetylchitobiose did not form (treatment 3, Figure 3). Interestingly, the presence of glucose (treatment 2, Figure 3) can block the induction of *N,N,N'*-diacetylchitobiose in cells grown in Dialyzed FBS. Consistent with these observation, and those of Zou and co-workers [23], supplementing starvation media with 5mM Glucose, 5mM Mannose, or 100 μ M glucosamine could prevent the formation of *N,N,N'*-diacetylchitobiose (Supplemental Figure 2). However, supplementing starvation media with 500 μ M glucose or mannose was ineffective at blocking the formation of *N,N,N'*-diacetylchitobiose.

As glucose starvation alone did not lead to the induction of *N,N,N'*-diacetylchitobiose, we assessed hexose concentrations in the media used to feed cells using the QuantiChrom Kit which detects Glucose, Galactose and Mannose. We noted that FBS itself contributed to approximately 0.79mM hexose, a non-significant source of hexose (Table 1), and this is consistent with the reported concentration of glucose in FBS, which range from 109-130mg/dL. We then assessed the remaining hexose concentration in the media after each time point (Figure 3F). The cells treated with DMEM containing 0g/L glucose and 10% (v/v) FBS grew without a significant source of carbohydrate for 48h without induction of *N,N,N'*-diacetylchitobiose. Together, these data suggest that glucose deprivation alone is not sufficient to induce *N,N,N'*-diacetylchitobiose formation and that withdrawal of some unidentified factor in the FBS is also required to induce *N,N,N'*-diacetylchitobiose formation.

O-GlcNAc levels increase in response to nutrient deprivation

To differentiate between O-GlcNAc and *N,N,N'*-diacetylchitobiose, we used the strategy presented in Figure 1 and a previously described inducible OGT knockout cell line [24].

First, we explored whether *N,N,N'*-diacetylchitobiose would accumulate in the OGT knockout cell line by treating OGT wild-type and null cells with complete media or starvation media. Despite the loss of OGT, CTD110.6 reactivity was elevated in all of the starvation treated samples (Figure 4A) suggesting that the induction of CTD110.6 reactivity upon nutrient deprivation is due to *N,N,N'*-diacetylchitobiose and not O-GlcNAc. Consistent with this conclusion, upon β -elimination much of the CTD110.6 reactivity was retained in the starved samples (Figure 4B). Additionally, when probed for α -mannose residues with ConA, we observed a decrease in reactivity in the starvation treated samples consistent with the formation of *N,N,N'*-diacetylchitobiose and attenuation of *N*-glycan biosynthesis (Figure 4C and 4D). Together, these data suggest that *N,N,N'*-diacetylchitobiose forms both in the OGT wild type and null cells under conditions of severe nutrient deprivation.

We then treated our samples with PNGase F and probed with CTD110.6. In OGT wild-type cells we observed an initial increase in CTD110.6 signal after 16h of starvation but this signal was reduced at 24h and decreased below basal levels at 40h (Figure 4E). In OGT knockout cells, CTD110.6 reactivity is greatly diminished, suggesting that the observed changes in reactivity in the OGT wild type are due to the activity of OGT and the reactivity of CTD110.6 to O-GlcNAc. Consistent with this conclusion, upon β -elimination, CTD110.6 reactivity is abolished in all of the PNGase F treated extracts, further indicating that this reactivity is due to an *O*-linked glycan such as O-GlcNAc (Figure 4F). To confirm that our PNGase F treatments were successful, we probed for α -mannose residues in *N*-glycans through using ConA and found that ConA reactivity was diminished upon PNGase F treatment indicating that *N*-glycans were removed (Figure 4G and 4H). Together, these data demonstrate that upon nutrient starvation, there is an induction of O-GlcNAc levels which is accompanied by the appearance of *N,N,N'*-diacetylchitobiose, which is consistent with previous reports [8, 12]. Having characterized the appearance of *N,N,N'*-diacetylchitobiose in OGT null cells, we tested the specificity of CTD110.6 in applications other than immunoblotting and during times of severe cellular stress.

CTD110.6 Will Detect *N,N,N'*-diacetylchitobiose In Other Applications

To determine if the specificity of CTD110.6 is compromised in other antibody based methodologies, we have assessed the reactivity of CTD110.6 by Immunofluorescence and Immunoprecipitation in OGT wild-type and null cells which have been exposed to starvation conditions (48h). As shown in figure 5 only faint CTD110.6 reactivity is detected in OGT null cells under control conditions. However, upon starvation a robust increase in CTD110.6 reactivity is observed and this is perinuclear. The residual CTD110.6 staining in OGT null cells grown in complete media is likely due to the small amount of OGT present at 48h (*discussed below*). Notably

Next we assessed the ability of CTD110.6 to immunoprecipitate proteins modified by *N,N,N'*-diacetylchitobiose. As shown in Figure 6, starvation induced an increase in the O-GlcNAc modification in OGT null cells. At least one starvation specific glycoprotein was immunoprecipitated by CTD110.6 (asterisk), suggesting that CTD110.6 will bind *N,N,N'*-diacetylchitobiose in an immunoprecipitation. These data are not surprising, as CTD110.6 can be purified using GlcNAc-immobilized to agarose through a β -O-glycosidic bond [13].

Blocking in Milk Reduces the Affinity of CTD110.6 for N-N,N'-diacetylchitobiose

While the technique described above (Figure 1) clearly delineates between O-GlcNAc and *N-N,N'*-diacetylchitobiose, it is somewhat cumbersome for everyday applications. In general, most reports using CTD110.6 suggest blocking membranes with either high tween or with BSA [17]. We have demonstrated that simply blocking membranes with 3% milk in TBS appears to significantly reduce the affinity of CTD110.6 for *N-N,N'*-diacetylchitobiose (Figure 6). Presumably, this is due to the high concentration of glycoproteins and sugar in Milk. It should be noted that unlike the experiments using on-blot β -elimination, in these experiments proteins were electroblotted to nitrocellulose rather than PVDF.

O-GlcNAc levels increase in response to stress

Many forms of stress increase O-GlcNAc levels [1] and significantly alter cellular metabolism, resulting in decreased ATP production [25]. To determine whether *N-N,N'*-diacetylchitobiose accumulates in response to other forms of cellular stress we used OGT wild type and null cells. We used many previously reported stressors that have been shown to increase CTD110.6 reactivity including: tunicamycin (Tun) and dithiothreitol (DTT), which are able to induce endoplasmic reticulum (ER) stress, as well as other inducers of the cellular stress response. Upon the induction of cellular stress CTD110.6 reactivity was significantly elevated. This reactivity was diminished in response to OGT knockout under all of the following conditions: nutrient deprivation (early timepoints), heat shock, cycloheximide (CHX), arsenite (NaAs), MG132, osmotic stress (NaCl), DTT, Tun and chloroquine (CQX) (Figure 7 and Supplemental Figure 3). As we have demonstrated that *N-N,N'*-diacetylchitobiose forms in the OGT knockout, these data suggest that the stress treatments used here induce O-GlcNAc and not *N-N,N'*-diacetylchitobiose. It is possible that if exposed to longer stress treatments that *N-N,N'*-diacetylchitobiose may form, however, longer stress treatments often lead to reduced O-GlcNAc signal and this is associated with cell death [26].

Finally, there is a small amount of reactivity with CTD110.6 is retained in the OGT null. This signal likely results from: 1) the small number of cells in this culture have not been transduced with Cre-ERT2, and as a result a small number of cells are still wild-type for OGT [24]. Consistent with this observation, in Figure 4, β -elimination completely ablates the CTD110.6 signal in OGT null cells treated with PNGase F (Figure 4F); 2) Our previous studies demonstrate that OGT levels are not completely suppressed until 96h [24]. We do not typically use OGT null cells beyond 48h, as OGT null cells display significant apoptotic and necrotic markers; and 3) Recently, CTD110.6 was demonstrated to react with extracellular O-GlcNAc which is added to proteins by eOGT [27]. Although, as these proteins need to be enriched to react with CTD110.6, this is the least likely explanation.

Final Comments and Conclusions

We have described an experimental workflow that is capable of distinguishing *O*-linked and *N*-linked glycans. Using this workflow, we were able to characterize the cross-reactivity of a variety of commercially available O-GlcNAc specific antibodies: CTD110.6, 9D1.E4(10), RL2, 18B10.C7(3), 1F5.D6(14), and HGACs 39, 85, 49. We demonstrated that RL2, 18B10.C7(3), 1F5.D6(14), and HGACs 39, 85, 49 were O-GlcNAc specific. As discussed

below, CTD110.6 and 9D1.E4(10) are only non-specific under what appear to be non-physiological conditions. Nonetheless, these data highlight the need to use both PNGase F and mild β -elimination treatments to confirm the specificity of O-GlcNAc probes especially in systems which are starved of nutrients including ischemic injury and in cell culture models utilizing rapidly growing cells.

We have also found that reduced formation of *N*-linked oligosaccharides, or increased *N*-glycan degradation, producing the *N*-*N*,*N*'-diacetylchitobiose structure was evident only under conditions of severe nutrient deprivation (loss of both glucose and FBS components) in U2OS, MEFs, HEK293, and HepG2 cell lines (Figure 1, Figure 4, Figure S1, Figure S2). We speculate that this unknown molecule(s) is a key metabolic substrate. Zho and co-workers have demonstrated that this phenotype can be rescued using glucosamine, and we were able to recapitulate this data using as little as 100 μ M glucosamine (Supplemental Figure 2) [23]. This unknown molecule(s) must have high potency as *N*-*N*,*N*'-diacetylchitobiose formation is not observed when cells are treated with 10% (v/v) FBS containing no glucose for over 48h.

As has been previously published, we have shown that O-GlcNAc levels respond dynamically to a variety of different stressors. Whereas *N*-*N*,*N*'-diacetylchitobiose only forms under conditions of severe nutrient deprivation. It is unclear if *N*-*N*,*N*'-diacetylchitobiose forms under physiologically relevant conditions, but one might predict that it may be induced during prolonged ischemia as is common during events such as myocardial infarction and stroke. If this modification does form, one is left wondering what the biological consequences are. Do proteins modified by *N*-*N*,*N*'-diacetylchitobiose reach the cell surface, or is this a mechanism for halting protein folding and secretion in the ER/Golgi? Our data suggest that some proteins modified by *N*-*N*,*N*'-diacetylchitobiose reach the cell surface, while others accumulate in the peri-nuclear region. These data may suggest that proteins modified by *N*-*N*,*N*'-diacetylchitobiose are eventually retained in the ER or engulfed by autophagosomes (Figure 5).

In conclusion, we have demonstrated that *N*-*N*,*N*'-diacetylchitobiose only forms under conditions of severe nutrient deprivation. While glucose is important, so are the components of serum. Further studies will delineate the physiological role of *N*-*N*,*N*'-diacetylchitobiose in the regulation of protein synthesis and secretion during nutrient deprivation.

Materials and methods

General Reagents

Unless specifically noted, general reagents were obtained from Thermo-Fischer and Sigma Aldrich.

Cell Culture

Cells were maintained at 37°C in a humidified incubator at 5% CO₂. MEFs were seeded ~48h prior to the beginning of stress/starvation treatments and were maintained in DMEM (1.0g/l glucose, Cellgro, Manassas, VA) supplemented with 10% (v/v) FBS (Gibco, Grand

Island, NY) and penicillin/streptomycin (Sigma, St. Louis, MO). MEFs were seeded at 5×10^5 in 100mm plates.

U2OS cells were plated for ~72h prior to being fed the starvation media. U2OS cells were maintained in DMEM (4.5g/l glucose; Cellgro) supplemented with 10% (v/v) FBS and penicillin/streptomycin. U2OS were seeded at 1×10^6 in 150mm plates. Starvation media utilized glucose free DMEM (0g/l glucose; Gibco) supplemented with 10% (v/v) dialyzed FBS (Gibco) and penicillin/streptomycin. The starvation media was added when the cells were between 85%-95% confluence. All media used either contained pyruvate or was supplemented with pyruvate (Cellgro) at a final concentration of 1mM. Starvation experiments utilized a variety of different forms of FBS including: Complete FBS (Gibco), delipidated FBS (Cocalito Biologicals; Reamstown, PA), and charcoal stripped FBS (Gibco).

OGT Knockout

Inducible OGT knockout MEFs were treated with 500nM (Z)-4-hydroxytamoxifen (4OHT; Stock 500 μ M in EtOH, Sigma) or EtOH ~8h after plating as previously described [24]. At 24h the media was removed, cells were washed with PBS, fed fresh media and were allowed to recover for 16h prior to stress treatments. The effectiveness of OGT knockouts was confirmed by Western blot.

Stress Treatments

During heat shock treatments, MEFs were placed in a humidified incubator at 5% CO₂ and 45°C for 1h, and at the end of the heat treatment cells were returned to 37°C for either 1h or 3h. Other stress treatments included: 20 μ g/ml CHX (stock 20mg/ml in ethanol; Sigma) for 1h or 3h; 100 μ M CQX (stock 10mM in PBS; Sigma) for 1h or 3h; 100 μ M NaAs (stock 50mM in H₂O, made fresh each day; Sigma) for 1h or 3h; 2.5 μ g/ml Tun and 1.0 μ g/ml Tun (stock 2.5mg/mL in DMSO;) for 24h; 1 mM DTT (stock 1M in H₂O; Sigma) for 2h or 4h; 100mM NaCl (stock 5M in H₂O; Sigma) for 1h or 3h; and 20 μ M MG132 (stock of 25mM in DMSO; Sigma) for 1h or 3h. Alternatively, cells were treated for an equivalent time with the appropriate vehicle for 1h or 3h. Experiments were performed a minimum of three times.

Protein Extraction

Cells were washed with ice-cold phosphate buffered saline (PBS, 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄, pH7.4), harvested, and extracted with 1% (v/v) Nonidet P-40 in Tris-HCl, pH 7.4, containing EDTA (2mM) and protease inhibitors: phenylmethylsulfonyl fluoride (0.1mM), Thiamet-G (500nM), KF (5mM), β -glycerophosphate (5mM), β -hexosaminidase inhibitor (10mM, Calbiochem, Gibbstown, NJ; Cat# 376820), protease inhibitor cocktail 1 (PIC1), and PIC2. Extracts were separated by SDS-PAGE using Bis-tris gels (Invitrogen). Proteins transferred to either nitrocellulose or PVDF membranes and blocked with either 3% (w/v) bovine serum albumin (BSA) in tris buffered saline (TBS, 50mM tris, 150mM NaCl, pH7.6) or 3% (w/v) skim milk in TBS.

Antibodies and Western Blotting

Blots were probed with the following antibodies and lectins: anti-O-GlcNAc (CTD110.6 antibody; RL2 antibody; 9D1.E4(10) antibody, Millipore Cat# 05-1245; 18B10.C7(3) antibody, Millipore Cat# 05-1244; 1F5.D6(14) antibody, Millipore Cat# 05-1246), anti-GlcNAc (HGAC 39; HGAC 85; HGAC 49, courtesy of Neil Greenspan), anti-OGT (AL28, gift Gerald W. Hart), anti- α -mannose (ConA, EY Laboratories, San Mateo, CA; Cat# H-1104-1), or anti-actin (Sigma). Secondary antibodies included: horseradish peroxidase-conjugated anti-mouse IgM, anti-mouse IgG and anti-rabbit IgG.

Densitometry was performed on non-saturated exposures and analyzed using the ImageJ software (NIH). The relative signal was captured from the entire lane when analyzing probes that yielded multiple bands. Signals were normalized to actin to control for loading differences. All graphs presented represent the average of three independent experiments and are shown as fold changes of the control. Error bars represent ± 1 S.E.

Hexose assay

Hexose assays were performed in a 96-well format using the Quantichrom (Cat: DIGL-100) reagent. Glucose standards or media was assayed in triplicate (10 μ l) by adding 100 μ l of the Quantichrom reagent. Samples were mixed and centrifuged before being heated to 85°C for 20min, allowed to cool at 25°C briefly before being read on a 96 well plate reader at an absorbance of 630nm. Data presented is the average of three experiments with error bars representing $\pm$ one SE of the mean.

PNGase F Treatment

Cell extracts (50 μ g) were treated according to the manufacturer's instructions with 500 units of Peptide: *N*-glycosidase F (New England BioLabs Cat# P0704S) for 3h at 37°C. Reactions were stopped with SDS-PAGE sample buffer and denatured at 85°C for 5min. Control samples were treated identically, but PNGase F was omitted. To confirm that the reaction ran to completion, ConA was used to detect whether *N*-linked mannose structures were still present.

Immunofluorescence

MEFs were plated on coverslips and the induction of OGT deletion and starvation was carried out as described above. Cells were fixed in 4% v/v paraformaldehyde for 15min, before washing 3x in PBS. Cells were permeabilized in 0.075% Triton X-100 in 2mg/ml BSA in PBS for 30minutes. Cells were washed 3x in PBS and Blocked in 2mg/ml BSA (2h, Room Temperature). CTD110.6 was incubated with cells at 2.5 μ g/ml in 2mg/ml BSA overnight at 4°C. Cells were washed (3x PBS) and incubated with a goat-anti mouse IgM conjugated to AlexaFluor 647 (Invitrogen) diluted 1/500 in 2mg/ml BSA. Cells were stained with DAPI before being washed (3xPBS) and mounted in Vectashield. Images were captured on a Zeiss 710NLO Multiphoton confocal microscope using the x63 objective at the Johns Hopkins University School of Medicine Microscope Facility. The Brightness and Contrast of the images for CTD110.6 and Merge were adjusted (Brightness +20%, Contrast -40%) to improve visibility on the printed page.

Immunoprecipitations

Proteins were extracted as described above, except that the concentration of NP-40 was dropped to 0.1% v/v in the extraction buffer. Cell lysates at 1mg/ml were diluted 1:1 with TBS to reduce the concentration of NP-40 to 0.05% v/v. 500µg of extract was incubated overnight with either 25 µg of CTD110.6 or a non-specific IgM covalently coupled to cyanogen bromide activated sepharose (Invitrogen). Samples were washed 3x in TBS, and O-GlcNAc modified proteins were eluted 2x 30minutes in 1M GlcNAc in TBS. Samples were mixed with SDS-PAGE loading buffer, boiled, and assessed by immunoblot.

On-blot β-Elimination

Identical samples were run in duplicate on a gel, blotted to the same PVDF membrane and the membrane was cut in half. One half was subjected to overnight treatment in Milli-Q H₂O at 40°C with rocking and the other was treated with 55mM NaOH at 40°C with rocking [14, 28]. Membranes were then washed 3x in TBS pH 7.4 and then blocked at room temperature for 1h with either 3% (w/v) milk or 3% (w/v) BSA in TBS. Membranes were washed 3x in TBST and then probed overnight. As an alternative, we also tested harsh β-elimination conditions: 100mM NaOH at 60°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

4OHT	(Z)-4-hydroxytamoxifen
BSA	Bovine serum albumin
CHX	Cycloheximide
ConA	Concanavalin A
CQX	Chloroquine
CTD110.6	O-GlcNAc recognizing monoclonal antibody
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
FBS	Fetal bovine serum
H9C2	Rat cardiomyoblast cell line
HBP	hexosamine biosynthetic pathway
HEK293	Human embryonic kidney cell line 293
HepG2	Liver hepatocellular carcinoma cell line
HG DMEM	DMEM containing 1 or 5g/L glucose, 10% (v/v) FBS
O/N	Overnight
MEF	Mouse embryonic fibroblast cell line
<i>N-N,N'</i>-diacetylchitobiose	<i>N</i> -linked GlcNAc β (1-4)GlcNAc
NaAs	Sodium arsenite
NG DMEM	DMEM containing 0g/L glucose
O-GlcNAc	<i>O</i> -linked β - <i>N</i> -Acetyl-D-glucosamine
O-GlcNAcase	Neutral β - <i>N</i> -acetylhexosaminidase
OGT	<i>O</i> -linked β - <i>N</i> -acetyl-D-glucosamine transferase
PBS	Phosphate-buffered saline

PNase F	Peptide <i>N</i> -glycosidase F
PVDF	Polyvinylfluoride
STV	Starvation Media: DMEM (0g/L Glucose), 10% Dialyzed FBS, Pen/Strep
TBS	Tris-buffered saline
TBST	Tris-buffered saline with tween
Tun	Tunicamycin
U2OS	Human osteosarcoma cell line
UDP-GlcNAc	Uridinediphosphate-GlcNAc

Figure 1.

A) Strategy used to distinguish between *O*-linked and *N*-linked glycans that react with CTD110.6. U2OS cells were plated at a density of 1×10^6 cells per 150mm plate and were grown in DMEM containing 5g/L glucose (HG DMEM), 10% (v/v) FBS. 24h before harvest, cells were fed HG DMEM, 10% (v/v) FBS or DMEM containing 0g/L glucose (NG DMEM), 10% (v/v) dialyzed FBS (+ STV). Total cell lysates were treated +/- PNGase F and then separated by SDS-PAGE and blotted to PVDF. Membranes were subjected to overnight (O/N) to either mild conditions (55mM NaOH at 40°C), harsh conditions (100mM NaOH at 60°C) or Milli-Q water at 40°C. **B)** OGT, actin, O-GlcNAc (CTD110.6), or **C)** mannose (ConA), actin and OGT were detected by immunoblot.

Figure 2. Various antibodies used to detect O-GlcNAc also react with *N,N,N'*-diacetylchitobiose U2OS cells were plated at a density of 1×10^6 cells per 150mm plate and were grown in HG DMEM, 10% (v/v) FBS. 24h before harvesting cells were fed either HG DMEM, 10% (v/v) FBS (- STV) or DMEM containing 0g/L glucose (NG DMEM) 10% (v/v) dialyzed FBS (+ STV). Total cell lysates were treated +/- PNGase F and then separated by SDS-PAGE and blotted to PVDF. Membranes were subjected to overnight to either 55mM NaOH or Milli-Q water at 40°C. OGT, actin, O-GlcNAc (CTD110.6, RL2, 18B10.C7(3), 1F5.D6(14), 9D1.E4(10), and GlcNAc (HGAC 39, HGAC 85, HGAC 49) were detected by immunoblot.

Figure 4. *N,N,N'*-diacetylchitobiose can be Induced in OGT Null Cells

MEF cells were plated at a density of 5×10^5 cells per 100mm plate and were grown in LG DMEM 10% (v/v) FBS. Cells were then fed either LG DMEM, 10% (v/v) FBS or starved in NG DMEM, 10% (v/v) dialyzed FBS and were harvested at 16h, 24h or 40h. Deletion of OGT was induced 32h after plating with (Z)-4-hydroxytamoxifen (4OHT). Extracts were treated +/- PNGase F, separated by SDS-PAGE and blotted to PVDF, and treated +/- β -elimination. **A)** CTD110.6, control blot; **B)** CTD 110.6, β -elimination; **C)** ConA, control blot; **D)** ConA, β -elimination; **E)** CTD110.6, PNGaseF treated, control blot; **F)** CTD110.6, PNGaseF treated, β -elimination; **G)** ConA, PNGaseF treated, control blot; and **H)** ConA, PNGaseF treated, β -elimination.

Figure 5. CTD110.6 will Recognize Proteins Modified by *N,N,N'*-diacetylchitobiose During Immunofluorescence

MEFs were plated onto coverslips, and grown in LG DMEM 10% (v/v) FBS. Cells were then fed either LG DMEM, 10% (v/v) FBS or starved in NG DMEM, 10% (v/v) dialyzed FBS and the Deletion of OGT was induced 32h after plating with (Z)-4-hydroxytamoxifen (4OHT). Cells were fixed at 48h and stained for DNA (Blue) and O-GlcNAc with CTD110.6 (Green). **A)** Wild-type, FED, **B)** Wild-type cells, nutrient deprived, **C)** OGT null cells, FED, **D)** OGT null cells, nutrient deprived, **E)** OGT null cells, nutrient deprived.

Figure 6. CTD110.6 will Immunoprecipitate Proteins Modified by *N,N,N'*-diacetylchitobiose
MEFs were plated at a density of 5×10^5 cells per 100mm plate and were grown in LG DMEM 10% (v/v) FBS. Cells were then fed either LG DMEM, 10% (v/v) FBS (F) or starved in NG DMEM, 10% (v/v) dialyzed FBS (S) and the deletion of OGT was induced with (Z)-4-hydroxytamoxifen (4OHT). Cells were harvested at 48h. Cell extracts were separated by SDS-PAGE and Immunoblot and probed with CTD 110.6. Membranes were blocked in either **A)** 3% BSA in TBST or **B)** 3% Milk in TBST. **C)** As a control, Actin and OGT were also detected by Immunoblot. **D)** Glycosylated proteins were immunoprecipitated using CTD110.6 or a non-specific IgM covalently coupled to agarose. Proteins eluted from the antibody in 1M GlcNAc in TBS were separated by SDS-PAGE and glycosylated proteins were detected by immunoblot with CTD110.6. * Indicates bands induced in response to starvation in OGT null cells that immunoprecipitate. As a control, Actin and OGT were also detected by immunoblot. F=Fed, S=Starved, and B=Buffer control.

Figure 7. Stressors Induce the O-GlcNAc Modification and not *N,N,N'*-diacetylchitobiose
MEF cells were plated at a density of 5×10^5 cells per 100mm plate and were grown in LG DMEM, 10% (v/v) FBS. OGT knockout was induced 8h after plating with 4OHT and were washed and refed 24h after 4OHT treatment. **A.** Cells were placed in either a 45°C incubator to induce heat shock (HS) or 37°C incubators as our control. Cells were then allowed to recover for 1 or 3h at 37°C. **B.** Cells were treated with 2mM DTT for either 2 or 4h. **C.** Cells were treated with 20μg/ml CHX for either 1 or 3h. **D.** Cells were treated with 100μM NaAs for 1h or 3h.

Table 1

Hexose Concentrations in Media used in this Study

Media	Hexose Concentration	Standard Deviation
HG DMEM, 10% FBS	21.3mM	1.74mM
NG DMEM, 10% Dialyzed FBS	0.79mM	0.31mM