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DETECTION AND ANALYSIS OF PROTEINS MODIFIED BY O-LINKED N-ACETYLGLUCOSAMINE

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Abstract

O-GlcNAc is a common post-translational modification of nuclear, mitochondrial, and cytoplasmic proteins that regulates normal physiology and the cell stress response. Dysregulation of O-GlcNAc cycling is implicated in the etiology of type II diabetes, heart failure, hypertension, and Alzheimer's disease, as well as cardioprotection. These protocols cover simple and comprehensive techniques for detecting proteins modified by O-GlcNAc and studying the enzymes that add or remove O-GlcNAc.

Basic Protocol 1: Increasing the Stoichiometry of O-GlcNAc on Proteins Before Analysis

Basic Protocol 2: Detection of Proteins Modified by O-GlcNAc Using Antibodies

Basic Protocol 3: Detection of Proteins Modified by O-GlcNAc Using the Lectin sWGA

Support Protocol 1: Control for O-Linked Glycosylation

Basic Protocol 4: Detection and Enrichment of Proteins Using WGA-Agarose

Support Protocol 2: Digestion of Proteins with Hexosaminidase

Alternate Protocol 1: Detection of Proteins Modified by O-GlcNAc Using Galactosyltransferase

Support Protocol 3: Autogalactosylation of Galactosyltransferase

Support Protocol 4: Assay of Galactosyltransferase Activity

Basic Protocol 5: Characterization of Labeled Glycans by β -Elimination and Chromatography

Basic Protocol 6: Detection of O-GlcNAc in 96 well plates

Basic Protocol 7: Assay for OGT Activity

Support Protocol 5: Desalting the O-GlcNAc Transferase

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Basic Protocol 8: Assay for O-GlcNAcase Activity

Keywords

signal transduction; glycosylation; O-GlcNAc; detection; analysis; galactosyltransferase; O-linked

INTRODUCTION

The modification of Ser/Thr residues of nuclear, cytosolic, and mitochondrial proteins by O-linked β -N-acetylglucosamine (O-GlcNAc) is a dynamic post-translational modification of metazoans. While O-GlcNAc has been identified in all metazoans studied, recent data suggest that plants use both O-fucose and O-GlcNAc (Xu et al., 2012; Zentella et al., 2017), protozoans use either O-GlcNAc or O-fucose (Banerjee et al., 2009), and that while filamentous yeast use O-GlcNAc (Schindler et al., 1987), *Saccharomyces cerevisiae* may use mannose (Halim et al., 2015).

O-GlcNAc is cycled on and off proteins by two dedicated enzymes; the O-GlcNAc transferase (OGT) that catalyzes the addition of O-GlcNAc and the O-GlcNAcase (OGA) that catalyzes the removal of O-GlcNAc. The reversible nature of the O-GlcNAc modification allows for multiple rounds of regulation/alteration of function over the lifetime of proteins. Indeed, protein O-GlcNAcylation has been demonstrated to impact protein function by altering structure and stability (Elbaum and Zondlo, 2014; Brister et al., 2014; Yuzwa et al., 2012; Levine et al., 2019), enzymatic activity (Han et al., 2017; Rao et al., 2015; Yi et al., 2012), protein-protein interactions (Toleman et al., 2018), and post-translational modifications (Comer and Hart, 2001; Chou et al., 1995). O-GlcNAcylation is responsive to stimuli/signaling including nutrient availability, cellular stress, insulin stimulation, or cellular stages such as the cell cycle suggesting that cells use O-GlcNAc to remodel cellular pathways (Hart et al., 2011).

Proteomic studies suggest that more than 4000 protein of diverse function are modified by O-GlcNAc. Proteins modified by O-GlcNAc include cytoskeletal proteins, nuclear pore proteins, RNA polymerase II (RNA Pol II), transcription factors, proto-oncogene products, tumor suppressors, hormone receptors, phosphatases, and kinases (Nandi et al., 2006; Vosseller et al., 2006; Chalkley et al., 2009; Khidekel et al., 2007; Trinidad et al., 2012). Not surprisingly, O-GlcNAc has been implicated in regulating key proteins within cellular pathways, including epigenetics, transcription, translation, protein homeostasis, autophagy, nutrient sensing and metabolism, immune signaling, and the cell cycle. Moreover, perturbations in the metabolism of UDP-GlcNAc, which alter the regulation of many O-GlcNAc-modified proteins, have been implicated in Alzheimer's disease, diabetes, and cancer (Hart et al., 2011; Zhu et al., 2014; Ma and Vosseller, 2014).

These protocols concentrate on techniques for the detection and analysis of proteins modified by O-GlcNAc, as well as methods for the analysis of enzymes responsible for the addition and removal of O-GlcNAc. The major focus is on methods that require standard laboratory equipment. First, a protocol for increasing the stoichiometry of O-GlcNAc on proteins is given (see Basic Protocol 1). This is followed by simple techniques for the

detection and screening of O-GlcNAc modified proteins either by immunoblotting or lectin affinity chromatography (see Basic Protocols 2 to 5). Separate protocols are used to verify that the glycan is O-linked GlcNAc (see Support Protocols 1 and 2). These methods are followed by protocols for a more comprehensive analysis of O-GlcNAc-modified proteins, including labeling of O-GlcNAc residues with [³H]Gal, and subsequent product analysis (see Alternate Protocol 1, see Basic Protocol 5, and see Support Protocols 3 and 4). There is also a protocol that enables the detection of O-GlcNAc in 96-well plate format (see Basic Protocol 6). The final two protocols assay for O-GlcNAc transferase and O-GlcNAcase activity, respectively (see Basic Protocols 7 and 8 and Support Protocol 5).

BASIC PROTOCOL 1 Increasing the Stoichiometry of O-GlcNAc on Proteins Before Analysis

In cultured mammalian cells, as well as tissue slices and tissues *in vivo*, the stoichiometry of O-GlcNAc moieties per protein molecule can be increased by treating cells/tissues/animals with inhibitors of O-GlcNAcase. Several inhibitors exist (Macauley et al., 2005; Dennis et al., 2006; Kim et al., 2006; Shanmugasundaram et al., 2006; Yuzwa et al., 2008), although only PUGNAc (Toronto Research Chemicals; (Haltiwanger, 1998)) and Thiamet-G (Cayman Chemicals; (Yuzwa et al., 2008)) are commercially available. Unlike Thiamet-G, PUGNAc also inhibits lysosomal hexosaminidases. Several OGT inhibitors have been developed (Gross et al., 2005). While these initial inhibitors were demonstrated to work in neonatal cardiomyocytes (1 to 5 μM (Ngoh et al., 2008)) and breast cancer cells (500 μM (Caldwell et al., 2010)), recent data suggests that these inhibitors react with the protein backbone limiting their utility (Jiang et al., 2011). Recently, high-throughput screening led to the development of a small-molecule OGT inhibitor, OSMI-1, which is cell-permeable (Ortiz-Meoz et al., 2015). Further structure-based evolution of OSMI-1 yielded three additional compounds OSMI -2, -3 and -4 with higher affinity for OGT (Martin et al., 2018). One alternative strategy has been to use cell-permeable compounds that are metabolized by the cell to generate non-hydrolyzable analogs of UDP-GlcNAc. Examples include Ac₄SSGlcNAc (effective in cells (Gloster et al., 2011)) or 5SGlcNH₆ (effective in animals (Liu et al., 2018)). However, it is important to note that OGT inhibitors that mimic UDP-GlcNAc are likely to impact other enzymes utilizing UDP-GlcNAc as a substrate. Streptozotocin (STZ; (Roos et al., 1998)), glucosamine (Han et al., 2000), and the glutamine fructose-6-phosphate amidotransferase (GFAT) inhibitors 6-diazo-5-oxonorleucine (DON) and Azaserine have also been used to alter the stoichiometry of O-GlcNAc on proteins. However, STZ has been shown to induce poly-(ADP-ribose) polymerase-mediated apoptosis in Min6 cells (Gao et al., 2000) and should be used with caution. STZ and glucosamine treatments are most effective in cells that express the glucose transporter GLUT-2 (Schnedl et al., 1994). Notably, inhibitors of GFAT can have off-target effects on other glutamine utilizing enzymes. Benzyl-α-GalNAc and benzyl-β-GlcNAc, which have been reported to inhibit OGT, should be avoided, as these have been characterized as inhibitors of prototypical O-GalNAc-type glycosylation. Care should be taken when using inhibitors of OGT and OGA, as treatment at too high a dose or for too long can impact the maturation of the mRNAs encoding OGT and OGA and thus protein expression (Park et al., 2017; Tan et al., 2020).

Materials

Cells of interest growing in monolayer culture, and appropriate culture medium

1 mM Thiamet-G (Millipore-SIGMA; SML0244) stock in 1 M HEPES, pH 7.4 (filter sterilize and store in aliquots up to 6 to 12 months at -80°C)

100 mM PUGNAc (Millipore-SIGMA; A7229) stock in PBS, pH 7.5 (filter sterilize and store in aliquots up to 6 to 12 months at -80°C)

500 mM glucosamine stock in 500 mM HEPES, pH 7.5 (make just prior to use; filter sterilize)

Ice

100-mm tissue culture dishes

Humidified water-jacket CO_2 incubator

Additional reagents and equipment for SDS-PAGE and electroblotting (Gallagher, 2001).

1. Grow cells in monolayer culture in a sufficient number of 100-mm dishes.
2. Add (or replace) growth medium with fresh medium containing 0.1 to 1 μM Thiamet-G (added from 1 mM stock) or 10 to 100 μM PUGNAc (added from 100mM stock) or 5 mM glucosamine (added from 500 mM stock). Incubate the cells in a humidified water-jacket CO_2 incubator at 37°C for 4 to 18 hr.

Treatments should be optimized for the desired cell type for 4–18h. Longer treatments alter the expression of OGT and OGA, as well as leading to significant toxicity (Slawson et al., 2005).

The vehicle is either 1 M HEPES, PBS, or 0.5 M HEPES, pH 7.5.

When using glucosamine, mannitol is often added to the controls at the same concentration. The use of mannitol controls for changes in osmolarity due to the additional sugar in the medium (Heart et al., 2000).

3. At the end of treatment, take the dishes out of the incubator and place on ice. Extract as desired. Separate proteins by SDS-PAGE and electroblot onto an appropriate membrane (Gallagher, 2001).

Alternatively, extract proteins and proceed with protein purification or immunoprecipitation (Immunoprecipitation., 2001).

BASIC PROTOCOL 2 Detection of Proteins Modified by O-GlcNAc Using Antibodies

Several antibodies have been developed that recognize terminal GlcNAc residues or the O-GlcNAc modification (Table 1). Additionally, site-specific O-GlcNAc antibodies have been raised against glycosylation sites on proteins including c-Myc (Kamemura et al.,

2002), Tau (Cameron et al., 2013; Yuzwa et al., 2011), SirT1 (Shan et al., 2018), CRMP2 (Muha et al., 2019), as well as IRS2 and a subset of histones (H2A, H2B, H3, H4; GlycoScientific). These tools allow researchers to probe the O-GlcNAc modification state of key proteins without purification of the protein first. Site-specific antibodies are not discussed here since they are not widely available. When using O-GlcNAc pan-specific antibodies (antibodies that recognize O-GlcNAc linked to Ser/Thr residues) or GlcNAc pan-specific antibodies (antibodies that recognize any terminal beta GlcNAc residue, including those linked to longer glycan structures), it is important to run appropriate controls, as some antibodies cross-react with peptide sequences that mimic GlcNAc residues (Shikhman et al., 1994), while others have some dependence on the peptide backbone (Holt et al., 1987; Snow et al., 1987). Appropriate controls include: (1) elevating O-GlcNAc levels in tissue culture using inhibitors of the O-GlcNAcase or glucosamine (see Basic Protocol 1); (2) lowering O-GlcNAcylation in tissue culture cells using inhibitors of OGT or the hexosamine biosynthetic pathway; (3) removing O-GlcNAc from samples *in vitro* using hexosaminidase (see Support Protocol 3); (4) elevating the levels of O-GlcNAc in tissue culture by overexpressing OGT or performing RNAi of O-GlcNAcase; (5) lowering O-GlcNAcylation by overexpressing O-GlcNAcase or performing RNAi of OGT; (6) treating lysates with PNGase F to remove N-linked glycans; (7) using appropriate purified control proteins, such as ovalbumin, which bears N-linked glycans with terminal GlcNAc residues, or synthetic neoglycoconjugates, such as BSA-aminophenyl-GlcNAc (BSA-AP-GlcNAc), or (8) competing away antibody binding with free GlcNAc which controls for non-specific binding and cross-reactivity with endogenous immunoglobulin.

Using the Millipore Sigma Immobilon Western Enhanced Chemiluminescent (ECL) Horse radish peroxidase (HRP) Substrate system the authors have found that 10 µg of nuclear or total cell extract is sufficient. However, 20 to 30 µg of cell extract provides the highest quality data. For the detection of O-GlcNAc from tissue samples, the authors have established that 25 to 30 µg of total tissue lysate is required for detection using CTD110.6. For detection using the RL2 antibody, 15 to 20 µg of tissue lysate is sufficient to be within the linear range of detection using the aforementioned ECL system. For purified proteins, Comer and co-workers found that 25 to 50 ng of a neoglycoconjugate was sufficient (Comer et al., 2001). The protocol given is for CTD110.6 (Comer et al., 2001), which appears to have the least peptide dependence and recognize the greatest number of O-GlcNAc modified proteins. A table summarizing conditions for a subset of commercial antibodies is included and use a similar protocol to CTD110.6 (Table 2).

Materials

Purified or crude protein (e.g., Basic Protocol 1) separated by SDS-PAGE and electroblotted to polyvinylidene difluoride (PVDF; (Gallagher, 2001)) or nitrocellulose (*preferred to PVDF*) (duplicate blots are required)

Blocking buffer (3% milk w/v in TBST)

Antibody: CTD 110.6 (Millipore SIGMA, MABS1254) diluted to 1 µg/mL in antibody dilution buffer (3% BSA w/v in TBST; see recipe for TBST in Reagents and Solutions)

N-acetylglucosamine (GlcNAc; Millipore SIGMA, A4106)

TBST (see recipe in Reagents and Solutions)

HRP-conjugated anti-mouse IgM (Millipore SIGMA, A8786) diluted 1/5000 in antibody dilution buffer (3% BSA w/v in TBST)

TBS (see recipe in Reagents and Solutions)

Immobilon Western Chemiluminescence HRP Substrate (Millipore SIGMA, WBKLS0500)

Additional reagents and equipment for visualization with chromogenic and luminescent substrates (Gallagher, 2001).

IMPORTANT NOTE: Volumes are given for 9 × 14-cm membranes washed in 10.5 × 15.5-cm boxes. Volumes can be scaled down or up, but membranes should be covered.

1. Block the blots by incubating with 25 ml blocking buffer for 60 min at room temperature.
2. Wash the blots with 25 ml TBST three times, each time for 10 min at room temperature.
3. Incubate the blots with CTD 110.6 (diluted to 1 µg/mL in antibody dilution buffer), in duplicate, with and without 100 mM GlcNAc, overnight at 4°C.

An antibody that has been antigen purified using a GlcNAc-agarose column yields higher quality blots.

Commercially available CTD110.6 is purified by ammonium sulfate precipitation. Thus, it may be necessary to titrate antibody concentration when using a new lot number. Typically, we find that 1 µg/mL is a good place to start. For the detection of O-GlcNAc in tissue lysates, we find that higher concentrations of CTD110.6 are necessary,

To control for specificity, it is important to perform a control blot. Here, the antibody is pre-incubated with 100 mM GlcNAc for ~5 min on ice before being applied to the control blot. This control is most important when probing immunoprecipitations and tissue lysates that may contain immunoglobulin. Table 2 reports the concentration of free GlcNAc to use for other O-GlcNAc antibodies.

4. Wash the blots with 25 ml TBST three times, each time for 10 min at room temperature.
5. Incubate the blots with HRP-conjugated anti-mouse IgM (1/5000 dilution in antibody dilution buffer) for 50 min at room temperature.

The concentration of secondary antibody varies from lot to lot and should be optimized each time with each new preparation.

6. Wash the blots with 25 ml TBST four times, each time for 10 min at room temperature.
7. Wash the blots with 25 ml TBS once, for 10 min at room temperature.
8. Develop the HRP reaction using, e.g., the ECL system(Gallagher, 2001).

CTD110.6 should bind BSA-AP-GlcNAc, but not BSA-AP. A number of bands in total cell lysate should be detected predominantly over 40 kDa. Treatment with Thiamet-G should elevate the levels of O-GlcNAc 2- to 3-fold on numerous proteins in total cell lysate. Reactivity toward both BSA-GlcNAc and total cell lysate should be completely competed away by free GlcNAc. Note that the antibody often cross-reacts with pre-stained markers.

BASIC PROTOCOL 3 Detection of Proteins Modified by O-GlcNAc Using the Lectin sWGA

Many lectins are reportedly specific for β -GlcNAc residues. The authors have typically used succinylated wheat germ agglutinin (sWGA), which is widely available and is derivatized with a number of useful functional groups including HRP. Before succinylation, WGA will recognize both sialic acid and GlcNAc (Monsigny et al., 1979). For additional information concerning lectin chromatography, see Lectin Affinity Chromatography (Freeze, 2001).

The amount of “test” protein used is dependent on the technique(s) used to develop the HRP reaction. Using the Millipore Sigma Immobilon Western Enhanced Chemiluminescent HRP Substrate system, the authors find that 15 μ g of cytoplasmic or nuclear extract is sufficient, but 20 to 30 μ g of protein produces the best data.

It is important to include an appropriate positive (100 ng ovalbumin) and negative (100 ng of BSA) control. As a control, a portion of the sample should also be treated with hexosaminidase (see Support Protocol 2), to show that reactivity is toward GlcNAc. Alternatively, the levels of O-GlcNAc can be manipulated in cell culture (see Basic Protocol 1). An additional control is to subject the blot to mild reductive β -elimination to verify that lectin/antibody reactivity is towards O-linked glycans (see Support Protocol 1), rather than N-linked glycans.

Materials

Purified or crude protein separated by SDS-PAGE and electroblotted (Gallagher, 2001) to polyvinylidene difluoride (PVDF) or nitrocellulose (duplicate blots are needed)

5% (w/v) BSA in TBST (see recipe for TBST)

TBST (see recipe in Reagents and Solutions)

0.1 μ g/ml HRP-conjugated sWGA (EY Labs; H-2102) in TBST (see recipe for TBST): the lectin can be stored at 1 mg/ml in 0.01 M PBS, pH 7.4 (APPENDIX 2E), at -20°C for at least 1 year

N-acetylglucosamine (GlcNAc; Millipore Sigma; A4106)

High-salt TBST (HS-TBST): TBST (see recipe in Reagents and Solutions) containing 1 M NaCl

Tris-buffered saline (TBS; see recipe in Reagents and Solutions)

ECL kit (Millipore Sigma Immobilon Western Chemiluminescent HRP Substrate, WBKLS0500)

Additional reagents and equipment for visualization with chromogenic and luminescent substrates (Gallagher, 2001)

IMPORTANT NOTE: Volumes are given for 9 × 14-cm membranes washed in 10.5 × 15.5-cm boxes. Volumes can be scaled down or up, but membranes should be covered.

1. Wash the duplicate blots for 10 min in 25 ml TBST at room temperature.
2. Block by incubating the blots in 25 ml 5% (w/v) BSA/TBST for at least 60 min at room temperature.

IMPORTANT NOTE: *Milk cannot be used as the blocking agent since many of the proteins in milk are modified by glycans that react with sWGA.*

3. Wash the blots three times, each time for 10 min with 25 ml TBST at room temperature.
4. Incubate the blots in 0.1 µg/ml sWGA-HRP in HS-TBST, in duplicate, with and without 1 M GlcNAc, overnight at 4°C.

To control for lectin specificity, it is important to perform a control blot. Here, the lectin is pre-incubated with 1 M GlcNAc for ~5 min on ice before being applied to the control blot.

5. Wash the blots six times, each time for 10 min, with 25 ml HS-TBST at room temperature.
6. Wash the blots once with 25 ml TBS for 10 min at room temperature.
7. Develop the HRP-reaction using, e.g., the ECL system (Gallagher, 2001).

sWGA should bind BSA-AP-GlcNAc and ovalbumin, but not BSA-AP. A number of bands, predominantly over 40 kDa, in total cell lysate should be detected. Treatment with Thiamet-G should elevate the levels of O-GlcNAc 2- to 3-fold on numerous proteins in total cell lysate. Reactivity toward BSA-GlcNAc, ovalbumin, and total cell lysate should be completely competed away by free GlcNAc. Using the ECL system described, 100 ng of ovalbumin should be visualized in 5 to 15 sec.

SUPPORT PROTOCOL 1 CONTROL FOR O-LINKED GLYCOSYLATION

Traditionally, mild alkaline reduction (reductive β -elimination) has been used to release O-linked carbohydrates from proteins (Amano and Kobata, 1989). This method has been adapted for blots to show that lectin/antibody reactivity is toward O-linked rather than N-linked glycans (Duk et al., 1997). Proteins blotted to PVDF are treated with 55 mM NaOH overnight (releasing O-linked sugars) and then probed using lectins or antibodies (Reeves et al., 2014). The authors note that this protocol will not differentiate between O-GlcNAc and other forms of O-linked glycosylation.

There are a number of reasons why lectin/antibody reactivity could be lost after NaOH treatment, e.g., the sugars were destroyed instead of being released, or the protein was degraded. To control for these, it is important to have control proteins with N- and O-linked sugars, and to stain one blot for protein after treatment, preferably with an antibody. The authors suggest a control blot of bovine asialofetuin (Millipore Sigma), which contains both N- and O-linked sugars terminating in GlcNAc, treated and not treated with PNGase F (Powell, 2001).

Materials

Protein samples and controls blotted to PVDF (triplicate blots are needed; nitrocellulose is not suitable as it dissolves in 55 mM NaOH)

Tris-buffered saline (TBS; see recipe in Reagents and Solutions)

55 mM NaOH

Milli-Q water

TBST (see recipe in Reagents and Solutions)

3% (w/v) BSA in TBST (see recipe for TBST in Reagents and Solutions)

40°C water bath

Additional reagents and equipment for probing protein blots with protein-specific antibodies (see Basic Protocol 2) or lectins (see Basic Protocol 3)

IMPORTANT NOTE: Volumes are given for 9 × 14-cm membranes washed in 10.5 × 15.5-cm boxes. Volumes can be scaled down or up, but membranes should be covered.

1. Wash the blots once with 25 ml TBS for 10 min at room temperature.
2. Incubate the two blots in 25 ml 55 mM NaOH at 40°C overnight; incubate the control blot in 25 ml Milli-Q water at 40°C overnight.

The blots treated with NaOH will yellow slightly.
3. Wash the blots three times, each time for 10 min at room temperature, with 25 ml TBST.

4. Block by incubating the blots in 25 ml of 3% (w/v) BSA/TBST for 60 min at room temperature.
5. Probe the blots (one treated and one untreated) with carbohydrate-specific lectins (see Basic Protocol 3) or antibodies (see Basic Protocol 2). Probe the second NaOH-treated blot with a protein-specific antibody (see Basic Protocol 2).

On the untreated blot, asialofetuin \pm PNGase F should react with sWGA, as both the N- and O-linked sugars contain terminal GlcNAc residues. On the treated blot, only the asialofetuin – PNGase F should react with sWGA.

BASIC PROTOCOL 4 DETECTION AND ENRICHMENT OF PROTEINS USING WGA-AGAROSE

WGA lectin affinity chromatography provides a convenient method for enriching and detecting O-GlcNAc modified proteins. This procedure has been adapted for detecting proteins that are difficult to purify or are present in low copy numbers, such as transcription factors. In this protocol, the protein of interest is synthesized in a rabbit reticulocyte lysate (RRL) *in vitro* transcription-translation (TNT) system (Promega) and labeled with either [³⁵S]Met, [³⁵S]Cys, or [¹⁴C]Leu. After desalting, the proteins are tested for their ability to bind WGA-agarose in a GlcNAc-specific manner (Roquemore et al., 1994). This protocol is readily adapted to purifying proteins from cell extracts, but, as WGA binds proteins with both terminal GlcNAc and sialic acid residues, typically, one would purify proteins from nuclear and cytoplasmic extracts to avoid co-purifying proteins with prototypical glycans.

Alternatively, the lectin *Ricinus communis* agglutinin 1 (RCA1) has been used to select for O-GlcNAc proteins that have previously been labeled by galactosyltransferase (see Alternate Protocol 1). Proteins modified by terminal Gal are specifically retained on an RCA1 affinity column. Labeled O-GlcNAc-modified proteins are released under mild conditions, while those containing N-linked structures require lactose addition to the buffer before elution results (Hayes et al., 1995; Greis and Hart, 1998). The method described in this protocol can be adapted for RCA1 affinity chromatography by substituting RCA1-agarose (Vector Laboratories) for WGA-agarose and changing the order of the Gal and GlcNAc elution buffer.

Materials

cDNA subcloned into an expression vector with an SP6 or T7 promoter (~0.5 to 1 $\mu\text{g}/\mu\text{l}$)

TNT \otimes Lysate systems kit system kit (Promega)

Label: [³⁵S]Met *or* [³⁵S]Cys, *or* [¹⁴C]Leu

Ensure that the label used is well represented in your protein of interest. Although [³⁵S]Met is usually the most cost-effective and sensitive choice.

WGA-agarose (Vector Laboratories; AL1023) WGA wash buffer: PBS (*APPENDIX 2E*) containing 0.2% (v/v) NP-40

WGA Gal elution buffer (see recipe in Reagents and Solutions)

WGA GlcNAc elution buffer (see recipe in Reagents and Solutions)

TCA *or* methanol

1-ml tuberculin syringe with glass wool plug at the bottom to support chromatography matrix *or* Bio-Rad Bio-Spin disposable chromatography column

Liquid scintillation counter

Additional reagents and equipment for digesting proteins with hexosaminidase (see Support Protocol 2), desalting (see Support Protocol 5), SDS-PAGE, and autoradiography (Gallagher, 2001).

Prepare the proteins

1. Synthesize the proteins to incorporate the desired label ($[^{35}\text{S}]\text{Met}$, $[^{35}\text{S}]\text{Cys}$, or $[^{14}\text{C}]\text{Leu}$) using the TNT ® system according to the manufacturer's instructions. Include the protein of interest, a positive control for WGA binding (e.g., the nuclear pore protein p62), a negative control (luciferase, supplied with kit), and a no-DNA control.
2. Treat half of each sample with hexosaminidase (see Support Protocol 2).
3. Desalt the samples using spin filtration (e.g., *Cytiva Life Sciences* Microspin G-50 columns) or a 1-ml G-50 desalting column (as for desalting O-GlcNAc transferase; see Support Protocol 5).

Apply the protein samples to chromatography columns

The following procedure is carried out at 4°C.

4. Equilibrate WGA-agarose and pack column as follows:
 - a. If the resin is supplied as 50% slurry (i.e., 50% resin/50% storage solution), remove 300 µl (double the volume required) and pipet into a 1-ml tuberculin syringe or disposable chromatography column.

WGA is used here rather than sWGA as it has a higher affinity for GlcNAc and O-GlcNAc
 - b. Let the storage solution drain from resin.
 - c. Equilibrate resin by washing column four times, each time with 1 ml of WGA wash buffer. Cap the column.

The volumes given are appropriate for a sample derived from an TNT. For enrichment of other protein samples, the volume of WGA should be optimized for the protein sample applied. The authors find that 50 mg of

cell extract requires 5 ml of WGA-agarose, assuming that 1% to 2% of the total cell extract is modified by O-GlcNAc.

5. Apply the sample (~30 μ l of an TNT reaction) to the column and let stand at 4°C for 30 min, or cap and incubate at 4°C for 30 min with rotating or rocking.

Save a small amount (1%) of the starting material as a control to count and to run on a gel.

Wash the column and elute GlcNAc

6. At the end of the 30 min incubation, uncap the column and allow the sample to “run through” the resin. Collect this as the “run through” fraction. Wash the column with 15 ml WGA wash buffer at 10 ml/hr, collecting 0.5 ml fractions.
7. Load the column with 300 μ l WGA Gal elution buffer and let stand at 4°C for 20 min.
8. Wash the column with 5 ml WGA Gal elution buffer, collecting 0.5 ml fractions.
9. Repeat steps 6 to 8 using GlcNAc elution buffer.
10. Count 25 μ l of each fraction using a liquid scintillation counter.

Depending on the stoichiometry, or the number of O-GlcNAc residues per molecule of protein, 1% to 10% of the labeled protein should bind the WGA.

11. Pool positive fractions that elute in the presence of GlcNAc and precipitate using TCA or methanol.

To precipitate proteins with methanol, mix 1 vol of sample with 10 vol of ice-cold methanol. Incubate overnight at -20°C. Recover protein by centrifuging for 10 min at 16,000 \times g, 4°C, in a microcentrifuge tube (which is the most efficient procedure) or in 15-ml conical centrifuge tubes for 10 min at 3000 \times g, 4°C. Resuspend samples in SDS-PAGE sample buffer (Gallagher, 2001).

As many proteins in rabbit reticulocyte lysate contain O-GlcNAc and bind WGA, the authors do not recommend the addition of carrier proteins at this point. Typically, a fraction of the GlcNAc elution containing a total of 1000 to 2000 dpm [³⁵S]Met is precipitated and analyzed by SDS-PAGE and autoradiography (Gallagher, 2001).

The use of acetone to precipitate proteins is not recommended, as free GlcNAc will also precipitate.

12. Analyze the pellet by SDS-PAGE and autoradiography (Gallagher, 2001).

Expect a band at the approximate molecular weight of the protein of interest.

SUPPORT PROTOCOL 2 DIGESTION OF PROTEINS WITH HEXOSAMINIDASE

Terminal GlcNAc and O-GlcNAc can be removed from proteins using commercially available hexosaminidases; these enzymes will also cleave terminal GalNAc residues. Unlike O-GlcNAcase, commercial hexosaminidases have low pH optima, typically pH 4.0 to 5.0.

Materials

Protein sample for digestion (include a positive control, e.g., ovalbumin)

2% (w/v) SDS (see *APPENDIX 2E* for 20% stock)

2× hexosaminidase reaction mixture (see recipe in Reagents and Solutions)

Additional reagents and equipment for SDS-PAGE and electroblotting (Gallagher, 2001)

1. Mix the sample 1:1 with 2% SDS and boil for 5 min.
2. Mix the sample 1:1 with 2× hexosaminidase reaction mixture and incubate at 37°C for 4 to 24 hr.
3. To assess the completeness of the digestion, separate an aliquot of the reaction by SDS-PAGE and electroblot (Gallagher, 2001) onto an appropriate membrane. Probe blots with carbohydrate-specific lectins or antibodies.

Ovalbumin should move several kilodaltons on a 10% to 12% gel, and reactivity toward WGA should be ablated. Reactivity of O-GlcNAc modified proteins to WGA and CTD110.6 should be reduced significantly.

ALTERNATE PROTOCOL 1 Detection of Proteins Modified by O-GlcNAc Using Galactosyltransferase

The enzyme β -1,4-galactosyltransferase (from bovine milk) will label any terminal GlcNAc residue with Gal, using uridine diphospho-D-Gal (UDP-Gal) as a donor substrate (Brew et al., 1968). Hart and colleagues have exploited this property, using the enzyme to label terminal GlcNAc residues on proteins with [6-³H]Gal, forming a [³H]- β Gal1-4 β GlcNAc (Torres and Hart, 1984; Roquemore et al., 1994; Greis et al., 1996). The labeled sugar can be chemically released (via β -elimination) and analyzed by size-exclusion chromatography on a BioGel-P4 column, using the ³H radiolabel to detect the fraction of interest (Roquemore et al., 1994). Labeling the O-GlcNAc residue allows for the subsequent detection of proteins and peptides of interest during SDS-PAGE, HPLC, protease digestion, and Edman degradation steps. Researchers have been able to identify glycosylation sites on as little as 10 pmol using these methods (Greis et al., 1996). Recently, this technique has been adapted to allow the incorporation of unnatural sugar analogs that can be derivatized to facilitate either the purification or detection of O-GlcNAc-modified proteins and peptides (Kim, 2018), a technique that is marketed by Invitrogen under the “Click-it” brand (Invitrogen).

To achieve efficient labeling of some proteins, it is necessary to denature samples, for example, by boiling in the presence of 10 mM DTT and 0.5% (w/v) SDS. Galactosyltransferase has been shown to be active in solutions containing 5 mM DTT, 0.5 M NaCl, up to 2% (v/v) Triton X-100, up to 2% (v/v) NP-40, and 1 M urea. Up to 0.5% (w/v) SDS can be used if it is titrated with a 10-fold molar excess of either Triton X-100 or NP-40 in the final reaction mixture. Digitonin, which is commonly used to solubilize cells, should be used with caution, as it is a substrate for galactosyltransferase. The total ionic strength should be less than 0.2 M.

Galactosyltransferase requires 1 to 5 mM Mn^{2+} for activity but is inhibited by Mg^{2+} and concentrations of $Mn^{2+} > 20$ mM. EDTA (or analogs) should be avoided unless titrated with appropriate levels of Mn^{2+} . Note that 1 mole of EDTA binds 2 moles of Mn^{2+} .

Free UDP is also an inhibitor of galactosyltransferase. For studies where complete labeling of the GlcNAc is preferable, such as site mapping, calf intestinal alkaline phosphatase is included in the reaction, as it degrades UDP (Unverzagt et al., 1990). While this increases the efficiency of the reaction, it is important to add this to the control as some preparations of alkaline phosphatase contain proteins that will label with galactosyltransferase (R.N. Cole, pers. commun.).

NOTE: Protease inhibitors, such as PIC 1, PIC 2, and PMSF (see the recipe for 1000× protease inhibitors in Reagents and Solutions), can be included (final concentrations, 1×), but GlcNAc and 1-amino GlcNAc should be removed prior to labeling by spin filtration or another method of desalting.

Materials

Protein sample(s)

Dithiothreitol (DTT)

Sodium dodecyl sulfate (SDS; see *APPENDIX 2E* for 20% stock solution)

Label: 1.0 mCi/ml UDP- $[^3H]$ Gal, (17.6 Ci/mmol; American Radiolabeled Chemicals) in 70% (v/v) ethanol

Nitrogen source

25 mM 5'-adenosine monophosphate (5'-AMP) in Milli-Q water, pH 7.0

Buffer H (see recipe in Reagents and Solutions)

10× galactosyltransferase labeling buffer (see recipe in Reagents and Solutions)

Galactosyltransferase, autogalactosylated (see Support Protocol 3)

Calf intestinal alkaline phosphatase

Unlabeled UDP-Gal

Stop solution: 10% (w/v) SDS/0.1 M EDTA

Speed-Vac evaporator

37°C incubator

100°C water bath

30 × 1-cm Sephadex G-50 column equilibrated in 50 mM ammonium formate/0.1% (w/v) SDS

Liquid scintillation counter

Additional reagents and equipment for acetone precipitation of protein (Lovrien and Matulis, 2001), PNGase F digestion of proteins (Powell, 2001), SDS-PAGE (Gallagher, 2001), and product analysis (see Basic Protocol 5)

Prepare the reaction

1. Denature the protein sample by adding DTT to 10 mM and SDS to 0.5% (w/v), then boiling the sample for 10 min.
2. Decide how many reactions are going to be carried out and thus how much label will be needed (~1 to 2 μ Ci/reaction).

A positive control (ovalbumin, 2 μ g), a negative control (because galactosyltransferase can label itself), and a sample-minus-enzyme control will be needed.

3. Remove solvent from label in a Speed-Vac evaporator or under a stream of nitrogen.

Ethanol can inhibit the galactosyltransferase reaction, but if <4 μ l is required, the label can be added directly to the reaction (final reaction volume, 550 μ l).

4. Resuspend an appropriate amount of label in 50 μ l per reaction of 25 mM 5'-AMP.

The AMP is included to inhibit possible phosphodiesterase reactions, which might compete for the label during the labeling experiment.

5. Set up reactions as follows:

Up to 50 μ l protein sample (final concentration 0.5 to 5 mg/ml)

350 μ l buffer H

55 μ l 10 \times galactosyltransferase labeling buffer

50 μ l UDP-[³H]Gal/5'-AMP mixture from step 4

30 to 50 μ l autogalactosylated galactosyltransferase

1 to 4 U calf intestinal alkaline phosphatase

Milli-Q water to a final volume of 550 μ l.

Reaction volumes can be scaled down to 55 μ l.

6. Incubate 2 hr at 37°C or overnight at 4°C.

These are the typical conditions. Galactosyltransferase is active over a range of temperatures.

7. Add unlabeled UDP-Gal to a final concentration of 0.5 to 1.0 mM and another 2 to 5 μ l of galactosyltransferase. Incubate for an additional 2 hr at 37°C.

This step is used when complete labeling of the GlcNAc is required, such as site mapping.

8. Add 50 μ l stop solution to each sample and heat to 100°C for 5 min in a water bath.

Isolate the product

9. Resolve the protein from unincorporated label using a Sephadex G-50 column equilibrated in 50 mM ammonium formate/0.1% w/v SDS. Collect 1-ml fractions.

The column dimensions should be 1 \times 30-cm, and the flow rate between 0.4 to 1 ml/min. Note, slower flow rates result in greater resolution. This step should be performed at room temperature.

Size-exclusion chromatography using Sephadex G-50 (~30 cm) is traditionally used to desalt samples. However, TCA precipitation, spin filtration/buffer exchange, or other forms of size-exclusion chromatography (e.g., Cytiva Life Sciences PD-10 desalting column) can be used. The addition of carrier proteins, such as BSA (~67 kDa) and cytochrome c (~12.5 kDa), to samples and buffers will reduce the amount of protein lost due to nonspecific protein adsorption.

10. Count a 50- μ l aliquot of each fraction using a liquid scintillation counter.

Approximately 2×10^6 dpm of [³H]Gal should be incorporated into 2 μ g of ovalbumin.

11. Combine the void volume and lyophilize to dryness.

12. Resuspend the samples in 1 volume of Milli-Q water (200ml) and precipitate with 9 volumes of ice cold acetone (1800ml) (Lovrien and Matulis, 2001).

13. Treat the samples with PNGase F (Powell, 2001), separate by SDS-PAGE, and detect by autoradiography (Gallagher, 2001). Alternatively, subject the samples to “product analysis” to confirm that the label was incorporated onto O-GlcNAc (see Basic Protocol 5).

Numerous proteins, including ovalbumin, should be labeled by Gal-transferase and detected by autoradiography. O-GlcNAc-modified proteins should be reactive before and after PNGase F treatment.

PNGase F treatment can be applied before or after Galactosyltransferase labeling.

SUPPORT PROTOCOL 3 AUTOGALACTOSYLATION OF GALACTOSYLTRANSFERASE

As galactosyltransferase contains N-linked glycosylation sites, it is necessary to block these before using this enzyme to probe other proteins for terminal GlcNAc.

Materials

10× galactosyltransferase labeling buffer (see recipe in Reagents and Solutions)

Galactosyltransferase (Millipore Sigma, G5507)

10,000 U/ml aprotinin

2-mercaptoethanol

UDP-Gal

Saturated ammonium sulfate: >17.4 g $(\text{NH}_4)_2\text{SO}_4$ in 25 ml Milli-Q water

85% ammonium sulfate: 14 g $(\text{NH}_4)_2\text{SO}_4$ in 25 ml Milli-Q water

Galactosyltransferase storage buffer prechilled (see recipe in Reagents and Solutions)

30- to 50-ml centrifuge tubes

Refrigerated centrifuge

1. Resuspend 25 U of galactosyltransferase in 1 ml of 1× galactosyltransferase labeling buffer.
2. Transfer the sample into a 30- to 50-ml centrifuge tube.

The centrifuge tubes selected should be able to withstand a centrifugal force of $15,000 \times g$.
3. Remove a 5 μl aliquot for an activity assay.

This is the “pre-gal” sample to be used in Support Protocol 4.
4. Add 10 μl of 10,000 U/ml aprotinin, 3.5 μl of 2-mercaptoethanol, and 1.5 to 3.0 mg of UDP-Gal.
5. Incubate the sample on ice for 30 to 60 min.
6. Add 5.66 ml of prechilled saturated ammonium sulfate in a dropwise manner over 10 minutes. Incubate on ice for 30 min.
7. Centrifuge 15 min at $>10,000 \times g$, 4°C, and pour off the supernatant. Resuspend the pellet in 5 ml cold 85% ammonium sulfate and incubate on ice for 30 min.

8. Centrifuge 15 min at $>10,000 \times g$, 4°C, and pour off the supernatant.
9. Resuspend the pellet in 1 ml of galactosyltransferase storage buffer and divide into 50- μ l aliquots, saving 5 μ l for an activity assay as the “auto-gal” sample. Assay that aliquot for activity (see Support Protocol 4).
10. Store the remaining aliquots up to 1 year at -20°C pending use in Alternate Protocols.

SUPPORT PROTOCOL 4 ASSAY OF GALACTOSYLTRANSFERASE ACTIVITY

As sample and activity may be lost during the autogalactosylation procedure, it is important to assess the activity of the enzyme.

Materials

1.0 mCi/ml UDP- ^{3}H Gal, (17.6 Ci/mmol; American Radiolabeled Chemicals) in 70% (v/v) ethanol

Nitrogen source

1 \times galactosyltransferase dilution buffer: galactosyltransferase storage buffer (see recipe in Reagents and Solutions) supplemented with 5 mg/ml BSA

10 \times galactosyltransferase labeling buffer (see recipe in Reagents and Solutions)

25 mM 5'-adenosine monophosphate (5'-AMP) in Milli-Q water, pH 7.0

“Pre-gal” sample aliquot (see Support Protocol 3, step 3) and “auto-gal” sample aliquot (see Support Protocol 3, step 9)

200 mM GlcNAc

Dowex AG1-X8 resin (PO₄ form) slurry in 20% (v/v) ethanol

Speed-Vac evaporator

37°C incubator

Pasteur pipets

Glass wool

15-ml scintillation vials

Liquid scintillation counter

1. Dry 40 μ l of 0.1 $\mu\text{Ci}/\mu\text{l}$ of UDP- ^{3}H Gal in a Speed-Vac evaporator or under a stream of nitrogen.
2. Resuspend in 90 μ l of 25 mM 5'-AMP.

3. Make 1/1000, 1/10,000, and 1/100,000 serial dilutions of the “pre-gal” and “auto-gal” sample aliquots, in $1\times$ galactosyltransferase dilution buffer. Using these dilutions, 200 mM GlcNAc, and $10\times$ galactosyltransferase labeling buffer, prepare reaction mixtures as described in Table. 3.
4. Start the reaction by adding 10 μ l of 0.05 μ Ci/ μ l UDP- 3 H]Gal (see step 2) to each tube.
5. Incubate the samples 30 min at 37°C.
While the samples are incubating, prepare the columns.
6. Pour 1 ml of Dowex AG1-X8 slurry (PO₄ form) into 13 Pasteur pipets, each plugged with a small amount of glass wool.
The glass wool prevents the resin from flowing out of the column. If too much glass wool is used, it will reduce the flow rate of the column. The glass wool plug should be 0.3- to 0.5-mm long and should not be over-compressed.
7. Wash with at least 3 ml of Milli-Q water. Do not let the columns run dry.
8. When almost all the Milli-Q water has eluted, place each column over a separate 15-ml scintillation vial.
9. Stop the reaction (still incubating from step 5) by adding 500 μ l of Milli-Q water.
10. Load each sample onto the corresponding column and add a 500 μ l water wash of the tube. Collect eluate as fraction A.
11. Elute with two 1 ml additions of Milli-Q water. Collect eluates as fractions B and C, respectively.
12. Count 100 μ l of the sample (Fractions A, B, and C) using a liquid scintillation counter. The activity can be expressed either as dpm 3 H incorporation onto the GlcNAc residue, or as μ mol 3 H incorporation (1μ Ci = 2.22×10^6 dpm).

Notably, for UDP-Galactose with a specific activity of 17.6 Ci/mmol, 1000 DPM represents 25.8 fmol of Galactose added.

Calculate the activity; one unit of activity (U) is defined as 1 μ M of Gal transferred to GlcNAc per minute at 37°C.

BASIC PROTOCOL 5 CHARACTERIZATION OF LABELED GLYCANS BY β -ELIMINATION AND CHROMATOGRAPHY

This protocol has three steps: (1) the release of carbohydrates as sugar alditols by reductive β -elimination; (2) desalting the sample, while confirming the size of the labeled sugar alditol(s); and (3) confirmation that the product is [3 H] β Gal1-4 β GlcNAcitol (from galactosyltransferase labeling).

Materials

Radiolabeled glycoproteins (Alternate protocol 1)

β -elimination reagent: 1 M NaBH₄/0.1 M NaOH (prepare fresh)

4 M acetic acid, pre-chilled on ice

1.5-ml screw-cap microcentrifuge tubes

37°C incubator

Additional reagents and equipment for acetone or methanol precipitation of proteins (Lovrien and Matulis, 2001), size-exclusion (gel-filtration) chromatography (Boysen and Hearn, 2001), and Dionex chromatography (Townsend et al., 1989; 1996; Townsend and Hardy, 1991).

1. Acetone- or methanol-precipitate the labeled proteins (Lovrien and Matulis, 2001) in 1.5-ml screw-cap microcentrifuge tubes.

The β -elimination reaction is performed in screw-cap microcentrifuge tubes to prevent the lids from popping open during the lengthy incubation, which would result in evaporation of the sample. The tubes should be tightly sealed. Take care in opening the tubes at the end of the reaction, as gas generated during the reaction will escape.

2. Resuspend the sample in 500 μ l β -elimination reagent and incubate 18 hr at 37°C.

After several hours, check that the pH is >13. Add more β -elimination reagent if needed.

3. Cool the sample on ice.
4. Neutralize the reaction by adding 5 μ l cold 4 M acetic acid in a stepwise manner. Check that the pH is between pH 6 and 7.

Samples can be desalted either by chromatography on a Sephadex G-50 column (1 \times 30 cm, equilibrated in 50 mM ammonium formate, 0.1% SDS) or by anion-exchange chromatography on a 1-ml Bio-Rad Dowex AG 50W-X2 200–400 mesh (H⁺ form) column equilibrated in water. Fractions containing [³H]GlcNAc or [³H]Gal are pooled and lyophilized. Residual NaBH₄ is removed by washing the sample with methanol; NaBH₄ is volatile in the presence of methanol and is removed in a Speed-Vac evaporator or under a stream on nitrogen (Fukuda, 1989).

5. Resuspend the sugar alditols in Milli-Q water. Analyze by size-exclusion chromatography (Boysen and Hearn, 2001) or by Dionex chromatography (Townsend and Hardy, 1991; Townsend et al., 1996; 1989).

To determine the size of the oligosaccharide, labeled glycans released by β -elimination are subjected to size-exclusion chromatography. Readers are referred to several standard methods using BioGel P4 (Kobata, 1994) or TSK Fractogel (Fukuda, 1989) chromatography. Alternatively, the Cytiva Life Sciences Superdex Peptide column, equilibrated in 30% (v/v) CH₃CN, 0.1% (v/v) TFA, has been used to determine the sizes of oligosaccharides (R.N. Cole, pers. commun.). Gal β 1–4GlcNAcitol should have a similar retention time to a disaccharide alditol standard.

To determine the nature of the monosaccharide alditol or disaccharide alditol generated from either metabolic labeling or galactosyltransferase labeling, samples released by β -elimination can be analyzed by high-voltage paper electrophoresis or high-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection on a Dionex CarboPac PA100 column (Townsend et al., 1989; Hardy and Townsend, 1994).

BASIC PROTOCOL 6 DETECTION OF O-GLCNAC IN 96 WELL PLATES

The ability to detect O-GlcNAc in a 96 well plate format allows for the rapid optimization of treatments that alter protein O-GlcNAcylation levels *in vivo*. The method described below, a quantitative immunofluorescence assay, can be used directly in a 96 well plate reader or alternatively in cell imaging platforms. This protocol was optimized using three separate cell types (U2OS, mouse embryonic fibroblast, Cos-7); however, it is ideal to ensure that the O-GlcNAc levels of new cell types are within the linear range prior to assessing treatments that alter O-GlcNAc levels.

Materials

Wash buffer: 1x PBS

MES Buffer: 100mM MES, 1mM EDTA, 1mM MgCl₂ pH6.9 (stored at 4°C).

Fixation Buffer 1: 1:10 solution of MES buffer:methanol (stored at –20°C).

This buffer rapidly dehydrates/precipitates protein while maintaining secondary structure. As methanol does not crosslink proteins there is less likelihood that protein epitopes are altered reducing antibody binding. Methanol also has the added effect of permeabilizing the cell membrane which leads to loss of some cytoplasmic content, particularly small molecules and lipophilic species.

Fixation Buffer 2: 4% v/v paraformaldehyde in 1X PBS (chill to 4°C).

Paraformaldehyde crosslinks proteins and nucleic acids with good preservation of the cellular spatial information and composition. Prolonged fixation increases the risk of epitope alteration and reduced antibody binding. Methanol-free paraformaldehyde is the recommended fixative for phalloidin stains.

Permeabilization solution: 0.5% (v/v) Triton-X-100 in 1X PBS.

Tween Wash Buffer (1X TBST): 1X TBS with 0.05% v/v Tween® 20.

Blocking buffer: 3% (w/v) BSA in TBST.

Competitive blocking buffer: 3% (w/v) BSA in TBST with 500mM GlcNAc.

Primary antibody dilutions:

CTD110.6 (Millipore SIGMA, MABS1254): 2µg/ml in 3% (w/v) BSA in 1X TBST.

RL2 (Millipore SIGMA, MABS157): 2µg/ml in 3% (w/v) BSA in 1X TBST.

Nuclear counter stain: SYTO 62 (5mM) Red Fluorescent Nucleic Acid Stain (Thermo Fisher Scientific, S11344), 1:2000 – 1:10000 (2.5µM – 0.5µM).

Optional: Actin counter stain – Alexa Fluor 647 Phalloidin (Thermo Fisher Scientific, A22287). Use only with paraformaldehyde fixed cells and use the manufacturer's recommended working concentration. The working solution is made in the blocking buffer.

Fluorescent secondary antibody dilutions: Anti-mouse IgM Alexa Fluor 488 (Thermo Fisher Scientific, A-21042) and anti-rabbit IgG Alexa Fluor 488 (Thermo Fisher Scientific, A-32731/ Bethyl Laboratories A120–201D2) 2µg/mL – 4µg/mL – in 3% (v/w) BSA in 1X TBST. *NOTE: Some of the secondary antibodies are at different concentrations (mg/mL). When choosing different secondary conjugates, it may be useful to try multiple dilutions or adjust the µg/mL concentration to match a previously validated dilution.*

Aspirator, cell culture hood, incubator

Cells arrayed in a 96 well black wall clear bottom plate format (Corning, 3603).

Vortex with shaking attachment (or alternative)

Plate reader such as Spectramax plate reader (Molecular Devices)

Optional: Minimax 300 cell imager system

Aluminum Foil or plate covers

Ice

Prepare and plate the cells

1. Split cells and resuspend to a concentration of 5×10^4 cells/ml.

Note: Initially, it is critical to plate a dilution series of cells to ensure that signal will be within the linear range.

2. Plate 100 µl in 96-well format (Black walled plate) including wells with media alone (blank).

NOTE: It is critical to include blank wells as negative controls for nonspecific secondary antibody binding, and wells in which the signal

can be competed away with free GlcNAc to provide a specificity control.

3. Allow cells to grow to 70–90% confluence.
4. Perform experimental manipulations as necessary, such as treatment with an inhibitor that would alter protein O-GlcNAcylation (See Basic Protocol 1).

Cell Fixation and Staining

5. Wash away the media:
 - a. Chill the wash buffer to 4°C.
 - b. Remove media from the microwell plate by aspiration.

NOTE: Be sure to use a plastic 10 μ l pipette tip to cover the glass Pasteur pipette end. This helps prevent the loss of cells during aspiration.

NOTE: Inverting the plate to remove the media and blotting the open face of the plate also reduces cell loss.
 - c. Wash with 100 μ l of wash buffer for 3 min and aspirate.

NOTE: Add the solution carefully by pipetting down the side of the wells to avoid detaching the cells.
6. Fix the cells:
 - a. Immediately fix cells by addition of 100 μ l of fixing solution (MES:Methanol or 4% (v/v) paraformaldehyde) and incubate the microwell plate on ice for 15 min.
 - b. Remove the Fixation Solution by aspiration.
 - c. Wash the cells with 100 μ l wash buffer for 3 min (X 3).
 - d. Proceed to the permeabilization step.

NOTE: If the cells are not processed with primary antibodies on the same day, you can store the fixed cells in 100 μ L of 1X PBS at 4°C (96 hours) until ready for immunostaining. When ready to process the cells aspirate the storage buffer and start from the permeabilization step.
7. Permeabilize the cells:
 - a. Add 100 μ l of permeabilization buffer and incubate for 10 min at room temperature.
 - b. Do not shake the plate
 - c. Aspirate buffer.
 - d. Add 100 μ l of 1X TBST to the well, incubate for 3 min and aspirate (X 3).

8. Block the cells:
 - a. Carefully add 150 μ l of Blocking Buffer down the side of the wells and incubate for 1.5 hr at room temperature with mild rocking.
9. Wash the cells:
 - a. Remove the blocking buffer by aspiration.
 - b. Add 200 μ l of 1X TBST to the well, incubate for 3 min and aspirate (X3).
10. Counterstain the cells:
 - a. Prepare the nuclear fluorescent stain as necessary. Some cells do not contain nuclei (red blood cells), whereas others can be multinuclear. In this case, actin is a more appropriate counterstain.
 - b. Add 100 μ l of the diluted counterstain to the cells and incubate for 30 min at room temperature.
 - c. Aspirate the stain.
 - d. Add 200 μ l of 1X TBST to the well, incubate for 3 min, and aspirate (X3).

Optional: The nuclear counterstain can be performed simultaneously with the blocking step by making the stain dilution in the blocking buffer.
11. Add primary antibodies:
 - a. Prepare O-GlcNAc antibody dilutions in blocking buffer and *competitive blocking buffer* as necessary.
 - b. Add 100 μ l of the desired primary antibody combination to the desired wells. The antibody solution should cover the bottom of each well.
 - c. Incubate with primary antibody overnight at 4°C with gentle shaking. Seal the plate around the edges with parafilm to reduce evaporation.

NOTE: If you are using a primary antibody conjugated to a fluorophore, you should also cover the plate with foil to avoid photobleaching of the dye.
12. Wash the cells:
 - a. Add 200 μ l of the 1X TBST washing solution by gently adding buffer down the side of the wells to avoid detaching the cells. Incubate for 3 min and aspirate (X3).
 - b. *Optional:* Rock the plate gently during the wash steps.
13. Add the secondary antibody:

- a. Dilute the fluorescent dye-labeled secondary antibodies in blocking buffer.

NOTE: To lower nonspecific binding, add Tween® 20 to the diluted antibody to a final concentration of 0.2% v/v.

- b. Add 100 µl of the secondary antibody solution to each well and incubate for 60 min at room temperature.

NOTE: Protect the plate from light during incubation.

14. Wash the cells:

- a. Wash the plate with 1X TBST by gently adding 200 µL of buffer down the side of the wells to avoid detaching the cells.
- b. Incubate for 3 min at room temperature and aspirate the buffer (X 3).
- c. After the final wash turn the plate upside down and blot gently on paper towels to remove traces of wash buffer.
- d. Add 100 µL of 1X PBS per well for storage or imaging.

NOTE: For best results, scan plate immediately; plates may also be stored at 4°C in commercial mounting media with antifade (protected from light).

- e. Seal the plate in parafilm to prevent evaporation and protect plates from light before imaging to ensure the highest sensitivity.

15. Scan the plate:

- a. Before scanning, clean the bottom plate surface with a damp Kimwipe (water, not ethanol) to remove stains.
- b. Scan the plate using cell imaging functionality for fluorescence and transmitted light. Alternatively, scan the plate using the conventional fluorescence plate reader functionality.
- c. When storing plates after imaging, the plates should be sealed and remain protected from light at 4°C.
- d. The O-GlcNAc signal can be calculated by deducting signals from the equivalent competition well.

NOTE: Well-to-well variability arising from variations in cell number can be addressed by rescaling the O-GlcNAc signal to the average counter stain intensity (DNA or actin) for that well.

Note: When optimizing treatments, a signal to noise ratio of at least 3 is desirable.

BASIC PROTOCOL 7 ASSAY FOR OGT ACTIVITY

The detection and analysis of O-GlcNAc on proteins is only the first step in the analysis of O-GlcNAc and the protein(s) of interest. More important is determining the function of the modification. Protocols for the analysis of the enzymes that add and remove O-GlcNAc have been included in this unit, as they may aid in understanding the role of O-GlcNAc in a particular model. Recent examples where studies such as this have been critical include those which have shown the reciprocity between O-GlcNAc and O-phosphate on the C-terminal domain of RNA Pol II (Comer and Hart, 2001); studies showing elevated activity of enzymes in certain tissue/cell lines and tissue fractions (Whelan et al., 2008); and, finally, studies which have indicated that the enzymes responsible for the addition and removal of O-GlcNAc copurify with kinases and phosphatases (Wells et al., 2004).

O-GlcNAc transferase (OGT), or uridine diphospho-*N*-acetylglucosamine:polypeptide β -*N*-acetylglucosaminyltransferase, transfers GlcNAc to the hydroxyl groups of Ser and Thr residues of proteins and peptides using UDP-GlcNAc as a donor substrate (Haltiwanger et al., 1990; 1992; Kreppel et al., 1997). OGT activity is assayed by determining the rate at which [³H]GlcNAc is transferred to an acceptor peptide. A number of peptides have been identified as substrates for OGT *in vitro*, but a peptide (³⁴⁰PGGSTPVSSANMM³⁵²) from the α -subunit of casein kinase II (CKII) is an efficient *in vitro* substrate that is commonly used (Kreppel and Hart, 1999).

OGT activity can be assayed in crude preparations (Haltiwanger et al., 1990) or using recombinant protein (Kreppel and Hart, 1999). OGT activity is sensitive to salt inhibition and reducing agents, so it is important to desalt the preparation before assaying if high salt concentrations are present (see Support Protocol 5). Typically, 0.2 to 1 μ g of purified recombinant protein is used per assay. To assay OGT activity in cell/tissue lysate, 10 to 50 μ g of desalted lysate is required per assay.

Materials

Clear round-bottomed 96-well plate

0.1 mCi/ml UDP-[³H]GlcNAc (20 to 45 Ci/mmol; American Radiolabeled Chemicals) in 70% (v/v) ethanol

25 mM 5'-adenosine monophosphate (5'-AMP), in Milli-Q water, pH 7.0

Crude *or* purified OGT sample, desalted (see Support Protocol 5)

10 \times OGT assay buffer (see recipe in Reagents and Solutions)

CKII peptide substrate (⁺H₂N-PGGSTPVSSANMM-COO⁻): dissolve in H₂O to 10 mM and adjust to pH 7, if necessary

Note: OGT peptide substrates should be custom synthesized.

20U/ μ l Calf intestinal alkaline phosphatase (18009019; Thermo Fisher Scientific)

0.5mM Thiamet-G in 0.5M HEPES pH7.5

Quenching buffer: 50mM formic acid, 500mM NaCl

50 mM formic acid

Acetone

Methanol (HPLC-grade)

Scintillation fluid

Speed-Vac evaporator

Strata C₁₈ 96-well plate (25 mg/well; 8E-S001-CGB; Phenomenex)

Plates can be reused up to 10 times; however, plates should be precycled before use.

Scintillation counter

1. Dry down an aliquot of UDP-[³H]GlcNAc in a Speed-Vac evaporator or under a stream of nitrogen just prior to use. Resuspend in an appropriate volume of 25 mM 5'-AMP, so that the concentration is 1 μCi/μl.

5'-AMP is included in the assay to competitively inhibit any pyrophosphatase in the sample that will hydrolyze the UDP-GlcNAc.

2. Prepare a master mix containing each of the following per reaction:

5 μl of 10× OGT assay buffer

5 μl of 10 mM CKII peptide substrate

0.5 μl 1 μCi/μl UDP-[³H]GlcNAc (0.5 μCi per reaction)

1 μl of 0.5mM Thiamet-G

0.25 μl of Calf intestinal alkaline phosphatase (5U per reaction)

H₂O to 20 μl

OGT is feedback inhibited by a by-product of the reaction, UDP. Calf intestinal alkaline phosphatase degrades UDP and hence increases the efficiency of the reaction.

3. 3. Array mastermix in a round-bottomed 96 well plate and initiate reactions by adding 30 μl of desalted OGT.

It is critical to include a negative control. A mimic of the CKII peptide where the Ser and Thr residues are replaced with Ala is appropriate, or, simply, a “no-peptide” control can be included.

Recombinant His-OGT purified in-house diluted in desalting buffer is included as a positive control.

The results generated are variable and the reactions should be set up in triplicate.

4. Incubate for 30 min –1 hour at room temperature.
An incubation time of 15 to 30 min at room temperature is usually sufficient.
5. Stop the reaction by adding 150 μ l of quenching buffer, 50 mM formic acid, 500mM NaCl.
6. Activate a C₁₈ 96-well plate (Phenomenex; 25mg resin/well) with 100% Acetone (1.5 ml) and then 100% methanol (1.5 ml). Equilibrate the plate in 50mM formic acid, 500mM NaCl (three times, 1.5ml each).

It is also possible to use Sep-Pak C₁₈ cartridges. The method is similar to that described below.

7. Load the reaction (200 μ l total) onto the 96-well plate. Wash the plate sequentially with 50mM formic acid containing 500mM NaCl, water, and then 50mM formic acid (two times with 1.5 ml each).

The CKII peptide binds to the C₁₈ resin. Unincorporated UDP-[³H]GlcNAc is eliminated by the wash.

8. Elute the peptide with 1.5 ml of 100% methanol.
9. Add 0.75 mL of elution volume to 5 ml scintillation fluid and count ³H. Calculate OGT activity according to the following equations. The activity can be expressed either as dpm ³H incorporation onto the GlcNAc residue, or as μ mol GlcNAc incorporated per minute per mg of protein.

Notably, for UDP-GlcNAc with a specific activity of 60 Ci/mmol, 1000 DPM represents 7.54 fmol of GlcNAc added.

SUPPORT PROTOCOL 5 Desalting the O-GlcNAc Transferase

OGT activity is sensitive to salt inhibition ($IC_{50} = 40$ to 50 mM NaCl). It is important to desalt the enzyme preparation before setting up the assay if a high concentration of salt is present. Additionally, O-GlcNAcase activity is sensitive to detergent present in extraction buffers. The protocol described below is also used to desalt O-GlcNAcase prior to setting up assay reactions.

Materials

Zeba 96-well Spin Desalting Plates, 40K MWCO (87774; Thermo Fisher Scientific)

96-well wash plates

96-well collection plates

OGT desalting buffer (see recipe in Reagents and Solutions)

Sample volume range 20 – 100 μ l

Ice

1. Place the desalting plate on top of the wash plate and centrifuge (1000xg, 2 min) to remove the storage solution.
2. Discard the flow-through and replace the desalting plate onto the wash plate.
3. Add 250 μ l of desalting buffer on top of the resin. Centrifuge and discard flow-through. Repeat this step 2 additional times.

After each spin, make sure resin appears white and free of liquid. Poor sample recovery may result from incomplete centrifugation.

4. Blot the bottom of the desalting plate to remove excess liquid and place it on top of a new collection plate.
5. Load the protein sample (20 – 100 μ l) onto the resin. Centrifuge (1000xg, 3min) and retain the flow-through that contains the sample.

Alternatively, Zeba Spin Desalting Columns, 7K MWCO (Thermo Fisher Scientific), PD-10 columns (Millipore SIGMA), or G50 Sephadex (Cytiva Life Sciences) can be used.

BASIC PROTOCOL 8 Assay for O-GlcNAcase Activity

O-GlcNAcase, also known as *N*-acetylglucosaminidase or hexosaminidase C (EC 3.2.1.52), is a cytosolic glycosidase specific for O-linked β -GlcNAc. The activity of O-GlcNAcase can be conveniently assayed *in vitro* with a fluorogenic substrate, 4-Methylumbelliferyl-GlcNAc (4MU- β -GlcNAc). The cleavage product, 4MU, is fluorescent with an emission at 460nm (Macauley et al., 2005).

Materials

Purified O-GlcNAcase (0.2 to 1 μ g) or cell/tissue extract (10 to 50 μ g, desalted).

10 \times O-GlcNAcase assay buffer (see recipe in Reagents and Solutions)

1M GalNAc

100mM 4MU- β GlcNAc in DMSO (Millipore SIGMA, M2133)

100mM 4MU- β GalNAc in DMSO (Millipore SIGMA, M9659)

4MU- β GalNAc is used to confirm that lysosomal hexosaminidases have been effectively inhibited.

Black flat-bottomed 96-well plate and sealing tape

OGT Desalting buffer (see recipe in Reagents and Solutions)

Free 4MU (4-methylumbelliferone) standards at 100 mM in DMSO

β -N-acetylhexosaminidase_f at 5000 U/ml (New England Biolabs, P0721)

Quenching buffer: 200mM glycine pH 10.75

Centrifuge at 4°C

37°C incubator

Plate reader

1. Prepare O-GlcNAcase.

Native or recombinant O-GlcNAcase can be partially purified from animal tissues or cultured cells by several chromatographic steps (Dong and Hart, 1994; Gao et al., 2001). Alternatively, a crude enzyme preparation can be generated by passing cell extract over a 1 ml Con-A column. Most of the interfering acidic hexosaminidases are modified by N-linked sugars and bind to Con-A, while neutral O-GlcNAcase is in the flow-through (Izumi and Suzuki, 1983). Additionally, O-GlcNAcase activity is sensitive to detergent present in extraction buffers. Therefore, cell/tissue extracts or the enzyme preparation is desalted (see Support Protocol 5) prior to setting up the assay reaction. Free GalNAc is used in the assay to inhibit lysosomal hexosaminidases. The lysates are assayed against both 4MU- β GlcNAc and 4MU- β GalNAc.

2. Precool a 96-well plate or microcentrifuge tubes on ice.

3. Set up reactions in triplicate in the precooled plate wells or tubes as follows:

20 μ l partially purified O-GlcNAcase enzyme or cell/tissue extract

20 μ l of desalting buffer is used as a negative control. 4MU-GlcNAc breaks down chemically. A blank reaction without enzyme should be included to determine the background.

20 μ l of β -N-acetylhexosaminidase_f (diluted to 25U/mL in desalting buffer) is used as a positive control.

4. Prepare a master mix containing each of the following per sample:

5 μ l 10 \times O-GlcNAcase assay buffer

5 μ l 1M GalNAc

50 mM GalNAc (final) is included in the reaction to inhibit lysosomal hexosaminidases A and B which may be present in the enzyme preparation. O-GlcNAcase is not inhibited by 50 mM GalNAc.

0.5 μ l of 4MU-GlcNAc

Each of the above reactions are repeated with 4MU-GalNAc as a substrate instead of 4MU-GlcNAc. 4MU-GalNAc acts as a negative control substrate that allows for an assessment of the activity of any contaminating lysosomal hexosaminidases

H₂O 19.5 µl.

5. Add 30 µl of Mastermix to each sample
6. Prepare a 4MU standard curve in DMSO. Add 50 µL to each well and perform this in triplicate.
7. Quench one reaction per every triplicate reaction set up at time point zero with quenching buffer (200mM Glycine pH 10.75). This is included to determine the background cleavage of 4MU-GlcNAc that is not a result of O-GlcNAcase activity.
8. Mix well and cover.
9. Incubate for 30–60 min at 37°C.

Reactions should be optimized to keep the fluorescence intensity within the linear range of the plate reader. The authors find that 10 to 50 µg of cell/tissue extract used in a reaction of 50 µl, with a 1 hr incubation time, is appropriate.

10. At the end of the incubation, add 150 µL of Quenching buffer (200mM glycine pH 10.75) to the remaining reactions, including the 4MU standards.
11. Read the fluorescence intensity with an excitation wavelength at 368nm and an emission wavelength of 450 nm on a plate reader.
12. Calculate O-GlcNAcase activity according to the following equation:

moles of GlcNAc or GalNAc released = [(Average fluorescence intensity after 60min) – (fluorescence intensity at time point 0)]/slope of the 4MU standard curve

OGA activity is normalized by subtracting the fluorescence signal resulting from lysosomal hexosaminidase contamination, which was assessed using 4MU-GalNAc.

OGA activity is expressed in moles of GlcNAc released/mg of purified OGA or cell extract/min of total reaction time.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Biosynthetic labeling medium

Glucose-free culture medium containing:

50 µCi/ml D-[6-³H]glucosamine (22 Ci/mmol; American Radiolabeled Chemicals; ART 0110A)

10% (v/v) FBS

Prepare fresh

Buffer H

50 mM HEPES, pH 6.8

50 mM NaCl

2% (v/v) Triton X-100

Store up to 1 month at room temperature

Citrate-phosphate buffer, pH 4.0, 2x

Dissolve 12.9 g citric acid monohydrate (mol. wt. 210) and 20.6 g disodium hydrogen phosphate heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 6\text{H}_2\text{O}$) in 300 ml Milli-Q water. Bring volume to 500 ml. Divide into 10-ml aliquots and store up to 1 year at -20°C .

Galactosyltransferase labeling buffer, 10x

100 mM HEPES, pH 7.5

100 mM galactose

50 mM MnCl_2

Store up to 1 month at 4°C

Galactosyltransferase storage buffer

2.5 mM HEPES, pH 7.4

2.5 mM MnCl_2

50% (v/v) glycerol

Store up to 1 month at room temperature

Hexosaminidase reaction mixture, 2x

Per reaction:

25 μl 2x citrate-phosphate buffer (see recipe)

1 U *N*-acetyl- β -D-glucosaminidase (V-Labs)

0.01 U aprotinin

1 μg leupeptin

1 μg α_2 -macroglobulin

Prepare fresh

O-GlcNAcase assay buffer, 10×

500 mM sodium cacodylate, pH 6.4

3% (w/v) bovine serum albumin (BSA)

Prepare fresh

OGT assay buffer, 10×

500 mM sodium cacodylate, pH 6.0

10 mg/ml bovine serum albumin (BSA)

Prepare fresh

OGT desalting buffer

20 mM Tris·Cl, pH 7.8 (*APPENDIX 2E*)

1 mg/ml bovine serum albumin (BSA)

20% (v/v) glycerol

0.02% (w/v) NaN₃

Store up to 1 week at 4°C

Protease inhibitors, 1000×

PIC 1, 1000×:

Dissolve the following in 10,000 U/ml aprotinin solution (Millipore SIGMA)

1 mg/ml leupeptin

2 mg/ml antipain

10 mg/ml benzamide

PIC 2, 1000×:

Prepare in DMSO

1 mg/ml chemostatin

2 mg/ml pepstatin

PMSE, 1000×:

0.1 M phenylmethylsulfonyl fluoride in 95% ethanol

Tris-buffered saline (TBS)

10 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)

150 mM NaCl

Store up to 1 month at room temperature

TBST

10 mM Tris·HCl, pH 7.5 (*APPENDIX 2E*)

150 mM NaCl

0.05% (v/v) Tween 20

Store up to 1 month at room temperature

WGA buffer

25 mM Tris·HCl pH 7.8 (*APPENDIX 2E*)

300 mM NaCl

5 mM CaCl₂

1 mM MgCl₂

Store up to 6 months at room temperature

WGA Gal elution buffer

Phosphate-buffered saline (PBS; *APPENDIX 2E*) containing:

0.2% (v/v) NP-40

1 M D-(+)-galactose (Gal)

Store up to 1 week at 4°C

WGA GlcNAc elution buffer

Phosphate-buffered saline (PBS; *APPENDIX 2E*) containing:

0.2% (v/v) NP-40

1 M *N*-acetylglucosamine (GlcNAc)

Store up to 1 week at 4°C

WGA storage buffer

25 mM Tris·HCl, pH 7.8 (*APPENDIX 2E*)

300 mM NaCl

5 mM CaCl₂

1 mM MgCl₂

0.05% (w/v) sodium azide

Store up to 1 year at 4°C

COMMENTARY

Background Information

(β)-D-1-4-galactosylaminyltransferase from bovine milk recognizes terminal *N*-acetylglucosamine (GlcNAc) residues and modifies them by the addition of a single Gal residue. Torres and Hart first used this enzyme in combination with UDP-[³H]Gal to demonstrate that bovine lymphocytes contain proteins modified by O-linked GlcNAc (Torres and Hart, 1984). Further refinements of this experiment led them to propose that the product, β Gal1-4 β GlcNAc, was the result of the galactosyltransferase recognizing and modifying a single GlcNAc residue O-linked to Ser/Thr residues of nuclear and cytoplasmic proteins (Holt and Hart, 1986). Since this report, many cytosolic and nuclear proteins from mammalian cells were shown to be modified by O-GlcNAc. This method has remained the “gold standard” technique for the detection of O-GlcNAc-modified proteins, as the label provides a “tag” for subsequent analyses, such as those described under *Product characterization* below (see Critical Parameters and Troubleshooting). This approach has been modified to include sugars with biorthogonal handles that can be further modified by probes allowing the detection and enrichment of glycans. This approach, colloquially referred to as Click-Chemistry, is marketed by Invitrogen and has been reviewed in depth (Kim, 2018). As described in Basic Protocol 1, methods such as WGA affinity and immunoblotting with GlcNAc-specific lectins and antibodies have become popular as simple techniques for the initial characterization of target proteins.

Critical Parameters and Troubleshooting

Extraction of proteins from cells—The O-GlcNAc modification can be removed from proteins either by cytosolic O-GlcNAcase or lysosomal hexosaminidases. The inclusion of inhibitors during the extraction and purification process will preserve the levels of O-GlcNAc on proteins. Commonly used inhibitors (Dong and Hart, 1994) include 1-amino-GlcNAc (1 mM), GlcNAc (100 mM), PUGNAc (5 μ M), and Thiamet G (1 μ M). Note that these may have to be removed, as they will act as inhibitors in other methods.

Product characterization—Product characterization is a critical step to show that a protein is modified by O-GlcNAc and not other glycans. While many proteins modified by O-GlcNAc have been identified, there is evidence based on metabolic labeling (Medina et al., 1998) and lectin labeling studies (Hart et al., 1989) that suggests that O-GlcNAc is not the only intracellular carbohydrate post-translational modification. In addition, at least one peptide mimic of O-GlcNAc has been identified in cytokeratins (Shikhman et al., 1993; 1994). Moreover, many techniques used for breaking open cells also release proteins that are modified by complex N- and O-linked sugars, which may contain terminal GlcNAc. Many

of the techniques described in this unit will recognize any terminal β GlcNAc residue, and it is important to perform the described controls, such as PNGase F digestion, to demonstrate specificity.

Product analysis is critical for metabolic labeling with glucosamine. While UDP-GlcNAc is the major product, glucosamine can enter other biosynthetic pathways, such as those used for amino acid synthesis. This issue was highlighted by studies of the SV40 large T antigen. Some researchers have found that the SV40 large T-antigen labels with a number of different tritiated carbohydrates. However, O-GlcNAc is the only carbohydrate post-translational modification of the SV40 large T antigen. The incorporation of glucosamine into amino acid biosynthetic pathways could be reduced by growing cells in the presence of excess nonessential amino acids (Medina et al., 1998).

Lastly, while galactosyltransferase is specific for terminal GlcNAc residues, researchers (Elling et al., 1999) have shown that galactosyltransferase will modify GlcNAc linked in either the α - or β -anomeric conformation. The authors of this protocol have shown that proteins modified by α -O-GlcNAc will be labeled using the procedure described (N. Zachara, unpub. observ.). While α -O-GlcNAc has not been identified in complex eukaryotes, it is a common modification of cell surface proteins of simple eukaryotes, such as trypanosomes and *Dictyostelium*. Product analysis, such as HPAEC of the sugar alditols, will resolve many of the issues discussed.

Understanding Results

Modulating the abundance of O-GlcNAc is a useful control for detection methodologies, as well as for testing the impact of O-GlcNAc on protein or pathway function. It is ideal to choose the shortest treatment with the lowest dose, as these treatments are less likely to have pleiotropic effects. To optimize treatments (Basic Protocol 1), inhibitor dose or application time can be varied. Figure 1 presents data in which inhibitor dose (Thiamet-G) has been modulated over 4h. In this example, there is a linear relationship between O-GlcNAc and inhibitor dose from (75nM). As previously published, elevating O-GlcNAc levels also augments the abundance of OGA (Kazemi et al., 2010; Park et al., 2017; Tan et al., 2020).

When detecting O-GlcNAc by Immunoblot or lectin blot (Basic Protocol 2, Basic Protocol 3) it is critical to assess both linear range and specificity. Linear range will differ based on cell and tissue type, as well as the detection modality (ECL, fluorescence, reagents, film) and source of reagents (antibody concentration). Linear range is simply addressed by loading a dilution series of a protein of interest, detecting O-GlcNAc, and quantifying the O-GlcNAc and protein signals (Figure 2A). In this example, quantitation reveals that the signal is linear from 13–26 mg of protein. Specificity can be assessed by modulating O-GlcNAc levels (Basic Protocol 1, Support Protocol 1 and Support Protocol 2) and competing away primary antibody with free GlcNAc. This latter control is critical in immunoprecipitations and tissues, especially mouse tissues, in which immunoglobulin is present (Figure 2B). In the example shown (heart tissue) use of free GlcNAc or secondary alone, highlights two immune-reactive bands at ~70kDa that are mouse IgM heavy chain. A similar band at 55kDa is observed when probing with RL2, a mouse IgG.

There are numerous antibodies that can be used to detect the O-GlcNAc modification. Figure 3 presents representative data for 6 O-GlcNAc pan-specific antibodies. In addition to modulating O-GlcNAc levels (OGT WT and Null), the signal has been competed away with free GlcNAc. Confirmation of specific O-GlcNAc signals can be achieved by free GlcNAc competition and/or signal reduction with deletion of OGT. As previously reported, each of these antibodies displays different preferences for substrates and this is represented by the different binding patterns (Snow et al., 1987; Teo et al., 2010; Lee et al., 2016; Reeves et al., 2014). Analogous data and controls should be obtained when using sWGA (Basic Protocol 3). Screening of antibodies and lectins in different models is warranted as some proteins, such as α -synuclein, are not well recognized by antibodies (Levine et al., 2019). For such proteins the use of Galactosyltransferase or metabolic labeling is necessary.

A subset of pan-O-GlcNAc reactive antibodies cross-react with abbreviated N-linked glycans, as well as bisecting GlcNAc residues on N-linked glycans (Isono, 2011; Tashima and Stanley, 2014; Reeves et al., 2014). While the occurrence of the abbreviated N-linked glycan appears restricted to mammalian cells/tissues exposed to nutrient deprivation and protozoa, the ability to differentiate between an N- and O-linked GlcNAc residue is critical. Support protocol 1, represent such a method. In the example shown, cells have been exposed to nutrient deprivation (STV: 0mM Glucose, 1% FBS; 24h) which results in a robust increase in CTD110.6 signal that is sensitive to PNGase F (Figure 4A, lane 3). In control cells, the majority of the signal is sensitive to b-elimination suggesting that it arises from O-GlcNAc (Figure 4B). In contrast, a portion of the CTD110.6 signal in starved cells is resistant to b-elimination (Figure 4B). Sodium hydroxide will destroy protein epitopes. Thus, while a modest decrease in protein signals is expected (Compare actin in 4A and 4B), the signal should not be destroyed. Notably, a harsh b-elimination can impact N-linked glycans.

Galactosyltransferase labeling is a powerful approach for product characterization, identification of glycosylation sites, and of O-GlcNAc modified-proteins. Proteins labeled with ^3H Gal should first be separated from the majority of unincorporated label using size exclusion chromatography (Figure 5A). A negative control reaction should be used to identify the retention time of ^3H -UDP-Gal. Fractions with signal higher than the blank should be combined and the protein precipitated (TCA, Acetone, Methanol). In the example shown, better resolution from the unincorporated label could be achieved by increasing the length of the column. Proteins are subsequently separated by SDS-PAGE and the ^3H -Gal detected by autoradiography. Ovalbumin is a useful control, as it is both labeled by Galactosyltransferase and carries one glycan that is sensitive to PNGase F. Labeled proteins can be further assessed using a range of approaches such as product analysis (Basic Protocol 6), protein purification, and manual Edman degradation.

O-GlcNAc-modified proteins can be enriched using antibody or lectin affinity chromatography (Basic Protocol 4) (Lee et al., 2016). It should be noted that as these experiments are performed under native conditions, unmodified proteins that interact with O-GlcNAcylated proteins will also be enriched. Capturing O-GlcNAcylated proteins from nuclear and cytosolic lysates reduces the possibility of enriching proteins modified by N-linked glycans. When combining this approach with TNT®, fractions eluting from affinity

columns should be subsequently separated by SDS-PAGE and detected by autoradiography to confirm that radioactive signal arises from a protein with the appropriate molecular weight.

Basic Protocol 6 reports conditions for detecting O-GlcNAc in 96 well plates that should be readily adapted for immunofluorescence. In Figure 6A, OGT wild-type and null cells have been stained with CTD110.6. As expected, lower raw fluorescent signals are observed for O-GlcNAc in OGT null cells. As OGT null cells grow more slowly than OGT wildtype cells, the cell number is also reduced (Figure 6A, middle panel) (Kazemi et al., 2010). To generate the final data (Figure 6A, lower panel), raw CTD110.6 signals were corrected for total cell number using the nuclear stain. In Figure 6B, U2OS cells treated \pm Thiamet-G have been stained using CTD110.6 resulting in a stronger signal. Similar to immunoblotting, incubation of the primary antibody with free GlcNAc provides an excellent specificity control. In Figure 6, incubation with free GlcNAc significantly reduces the signal. To generate final data, the raw fluorescent signal for CTD110.6 +100mM GlcNAc is subtracted from the raw fluorescent signal for CTD110.6. Finally, as in Figure 6A, the specific CTD110.6 signal is corrected for cell number or total protein using a nuclear stain. Alternatively, the CTD110.6 signal could be normalized to total protein using a stain for a housekeeping protein such as actin.

Figure 7 presents the raw data, an example calculation, and processed data for both OGT and OGA assays (Figure 7 A-D). To calculate OGT activity:

- A. Triplicates are used to address variability and are averaged;
- B. To assess autoglycosylation a “no peptide control is used. OGT Activity (DPM) = (Signal: CK2-peptide) – (Signal: No-Peptide-control);
- C. To calculate DPM of GlcNAc added per minute per mg:
$$(\text{DPM}/\text{Assay time in minutes}) \times [1000/\text{protein per well in mg}].$$
- D. Based on the specific activity of the ^3H -UDP-GlcNAc – DPM are converted to fmoles.

Notably, a low signal to noise suggests that the OGT assay buffer has expired or that the assay length or protein amount should be increased. Initial experiments should assess linear range, by altering protein amounts present in the assay. In this example, and as we expect, OGT activity is depressed in the OGT null.

With regard to OGA assays:

- A. The reaction stopped at T=0min is used to assess the spontaneous breakdown of 4MU-GlcNAc/GalNAc and is background subtracted from the average of the duplicates;
- B. 4MU-GalNAc is used to assess activity from lysosomal hexosaminidases. As such, to assess OGA activity, the averaged 4MU-GalNAc signal is background subtracted from the 4MU-GlcNAc signal.

- C. Finally, activity is expressed in nmoles/min/mg by dividing the luminons by the assay length, adjusting for protein (here, multiplying by 100 as 10 μ g of lysate was used), and then converted to nmoles using an experimentally derived standard curve.

In this example, and as we expect, OGA activity is depressed in the OGT null. The importance of using the 4MU-GalNAc pseudo-substrate is highlighted in this example. First, readers will note that deletion of OGT which impacts OGA abundance only significantly alters total luminons in the 4MU-GlcNAc samples. Secondly, readers will note that in spite of the GalNAc included in the assay, there is a significant difference between the 4MU-GalNAc replicates and the time=0 sample. Like OGT assays, readers should ensure that they are within the linear range.

Time Considerations

Detection of O-GlcNAc proteins using antibodies (see Basic Protocol 2) and lectins (see Basic Protocol 3) will take ~2 to 3 days after the extraction of the proteins from cells. Samples and controls must be treated with PNGase F and/or hexosaminidase (1 hr to overnight), before SDS-PAGE and blotting. In either case, overnight incubation at 4°C provides the best signal-to-noise ratio.

WGA affinity chromatography of low-copy-number proteins will take 2 to 3 days. The TNT® protein expression and WGA affinity chromatography can be completed in 1 day; subsequent analysis of the product by SDS-PAGE will take 1 to 2 days depending on the label used and the amount of label incorporated into proteins eluting from the WGA-agarose.

Autogalactosylation of the galactosyltransferase and subsequent analysis of the activity will take 1 to 2 days. As the enzyme is stable for 6 to 12 months at -20°C, autogalactosylation does not need to be repeated for each analysis. Galactosyltransferase labeling of the proteins can take several hours to overnight, though optimization of the conditions may take a few days. The subsequent analysis, as well as desalting (dependent on the technique used), PNGase F digestion (1 hr to overnight), precipitation of protein (3 hr to overnight), SDS-PAGE, and autoradiography (1 to 10 days), can take up to 2 weeks. The length of time allotted for product analysis is dependent on the methods chosen but will almost certainly require 7 to 10 days. Further analysis, including digestion of labeled proteins and subsequent purification of peptides, will take at least 3 days.

Similar to indirect immunofluorescence methods, the O-GlcNAc In-cell western using antibodies will take 2–3 days to complete following user-defined experiments. The O-GlcNAc primary antibodies require overnight incubation for efficient binding but secondary antibody staining and analysis can be performed in a day.

OGT assays (Basic Protocol 7) are somewhat complicated and typically take 2 days to complete. Notably, after the OGT assay is stopped, it can be stored at -20°C for several weeks before desalting. O-GlcNAcase assays are straightforward and typically take 1 day.

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Abbreviations:

4MU	4 methylumberlliferyl
BSA	Bovine serum albumin
DON	diazo-5-oxonorleucine
ECL	enhanced chemiluminescence
Gal	Galactose
GFAT	glutamine fructose-6-phosphate amidotransferase
GlcNAc	N-acetylglucosamine
HRP	Horse radish peroxidase
HS-TBST	high salt TBST
OGA	O-GlcNAcase
O-GlcNAc	O-linked N-acetylglucosamine
OGT	O-GlcNAc transferase
PBS	Phosphate buffered saline
PVDF	polyvinylidene difluoride
sWGA	succinylated wheat germ agglutinin
RCA1	<i>Ricinus communis</i> agglutinin 1
RRL	Rabbit reticulocyte lysate
STZ	Streptozotocin,
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TNT	in vitro transcription translation
WGA	wheat germ agglutinin.

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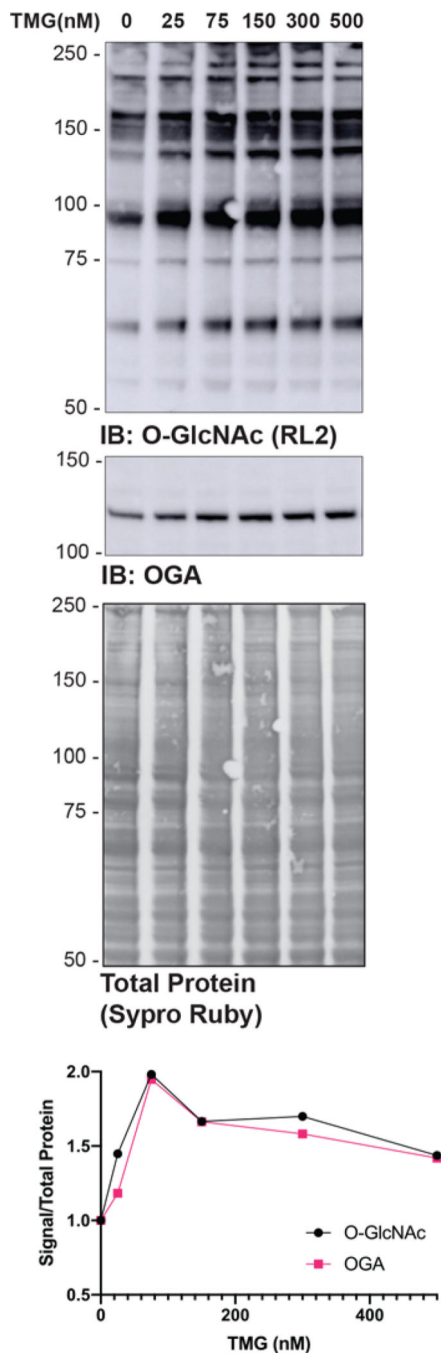


Figure 1. Determining inhibitor dose.

A) Mouse embryonic fibroblasts were treated with increasing doses of Thiamet-G (nM, 4h). Total cell lysates (10 μ g) were separated by SDS-PAGE, transferred to nitrocellulose, and total protein (SyproTM Ruby Protein Blot stain), O-GlcNAc (RL2), and OGA (345; (Butkinaree et al., 2008)) were detected. Blots were developed using ECL and images captured with a GE Healthcare AI600 RGB. **B)** Quantification of O-GlcNAc (•, black) and OGA (■, pink) signals normalized to total protein.

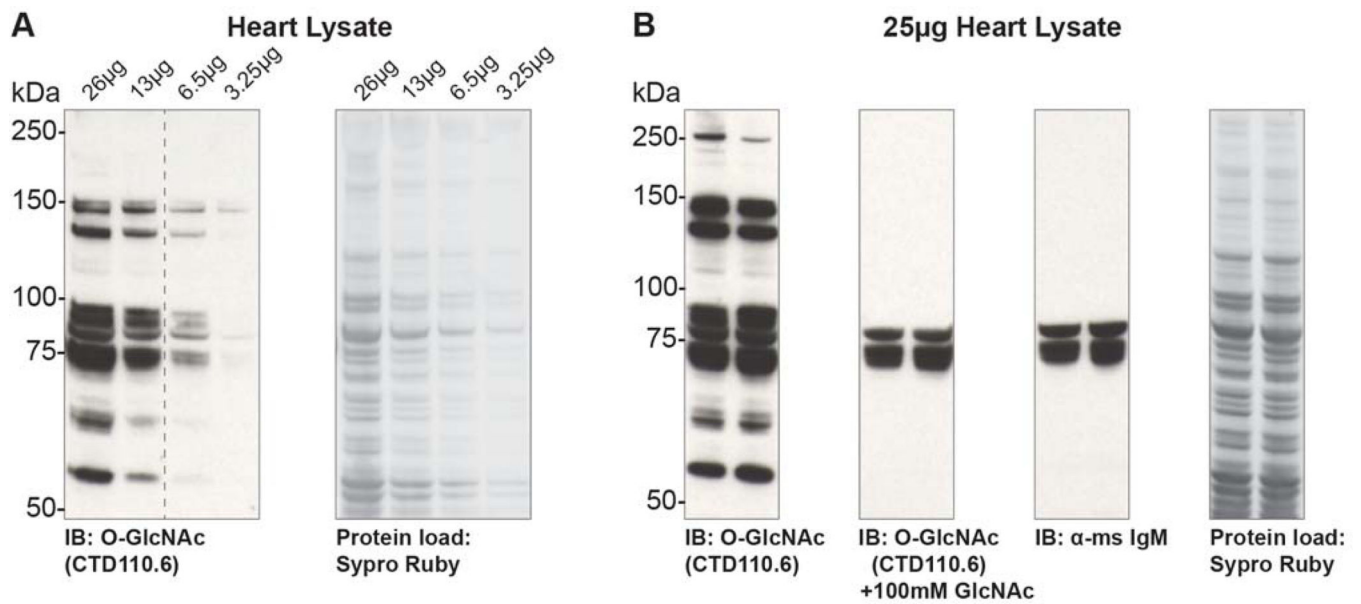


Figure 2. Critical controls when detecting O-GlcNAc.

A) A dilution series of heart lysate (indicated) was separated by SDS-PAGE, transferred to nitrocellulose, and total protein (Sypro™ Ruby Protein Blot stain) and O-GlcNAc levels were detected. The dashed line represents the point at which the signal is no longer linear.

B) 25µg of heart lysate was resolved by SDS-PAGE transferred to nitrocellulose, and total protein (Sypro™ Ruby Protein Blot stain), O-GlcNAc (CTD110.6), non-specific background (CTD110.6 + 100mM GlcNAc) and endogenous IgM-immunoglobulin (mouse IgM) were detected. Total protein was detected on a GE Healthcare AI600 RGB. Blots were developed using ECL and images captured on film.

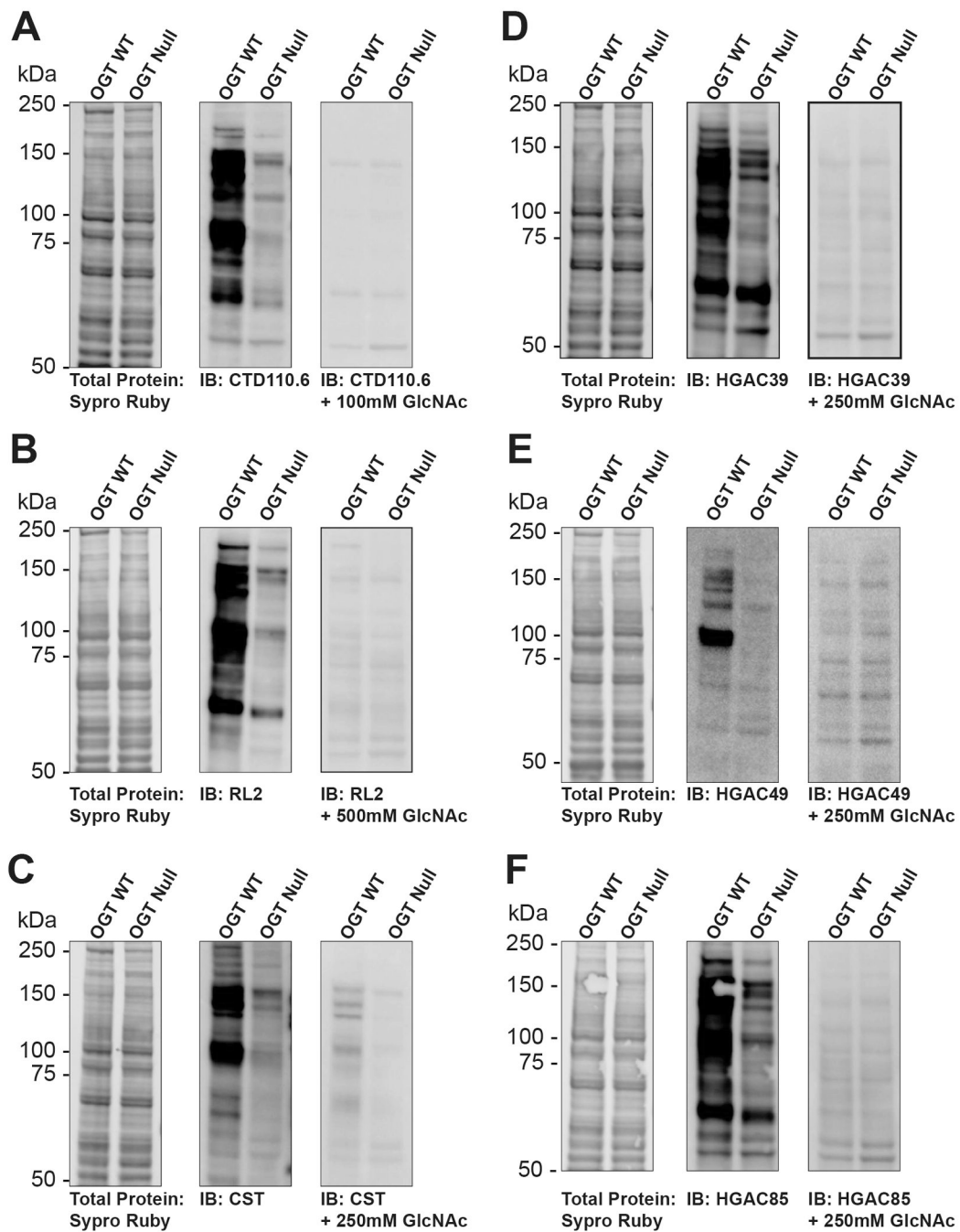


Figure 3 –. Antibody detection of O-GlcNAc.

10 μ g of cell lysate from OGT wild-type (WT) and null cells was resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were stained for total protein (Sypro™ Ruby Protein Blot stain) and **A**) CTD110.6 \pm 100mM GlcNAc; **B**) RL2 \pm 500mM GlcNAc; **C**) Multi-mAb – Cell Signaling Technologies (CST) \pm 250mM GlcNAc; **D**) HGAC39 \pm 250mM GlcNAc; **E**) HGAC49 \pm 250mM GlcNAc; and **F**) HGAC85 \pm 250mM GlcNAc. Total protein and immunoblots (developed with ECL) signals were captured with a GE Healthcare AI600 RGB.

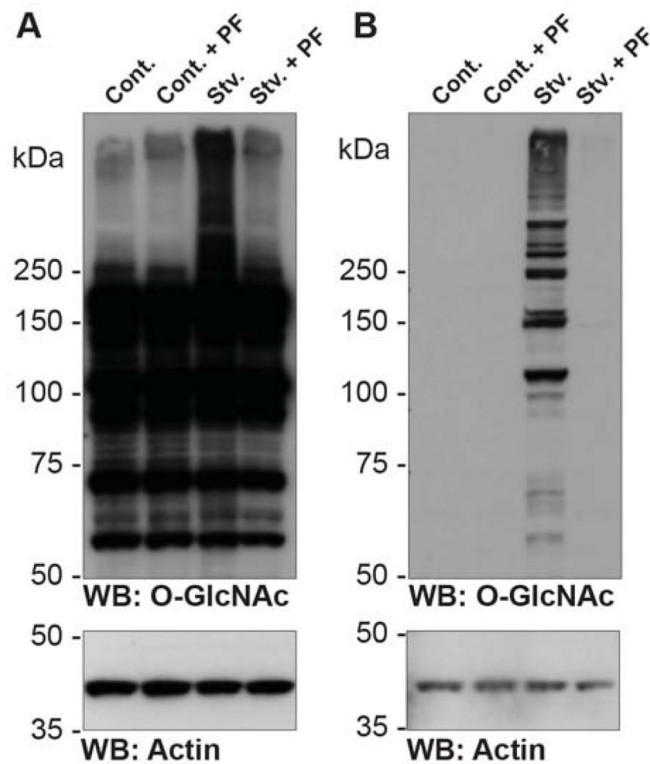


Figure 4. Differentiation of N-linked and O-linked glycans.

Lysates from cells exposed to control conditions or nutrient deprivation (STV: 0mM glucose, 1% dialyzed FBS) were treated with \pm PNGase F (PF), prior to separation by SDS-PAGE and transferred to PVDF. Membranes were incubated in **A**) water or **B**) 55mM NaOH overnight at 40°C. After blocking, membranes were probed for O-GlcNAc (CTD110.6) or Actin. Blots were developed using ECL and images captured on film. These data are adapted from Reeves et al., 2014 (Reeves et al., 2014).

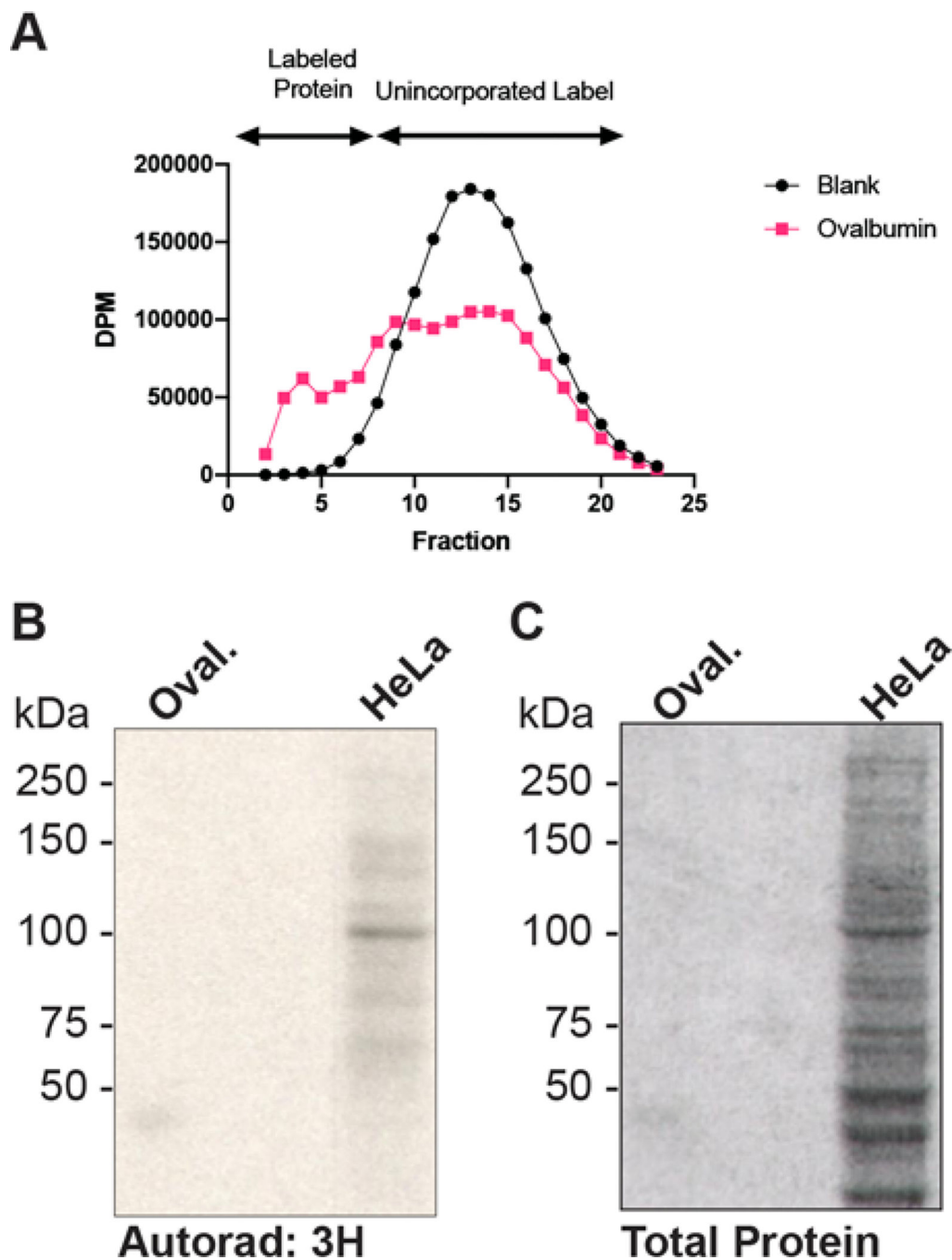


Figure 5. Galactosyltransferase Labeling.

A) Ovalbumin (●, pink) or a blank (■, black) were labeled with Gal-T and ^3H -UDP-Gal. Reactions were desalted using a G50 column. Fractions were counted and those with significant signal above the blank were combined and acetone precipitated. **B)** Ovalbumin (51,722 DPM) and HeLa cell lysates (18,295 DPM) labeled with Gal-T were separated by SDS-PAGE and stained with Coomassie blue (total protein). The resulting gel was dried and exposed to autoradiography film (1 week) with the aid of an intensifying screen.

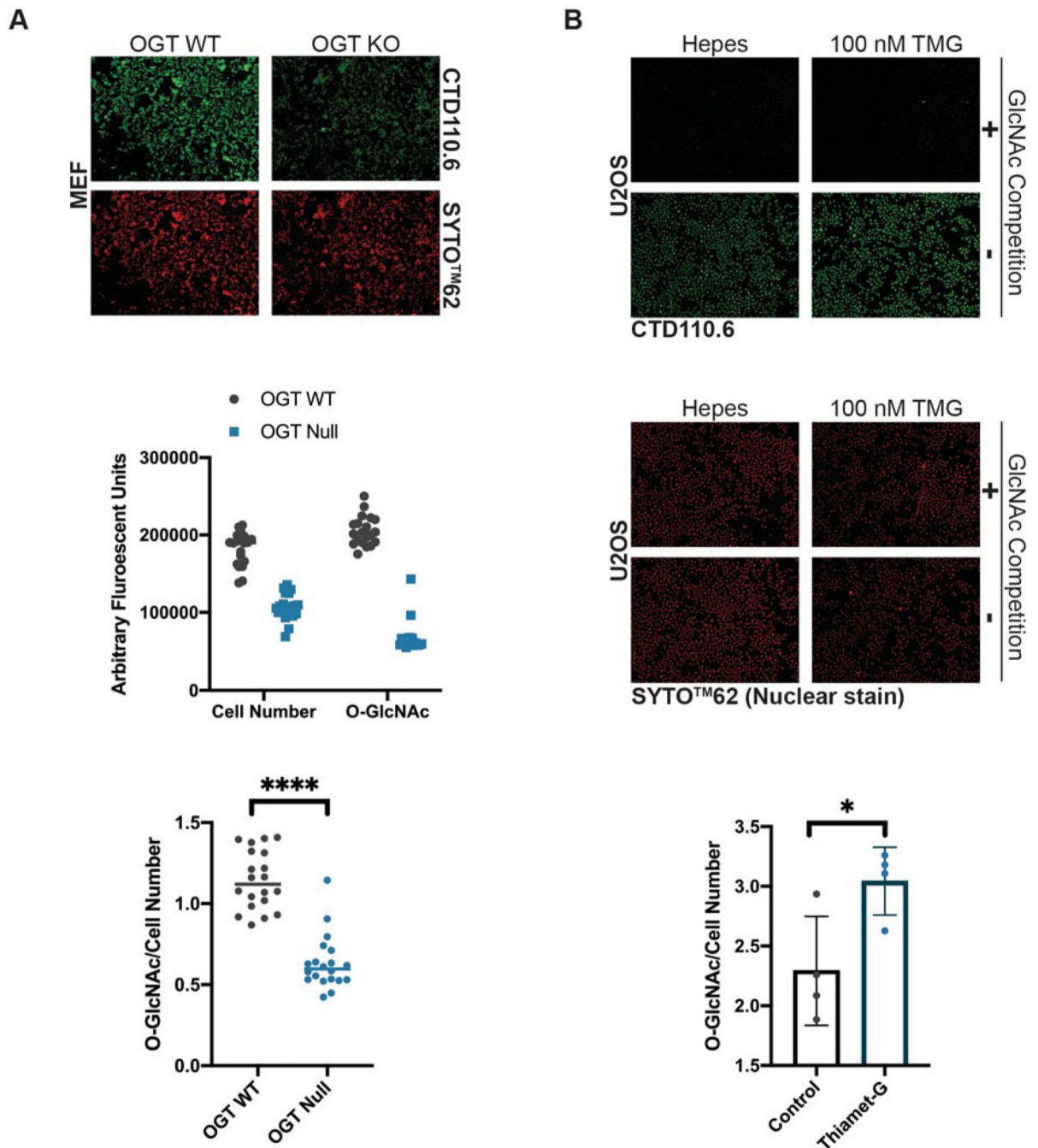


Figure 6. In Cell Western Blotting.

A) O-GlcNAc or cell number were detected in OGT WT (•, black) or null (•, blue) cells with CTD110.6 and SYTO™62. Raw fluorescent numbers for cell number and O-GlcNAc are plotted in the middle panel. O-GlcNAc levels corrected for cell number are plotted in the lower panel. P-values are the result of a student's T-test of 20 technical replicates. **** $p < 0.001$. **B)** O-GlcNAc or cell number were detected in U2OS cells treated with Vehicle (•, black) or TMG (100nM, 4h; •, blue) with CTD110.6 (upper panel) and SYTO™62 (middle panel). The O-GlcNAc signal, corrected for cell number is plotted in the lower panel. Error

bars represent one standard deviation. P-values are the result of a student's T-test of 4 technical replicates. * $p < 0.05$. Fluorescent intensity was assessed using a Molecular Devices SpectraMax i3x and fluorescent cell images (4x) with a Molecular Devices SpectraMax MiniMax 300™ Imaging Cytometer.

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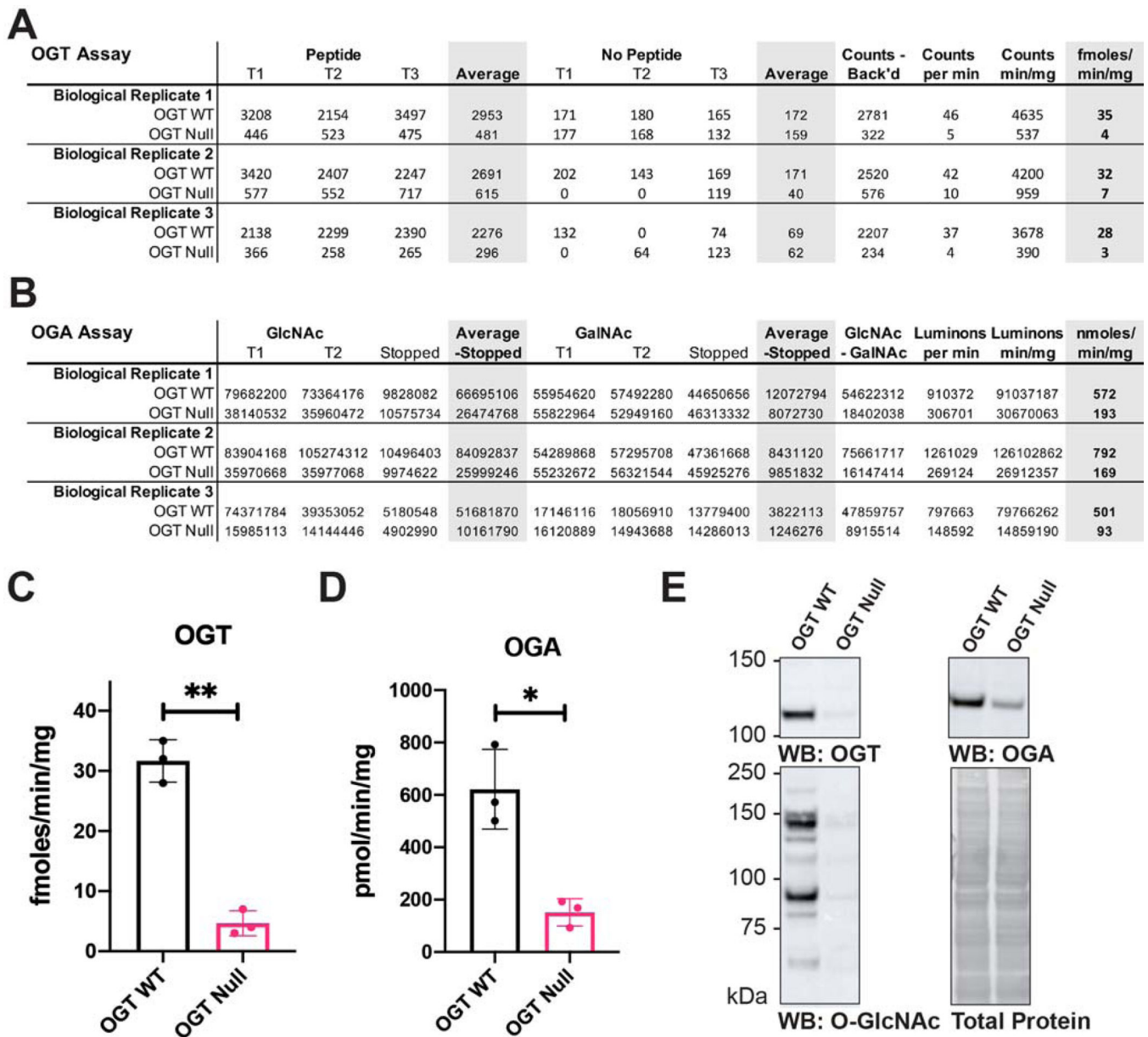


Figure 7 –. Representative data from OGT and OGA assays.

A) OGT activity in 10 μ g of desalted lysate from OGT Wild-type (WT) and null cells was assayed in triplicate in the presence and absence of CK2 substrate peptide. The incorporation of ^3H -GlcNAc was assessed using liquid scintillation counting (DPM). Activity was subsequently calculated using 10 μ g of protein, an assay length of 60 min, and 7.54fmol of GlcNAc per 1000 DPM. **B)** OGA activity in 10 μ g of desalted lysate from OGT Wild-type (WT) and null cells was assayed in triplicate using the pseudosubstrates 4MU- β -GlcNAc and 4MU- β -GalNAc. One replicate was stopped using glycine at time=0 (Stopped). 4MU released was assessed in a fluorescent plate reader and quantified using a 4MU standard curve. Activity was subsequently calculated using 10 μ g of protein, an assay length of 60 min, and 159222 luminons per nmol (experimentally derived standard curve). **C)** Plotted OGT activity assays. **D)** Plotted OGA activity assays; **E.** Lysates from OGT WT

and Null cells (10 μ g) were separated by SDS-PAGE and electroblotted to nitrocellulose. The following were detected using a GE Healthcare AI600 RGB: Total Protein (Sypro™ Ruby Protein Blot stain), O-GlcNAc (CTD110.6), OGT (Sigma, DM17), and OGA (345; (Butkinaree et al., 2008)). Error bars represent one Standard Deviation. P-values are the result of a student's T-test (2 tailed). * $p < 0.05$, ** $p < 0.01$.

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Table 1

Antibodies and Lectins that Recognize O-GlcNAc and GlcNAc

NAME	ANTIBODY ISOTYPE	RECOGNIZES	COMMERCIALY AVAILABLE	POSITIVE CONTROL	NEGATIVE CONTROL	REFERENCE
CTD110.6	IgM	β GlcNAc	Covance, Pierce, Millipore SIGMA, Cell Signaling, Santa Cruz Biotechnology	BSA-AP-GlcNAc	BSA-AP ^a or ovalbumin	(Comer et al., 2001)
RL2	IgG	O-GlcNAc	Abcam, Affinity Bioreagents, Santa Cruz Biotechnology	BSA-AP-GlcNAc	BSA-AP or ovalbumin	(Snow et al., 1987)
MY95	IgG	O-GlcNAc	No	BSA-AP-GlcNAc	BSA-AP or ovalbumin	(Matsuoka et al., 2002)
18B10.C7(3)	IgG	O-GlcNAc	Millipore	BSA-AP-GlcNAc	BSA-AP or ovalbumin	(Teo et al., 2010)
9D1.E4(10)	IgG	β GlcNAc	Millipore	BSA-AP-GlcNAc	BSA-AP or ovalbumin	(Teo et al., 2010)
1F5.D6(14)	IgG	O-GlcNAc	Millipore	BSA-AP-GlcNAc	BSA-AP or ovalbumin	(Teo et al., 2010)
HGAC 85	IgG	β GlcNAc	Novus Biologicals, Abcam, Pierce, Enzo life sciences, Affinity Bioreagents	BSA-AP-GlcNAc	BSA-AP or BSA	(Turner et al., 1990)
HGAC 49	IgM	β GlcNAc	No	BSA-AP-GlcNAc	BSA-AP or BSA	(Turner et al., 1990)
HGAC 39	IgG	β GlcNAc	No	BSA-AP-GlcNAc	BSA-AP or BSA	(Turner et al., 1990)
Multi-mAb ^b	IgG	O-GlcNAc	Cell Signaling Technology		BSA	N/A

^aBSA-AP, BSA-aminophenol.^bThis antibody was raised against glycoproteins. Modulating O-GlcNAc levels is an appropriate control.

Table 2

Conditions for Some Common Commercial Antibodies

ANTIBODY	BLOCK *	PRIMARY CONCENTRATION	PRIMARY BUFFER	SECONDARY	SECONDARY BUFFER *	GLCNAC COMPETITION
CTD110.6	3% Milk	1µg/ml	3% BSA	Mouse Anti-IgM	3% BSA	100mM
RL2	3% Milk	1µg/ml	3% BSA	Mouse Anti-IgG	3% BSA	500mM
HGAC 85	3% BSA	1µg/ml	3% BSA	Mouse Anti-IgG	3% BSA	250mM
HGAC 49	3% BSA	1µg/ml	3% BSA	Mouse Anti-IgM	3% BSA	250mM
HGAC 39	3% BSA	1µg/ml	3% BSA	Mouse Anti-IgG	3% BSA	250mM
Multi-mAB (CST)	3% Milk	1µg/ml	3% BSA	Rabbit Anti-IgG	3% BSA	250mM

* Milk and BSA are w/v and are dissolved in TBST

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Table 3

Reaction Mixtures for Assay of Galactosyltransferase Activity

SAMPLE	DILUTION	VOL. (μ l) OF DILUTED SAMPLE	VOL. (μ l) OF 200 MM GLCNAC	VOL. (μ l) 10 \times GAL LABELING BUFFER	VOL. (μ l) MILLI-Q WATER
Blank		0	10	10	70
Pre-Gal	1/1000	10	10	10	60
	1/10,000	10	10	10	60
	1/100,000	10	10	10	60
Auto-Gal	1/1000	10	10	10	60
	1/10,000	10	10	10	60
	1/100,000	10	10	10	60

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