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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 is implicated in the etiology of type II diabetes and Alzheimer's disease, as well as cardioprotection. This unit covers simple and comprehensive techniques for identifying proteins modified by O-GlcNAc, studying the enzymes that add and remove O-GlcNAc, and mapping O-GlcNAc modification sites.

Keywords

Signal transduction; Glycosylation; O-GlcNAc; Method(s); Detection; Analysis; Galactosyltransferase; O-linked; Glycosylation

INTRODUCTION

The modification of Ser and Thr residues by monosaccharides of O-linked β-N-acetylglucosamine (O-GlcNAc) is a common, dynamic, and essential modification of nuclear and cytoplasmic proteins. O-GlcNAc is ubiquitous, having been identified in all complex eukaryotes studied, including filamentous fungi, plants and viruses. Recent proteomic studies suggest that more than 1500 proteins in the cell are modified by O-GlcNAc, and that these proteins have diverse functions including cytoskeletal proteins, nuclear pore proteins, RNA polymerase II (RNA Pol II), transcription factors, proto-oncogene products, tumor suppressors, hormone receptors, phosphatases, and kinases (Khidekel et al. 2007; Nandi et al. 2006; Vosseller et al. 2006; Wang et al. 2008).

The functional consequences of modifying most proteins with O-GlcNAc is unclear, but it is required for survival at the single-cell level (Shafi et al. 2000). Three features suggest that O-GlcNAc performs a regulatory role: (1) O-GlcNAc occurs at sites on the protein backbone that are similar or identical to those modified by protein kinases; (2) O-GlcNAc is reciprocal with phosphorylation on some well-studied proteins, such as RNA Pol II, estrogen receptor-β, SV40 large T-antigen, and the *c*-Myc proto-oncogene product; and (3) like phosphorylation, O-GlcNAc is highly dynamic, with rapid cycling in response to cellular signals such as cellular stress or cellular stages such as the cell cycle (Hart et al. 2007). Perturbations in the metabolism of UDP-GlcNAc, which alter the regulation of many O-

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GlcNAc modified proteins, have been implicated in Alzheimer's disease, diabetes, and cancer (Dias and Hart 2007; Hanover et al. 2010; Hart et al. 2007; Liu et al. 2009; Skorobogatko et al.; Wang et al. 2008; Wells et al. 2003). Recently, O-GlcNAc levels have been shown to be elevated in response to different forms of cellular injury (Zachara et al. 2004), and elevating O-GlcNAc levels is protective in both in vivo and in vitro models of heat stress, oxidative stress, hypoxia, ischemia reperfusion injury, and trauma hemorrhage (Hart et al. 2007; Laczy et al. 2009; Ngoh et al. 2009).

This unit concentrates 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. We have focused on methods that require standard laboratory equipment. However, in some cases we also provide protocols that require more specialized technology for mapping sites of O-GlcNAc modification.

The unit is set out in a stepwise manner. 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/screening of O-GlcNAc modified proteins either by western blotting or lectin affinity chromatography (see Basic Protocols 2 to 5). Separate protocols verify that the glycan is O-linked GlcNAc (see Support Protocols 1 and 2). These methods are followed by protocols for more comprehensive analysis of O-GlcNAc modified proteins, including labeling of O-GlcNAc residues with [^{3}H]Gal, and subsequent product analysis (see Alternate Protocol 1, see Basic Protocols 5 and 6, and see Support Protocols 3 and 4). There are also two protocols for the enrichment of O-GlcNAc modified peptides, that are ideal for studies designed to site-map O-GlcNAc (see Basic Protocols 7 and 8. The final two protocols assay for O-GlcNAc transferase and O-GlcNAcase activity, respectively (see Basic Protocols 9 and 10 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 number of O-GlcNAc moieties per protein molecule can be increased by treating cells/tissues/animals with inhibitors of the O-GlcNAcase. Several inhibitors exist (Choubdar et al. 2008; Dennis et al. 2006; Dorfmueller et al. 2006; Dorfmueller and van Aalten; Kim et al. 2007; Kim et al.; Kim et al. 2006; Laczy et al.; Lee et al. 2006; Macauley et al. 2005; Scaffidi et al. 2007; Shanmugasundaram et al. 2006; Stubbs et al. 2006; Whitworth et al. 2007; Yuzwa et al. 2008), although only PUGNAc (Toronto Research Chemicals; (Haltiwanger et al. 1998) and Thiamet-G (Cayman Chemicals; (Yuzwa et al. 2008)) are commercially available. Unlike Thiamet-G, PUGNAc also inhibits lysosomal hexosaminidases. 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. In addition, STZ is only effective in cells that express the glucose transporter GLUT-2 (Schnedl et al., 1994). Recently, several specific OGT inhibitors have been developed (Gross et al. 2005). These inhibitors work well *in vitro*, but are of limited use *in vivo*. These inhibitors have been reported to work in isolated neonatal cardiomyocytes (1–5μM) (Ngoh et al. 2008) and breast cancer cells (500μM) (Caldwell et al.), but in the authors hands have not been effective in Cos-7 cells, HeLa cells, U2OS cells, and mouse embryonic fibroblasts. 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.

Materials

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

1 mM Thiamet-G (Caymen Chemicals) stock in 1M HEPES pH7.5 (filter sterilize and store in aliquots up to 6 to 12 months at −80°C)

PUGNAc (Toronto Research Biochemicals) 100mM stock in PBS pH7.5 (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)

100-mm tissue culture dishes

1. Grow cells in monolayer culture in a sufficient number of 100-mm dishes.
2. Add (or replace growth medium with fresh medium containing) 0.1–1μM Thiamet-G (added from 1mM stock) or 10–100μM PUGNAc or 5 mM glucosamine (added from 500 mM stock). Incubate cells in an incubator for 4 to 18 hr. The vehicle is either 1M HEPES, PBS, or 0.5M HEPES pH7.5.

   *When using glucosamine, mannitol is often added to the controls at the same concentration. This 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 (*UNIT 10.1*) and electroblot onto appropriate membrane (*UNIT 10.7*).

   Alternatively, extract proteins and proceed with protein purification or immunoprecipitation.

BASIC PROTOCOL 2 DETECTION OF PROTEINS MODIFIED BY O-GlcNAc USING ANTIBODIES

Several antibodies have been developed that recognize terminal GlcNAc residues (*below*) or the O-GlcNAc modification (*below*). Recently, several site-specific O-GlcNAc antibodies have recently been developed (c-myc, Keratin, vimentin), which allow researchers to probe the O-GlcNAc modification state of key proteins without purification of the protein first. These antibodies will not be discussed here since they are not widely available. When using O-GlcNAc pan-specific or GlcNAc pan-specific antibodies it is important to run appropriate controls as some peptides 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; or 6) using appropriate purified control proteins such as ovalbumin, which bears N-linked glycans with terminal GlcNAc residues, or synthetic neoglycoconjuagtes such as BSA-aminophenyl-GlcNAc (BSA-AP-GlcNAc).

Using the standard Amersham Pharmacia Biotech enhanced chemiluminescent (ECL) system, the authors have found that 10 μg of nuclear, or total cell extract is sufficient. However, 20–30μg of cell extract provides the highest quality data. 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. However, the authors use a similar protocol for RL2.

Materials

Purified or crude protein (e.g., Basic Protocol 1) separated by SDS-PAGE (*UNIT 10.1*) and electroblotted to polyvinylidene difluoride (PVDF; *UNIT 10.7*) or nitrocellulose (*preferred to PVDF*) (duplicate blots are needed)

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

Antibody dilution buffer (3% BSA w/v in TBST)

TBST (0.05% v/v Tween-20)

Antibody: CTD 110.6 ascites (Covance) diluted 1/2500 in Antibody dilution buffer (*antibody that has been antigen purified using a GlcNAc-Agarose column yields higher quality blots*)

N-acetylglucosamine (GlcNAc; Sigma)

HRPO-conjugated anti–mouse IgM diluted 1/5000 in TBS-HT

TBS

ECL kit (Amersham Pharmacia Biotech)

Additional reagents and equipment for visualization with chromogenic and luminescent substrates (*UNIT 10.10*)

1 Block blots by incubating with Blocking buffer for 60 min at room temperature.

2 Incubate blots with CTD 110.6 (1/2500 dilution in antibody dilution buffer), in duplicate, with and without 100 mM GlcNAc, overnight at 4°C.

To control for specificity it is important to perform a control blot. Here, the antibody is preincubated with 100 mM GlcNAc for ~5 min on ice before being applied to the control blot. Note that the concentration of antibody should be optimized with each new preparation. We find that 1/2500 is a good place to start with ascites (containing antibody in the 1mg/ml range). This control is most important when probing immunoprecipitations.

3 Wash blots in TBST thrice, each time for 10 min at room temperature.

5 Incubate blots with HRPO-conjugated anti–mouse IgM (1/5000 dilution in Antibody dilution buffer) for 50 min, room temperature.

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

6 Wash blots in TBST four times, each time for 10 min, room temperature.

7 Wash blots in TBS once, for 10 min, room temperature.

8 Develop the HRPO reaction using, e.g., the ECL system (UNIT 10.10).

Expected results*: CTD110.6 should bind BSA-AP-GlcNAc, but, not BSA-AP. A number of bands in total cell lysate should be detected predominantly over 40kDa. Treatment with thiamet-G should elevate the levels of O-GlcNAc 2–3 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 horseradish peroxidase (HRPO). Before succinylation, WGA will recognize both silaic acid and GlcNAc (Monsigny et al. 1980). For additional information concerning lectin chromatography, see *UNIT 9.1*.

The amount of "test" protein used is dependent on the technique(s) used to develop the HRPO reaction. Using the standard Amersham Pharmacia Biotech ECL system the authors find that 15 μg of cytoplasmic or nuclear extract is sufficient, but 20–30μg 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 (*UNIT 10.1*) and electroblotted to polyvinylidene difluoride (PVDF; *UNIT 10.7*) or nitrocellulose (duplicate blots are needed)

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

TBST (see recipe)

0.1 μg/ml HRPO-conjugated sWGA (EY Labs) 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; Sigma)

High-salt TBST (HS-TBST): TBST (see recipe) containing 1 M NaCl

Tris-buffered saline (TBS; see recipe)

ECL kit (Amersham Pharmacia Biotech)

Additional reagents and equipment for visualization with chromogenic and luminescent substrates (*UNIT 10.10*)

1. Wash duplicate blots for 10 min in 5% BSA/TBST, room temperature.
2. Block by incubating blots in 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 blots three times, each time for 10 min in TBST, room temperature.

4. Incubate blots in 0.1 μg/ml sWGA-HRPO in 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 preincubated with 1 M GlcNAc for ~5 min on ice before being applied to the control blot.

5. Wash blots six times, each time for 10 min, in HS-TBST.

6. Wash blots once in TBS for 10 min.

7. Develop the HRPO-reaction using, e.g., the the ECL system (*UNIT 10.10*).

   ***Expected results**: sWGA should bind BSA-AP-GlcNAc and ovalbumin, but not BSA-AP. A number of bands in total cell lysate should be detected predominantly over 40kDa. Treatment with thiamet-G should elevate the levels of O-GlcNAc 2–3 fold on numerous proteins in total cell lysate. Reactivity toward both 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.

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 (Sigma) which contains both N- and O-linked sugars terminating in GlcNAc, treated and not treated with PNGase F (*UNITS 12.4 & 12.6*).

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)

55 mM NaOH

3% (w/v) BSA in TBST (see for TBST)

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)

1. Wash blots once in TBS for 10 min.

2. Incubate two blots in 55 mM NaOH at 40°C overnight; incubate the control blot in Milli-Q water at 40°C overnight.

   The blots treated with NaOH will yellow slightly.

3. Wash blots three times, each time for 10 min at room temperature, in TBST.

4. Block by incubating blots in 3% w/v BSA/TBST for 60 min at room temperature.

5. Probe 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 ± 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 number, such as transcription factors. In this protocol, the protein of interest is synthesized in a rabbit reticulocyte lysate (RRL) in vitro transcription translation (ITT) system (Promega) and labeled with either [^{35}S]Met, [^{35}S]Cys, or [^{14}C]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 SILAC acid residues typically one would purify proteins from nuclear and cytoplasmic extracts to avoid co-purifying proteins with proto-typical 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 a RCA1 affinity column. Labeled O-GlcNAc proteins are released under mild conditions, while those containing N-linked structures require lactose addition to the buffer before elution results (Greis and Hart 1998; Hayes et al. 1995). The method described in this protocol can be adapted for RCA1 affinity chromatography by substituting RCA1-agarose (EY Labs) 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 μg/μl)

Kit for RRL ITT system (Promega)

Label: [^{35}S]Met, or [^{35}S]Cys, or

[^{14}C]Leu

WGA-agarose (Vector Laboratories)

Note, WGA is used here rather than sWGA as it has a higher affinity for GlcNAc and O-GlcNAc

WGA wash buffer: PBS (*APPENDIX 2E*) containing 0.2% (v/v) NP-40

WGA Gal elution buffer (see recipe)

WGA GlcNAc elution buffer (see recipe)

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

Additional reagents and equipment for digesting proteins with hexosaminidase (see Support Protocol 2), desalting (see Support Protocol 5), SDS-PAGE (*UNIT 10.1*), and autoradiography (*UNIT 10.11*)

Prepare proteins

1. Synthesize proteins to incorporate the desired label ([^{35}S]Met, [^{35}S]Cys, or [^{14}C]Leu) using the RRL ITT system according to the manufacturer's instructions. Include the protein of interest, a positive control for WGA binding (for example, 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 samples using spin filtration (e.g., Amersham Pharmacia Biotech Microspin G-50 columns) or a 1-mL G-50 desalting column (as for desalting O-GlcNAc transferase; see Support Protocol 5).

   The following procedure is carried out at 4°C.

Apply protein samples to chromatography columns

4 Equilibrate WGA-agarose and pack column as follows:

   a. If 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.

   b. Let storage solution drain from resin.

   c. Equilibrate resin by washing column four times, each time with 1mL of WGA wash buffer. Cap column.

   The volumes given are appropriate for a sample derived from an ITT. For enrichment of other protein samples, the volume of WGA should be optimized for the protein sample applied. The authors find that 50mg of cell extract requires 5mL of WGA-agarose, assuming that 1% to 2% of the total cell extract is modified by O-GlcNAc.

5 Apply sample (~30 μL of an ITT reaction) to the column and let stand at 4°C for 30min, 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 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 column with 15 mL of WGA wash buffer at 10 ml/hr, collecting 0.5-mL fractions.

7 Load the column with 300μL of WGA Gal elution buffer and let stand at 4°C for 20min.

8 Wash column with 5mL of WGA Gal elution buffer, collecting 0.5mL fractions.

9 Repeat steps 6 to 8 using GlcNAc elution buffer.

10 Count 25μL of each fraction using a liquid scintillation counter.

   Depending on the stoicheometry, or the number of O-GlcNAc residues per molecule of protein, 1–10% 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 microcentrifuging 10 min at 16,000 × g, 4°C, in a microcentrifuge tube (which is the most efficient procedure) or in 15-mL conical centrifuge tubes for 10min at 3000 ×g, 4°C. Resuspend samples in SDS-PAGE sample buffer (UNIT 10.1).

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 [^{35}S]Met is precipitated and analyzed by SDS-PAGE (UNIT 10.1) and autoradiography (UNIT 10.11). The use of acetone to precipitate proteins is not recommended, as free GlcNAc will also precipitate.

12 Analyze pellet by SDS-PAGE (*UNIT* 10.1) and autoradiography (*UNIT* 10.11). *Researchers should expect a band at the approximate molecular weight of their 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)

Additional reagents and equipment for SDS-PAGE (*UNIT 10.1*) and electroblotting (*UNIT 10.7*)

1. Mix sample 1:1 with 2% SDS and boil for 5 min.
2. Mix sample 1:1 with 2× hexosaminidase reaction mixture and incubate at 37°C for 4 to 24 hr.
3. To assess completeness of the digestion, separate an aliquot of the reaction by SDS-PAGE (*UNIT 10.1*) and electroblot (*UNIT 10.7*) onto an appropriate membrane. Probe blots with carbohydrate-specific lectins or antibodies.

   Ovalbumin should move several kilodaltons on a 10–12% 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-^{3}H]Gal, forming a [^{3}H]-βGal1-4βGlcNAc

(Greis et al. 1996; Roquemore et al. 1994; Torres and Hart 1984). The labeled sugar can be chemically released (via β-elimination) and analyzed by size-exclusion chromatography on a BioGel-P4 column, using the ^{3}H radiolabel to detect the fraction of interest (Roquemore et al. 1994). Labeling the O-GlcNAc allows for the subsequent detection of the 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 GlcNAc analogs that can be derivatized to facilitate either the purification or detection of O-GlcNAc modified proteins and peptides (Khidekel et al. 2003; Khidekel et al. 2007; Khidekel et al. 2004; Lamarre-Vincent and Hsieh-Wilson 2003; Rexach et al. 2008; Tai et al. 2004), a technique that is marketed by Invitrogen under the "Click-it" brand (Catalog C33368). This technique has been adapted with reagents that are not currently commercially available to enrich O-GlcNAc modified peptides (Wang et al. 2010; Wang et al. 2010) and to determine the number of O-GlcNAc moieties on a protein (Rexach et al.).

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 mol of EDTA binds 2 mol 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 what will label with galactosyltransferase (R.N. Cole, pers. commun.).

NOTE: Protease inhibitors, such as PIC 1, PIC 2, and PMSF (see 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-[^{3}H]Gal, (17.6 Ci/mM; GE Healthcare) 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)

10× galactosyltransferase labeling buffer (see recipe)

Galactosyltransferase, autogalactosylated (see Support Protocol 3)

Calf intestinal alkaline phosphatase

Unlabeled UDP-Gal

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

100°C water bath

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

Additional reagents and equipment for acetone precipitation of protein (*UNIT 4.5*), PNGase F digestion of proteins (*UNITS 12.4 & 12.6*), SDS-PAGE (*UNIT 10.1*), and product analysis (see Basic Protocol 6)

Prepare the reaction

1. Denature 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 μCi/reaction).

   A positive control (ovalbumin, 2 μg), 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 μl is required the label can be added directly to the reaction (final reaction volume, 500 μl).
4. Resuspend appropriate amount of label for each reaction, respectively, in 50 μl of 25 mM 5′-AMP.

   The AMP is included to inhibit possible phosphodiesterase reactions, which might compete for label during the labeling experiment.
5. Set up reactions as follows:

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

   350 μl buffer H

   50 μl 10× galactosyltransferase labeling buffer

   50 μl UDP-[^{3}H]Gal/5′-AMP mixture from step 4

   30 to 50 μl autogalactosylated galactosyltransferase

   1 to 4 U calf intestinal alkaline phosphatase

   Milli-Q water to final volume of 500 μl

   Reaction volumes can be scaled down to 50 μl.
6. Labeling at 37°C for 2 hr or at 4°C overnight.

   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 μl of galactosyltransferase. Incubate for an additional 2h at 37°C.

   For studies where complete labeling of the GlcNAc is required, such as site mapping, the reactions are chased with unlabeled UDP-Gal and fresh galactosyltransferase.

8. Add 50 μl of 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.

   Size-exclusion chromatography using Sephadex G-50 (~30cm) is traditionally used to desalt samples. However, TCA precipitation, spin filtration/buffer exchange, or other forms of size-exclusion chromatography (e.g., GE Healthcare 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-μl aliquot of each fraction using a liquid scintillation counter.

   Approximately 2×10^6 dpm of [^{3}H]Gal should be incorporated into 2μg of ovalbumin.

11 Combine the void volume and lyophilize to dryness.

12 Resuspend samples in Milli-Q water and precipitate with acetone (*UNIT 4.5*).

13 Treat samples with PNGase F (*UNITS 12.4 & 12.6*), separate by SDS-PAGE (*UNIT 10.1*) and detect by autoradiography (*UNIT 10.11*). Alternatively, subject samples to “product analysis” to confirm that the label was incorporated onto O-GlcNAc (see Basic Protocol 6).

   ***Expected data**: Numerous proteins should be labeled by Gal-transferase, as well as ovalbumin, and detected by autoradiography. O-GlcNAc modified proteins should be reactive before and after PNGAse F treatment.*

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)

10,000 U/ml aprotinin

2-mercaptoethanol

UDP-Gal

Saturated ammonium sulfate: >17.4 g $(NH_4)_2SO_4$ in 25 ml Milli-Q water

85% ammonium sulfate: 14 g $(NH_4)_2SO_4$ in 25 ml Milli-Q water

Galactosyltransferase storage buffer (see recipe)

30- to 50-ml centrifuge tubes

Refrigerated centrifuge

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

   The centrifuge tubes selected should be able to withstand a centrifugal force of 15,000 ×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 10μL of 10,000 U/ml aprotinin, 3.5μL of 2-mercaptoethanol, and 1.5 to 3.0mg of UDP-Gal.
5. Incubate the sample on ice for 30 to 60min.
6. Add 5.66 mL of prechilled saturated ammonium sulfate in a dropwise manner. Incubate on ice for 30min.
7. Centrifuge 15min at >10,000 × *g*, 4°C. Pour off supernatant.
8. Resuspend pellet in 5mL cold 85% ammonium sulfate and incubate on ice for 30min.
9. Centrifuge 15min at >10,000 × *g*, 4°C, and pour off supernatant.
10. Resuspend pellet in 1mL of galactosyltransferase storage buffer and divide into 50-μL aliquots, saving 5 μL for an activity assay as the “Auto-Gal” sample. Assay that aliquot for activity (see Support Protocol 4).
11. Store remaining aliquots up to 1 year at −20°C pending use in Alternate Protocol 1.

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/mM; GE Healthcare) in 70% v/v ethanol

Nitrogen source

1× galactosyltransferase dilution buffer: galactosyltransferase storage buffer (see recipe) supplemented with 5 mg/mL BSA

10× galactosyltransferase labeling buffer (see recipe)

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 10)

200 mM GlcNAc

Dowex AG1-X8 resin (PO_4 form) slurry in 20% (v/v) ethanol

Glass wool

1. Dry 40μL of 0.1 μCi/μL of UDP-[^{3}H]Gal in a Speed-Vac evaporator or under a stream of nitrogen.
2. Resuspend in 90μL 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× galactosyltransferase dilution buffer. Using these dilutions, 200 mM GlcNAc, and 10× galactosyltransferase labeling buffer, prepare reaction mixtures as described in Table 12.8.1.
4. Start the reaction by adding 10μL of 0.05 μCi/μL UDP-[^{3}H]Gal (see step 2) to each tube.
5. Incubate samples at 37°C for 30min.

   While the samples are incubating, prepare the columns.
6. Pour 1mL of Dowex AG1-X8 slurry (PO_4 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 3mL 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 500μL of Milli-Q water.
10. Load each sample onto the corresponding column and add a 500μL 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 100μL of the sample and 10μL of the UDP-[^{3}H]Gal using a liquid scintillation counter.
13. Calculate activity.

    a. Calculate the total moles of UDP-Gal transferred to GlcNAc as follows:

$$\{\text{cpm}_{(\text{with GlcNAc})} - \text{cpm}_{(\text{no GlcNAc})} / \text{total cpm}\} \times 1.76\ \text{nmol}$$

Note, $cpm_{(with\ GlcNAc)}$ represents counts in the sample, $cpm_{(no\ GlcNAc)}$ represents counts in the no-enzyme control, and total cpm represents the total counts available to transfer. $\{cpm_{(with\ GlcNAc)} - cpm_{(-GlcNAc)}/total\ cpm\}$ represents the portion of [^{3}H]Gal transferred in the reaction. This proportion is multiplied by the total number of moles of UDP-Gal in the reaction. If the specific activity of the UDP-[^{3}H]Gal is 17.6 Ci/mM and 10μL of label was used, then there are 1.76 nmol of UDP-Gal in 10μL.

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

BASIC PROTOCOL 5 DETECTION OF PROTEINS MODIFIED BY O-GLCNAC USING METABOLIC LABELING

Metabolic labeling of O-GlcNAc-bearing proteins provides a useful means to test if a protein of interest is modified by O-GlcNAc, to observe the gross dynamic changes in O-GlcNAc levels and to study the subcellular localization in response to stimulation or during cell cycle. In the protocol described below, cells are labeled with [^{3}H]glucosamine, which is metabolized to UDP-[^{3}H]GlcNAc in the hexosamine synthetic pathway. For labeling, it is critical that the labeled sugar compete with glucose import; this ensures efficient uptake of the label. While glucosamine is a good competitor, N-acetylglucosamine is not. For further discussions on metabolic labeling of glycoconjugates, readers are encouraged to consult (Varki 1994) for details.

Materials

Cells of interest, growing in culture

Biosynthetic labeling medium (see recipe)

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

Additional reagents and equipment for PNGase F digestion of proteins (*UNITS 12.4 &12.6*), SDS-PAGE (*UNIT 10.1*), and autoradiography (*UNIT 10.11*)

1. Replace growth medium with biosynthetic labeling medium. Label cells for 5 to 24 hr in an incubator.

   *The labeling is done in a glucose-free medium to maximize labeling efficiency. The addition of nonessential amino acids to the medium reduces the influx of glucosamine into the amino acid biosynthetic pathways (*Medina et al. 1998*).*

2. Wash labeled cells twice, each time by resuspending in 5 ml of PBS and centrifuging 10 min at 500 × *g*, 4°C.

3. Extract as desired.

   See Critical Parameters for a discussion on reducing O-GlcNAcase activity. There are many types of cell fractionation and extraction techniques available. Those that minimize plasma membrane and organelle contamination are preferable. If a particular study concerns one or a few proteins, and specific antibodies are available, the protein(s) can be isolated by immunoprecipitation after metabolic labeling.

4. Treat sample with PNGase F (*UNITS 12.4 &12.6*).

5. Separate proteins by SDS-PAGE (*UNIT 10.1*) and visualize labeled proteins by autoradiography (*UNIT 10.11*).

   Expected data*: Numerous proteins should be labeled, and the amount of label incorporated into proteins should increase if cells are treated with Thiamet G. The label should be insensitive to PNGAse F and sensitive to hexosaminidase.*

BASIC PROTOCOL 6 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] βGal1-4βGlcNAcol (from galactosyltransferase labeling) or [^{3}H]GlcNAcol (from metabolic labeling).

Materials

Labeled proteins

β-elimination reagent: 1 M $NaBH_4$/0.1 M NaOH (prepare fresh)

4 M acetic acid

Screw-cap microcentrifuge tubes

Additional reagents and equipment for acetone or methanol precipitation of proteins (*UNIT 4.5*), size-exclusion (gel-filtration) chromatography (*UNIT 8.3*), and Dionex chromatography (Townsend et al. 1996; Townsend and Hardy 1991; Townsend et al. 1989).

1. Acetone or methanol precipitate labeled proteins (*UNIT 4.5*) in 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 sample in 500 μl of β-elimination reagent and incubate at 37°C for 18 hr.

   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 μl 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 × 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 [^{3}H]GlcNAc or [^{3}H]Gal are pooled and lyophilized. Residual $NaBH_4$ is removed by washing the sample with methanol; $NaBH_4$ is volatile in the presence of methanol and is removed in a Speed-Vac evaporator or under a stream on nitrogen (Fukuda, 1990).

5. Resuspend the sugar alditols in Milli-Q water. Analyze by size-exclusion chromatography or by Dionex chromatography.

   *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 GE healthcare*

Superdex Peptide column, equilibrated in 30% v/v CH_3CN, 0.1% v/v TFA, has been used to size oligosaccharides (R.N. Cole, pers. commun.). Galβ1-4GlcNAcol should elute at the in a similar position 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 (*Hardy and Townsend 1994; Townsend et al. 1989*).*

BASIC PROTOCOL 7 BEMAD Change title of Basic Protocol 7. BEMAD is not descriptive. Suggest something like “Identification of Attachment sites”

There are several challenges associated with mapping sites of O-GlcNAc addition to the protein backbone, including: 1) like phosphorylation, O-GlcNAc is often sub-stoichiometric; 2) the detection of O-GlcNAc modified peptides is suppressed during mass spectrometry; and 3) in most mass spectrometers the O-GlcNAc modification is labile. As such, it is ideal to enrich peptides of interest and to either combine this with electron-transfer dissociation (ETD) or electron-capture dissociation (ECD) mass spectrometry in which the O-GlcNAc modification is stable (Chalkley et al. 2009; Khidekel et al. 2007; Skorobogatko et al.; Viner et al. 2009; Vosseller et al. 2006; Wang et al. 2010). One alternative to this approach is to mark the O-GlcNAc modification site with a more stable modification. The method described below, called β-elimination followed by Michael addition with dithiothreitol (BEMAD), is one such an approach (Vosseller et al. 2005; Wells et al. 2002; Zachara et al. 2004). Here, O-GlcNAc (as well as prototypical glycans and phosphorylation) is β-eliminated from the protein backbone and then dithiothreitol is added back in a Michael addition-type reaction. In addition to being more chemically stable, the DTT allows peptides of interest to be enriched by thiol affinity chromatography. The use of control peptides and phosphatase treatment are essential with this technique, as phosphate will also β-eliminate from the protein backbone.

Materials

Control peptides (Control phospho and O-GlcNAc modified peptides can be synthesized or are available from Invitrogen Catalog number #C33373)

~1–100pmol of protein sample in 40mM ammonium bicarbonate pH8.0

Microcentrifuge tubes rinsed in 50% (v/v) acetonitrile. Do NOT use autoclaved tubes

Trypsin, sequencing grade modified (Promega)

40mM ammonium bicarbonate, pH8.0

Trifluoroacetic acid (TFA)

Performic acid oxidation buffer: 45% (v/v) formic acid, 5% (v/v) hydrogen peroxide, in Milli-Q water (make fresh)

$MgCl_2$

Alkaline phosphatase (Promega)

DTT, high purity (GE Healthcare)

BEMAD solution: 0.1% (v/v) NaOH, 1% (v/v) Triethylamine, 10mM DTT (make fresh)

C_{18} reversed-phase macro spin columns (The Nest Group, Inc.; Southborough, MA)

Buffer A: 1% (v/v) TFA

Buffer B: 75% (v/v) acetonitrile, 1% (v/v) TFA

Thiol column buffer, degassed: PBS, 1mM EDTA (make fresh)

Thiol column elution buffer (made fresh), degassed: PBS, 1mM EDTA, 20mM DTT

Thiopropyl Sepharose™ 6B (GE Healthcare)

1% (v/v) acetic acid

Savant Speed-Vac Concentrator

Mass spectrometer, such as a Iontrap or Orbitrap mass spectrometer with nanospray source

Performic acid oxidation

1. Resuspend protein samples in performic acid oxidation buffer (~300μL)
2. Spike sample with control peptides (1–10pmol)
3. Incubate for 1h on ice
4. Dry down in a Speed-Vac vacuum concentrator

Trypsin digestion

1. Resuspend protein samples in 40mM ammonium bicarbonate, pH8.0
2. Digest the proteins by the addition of 1:10–1:100 (w/w) sequencing grade trypsin for 16–18h at 37°C
3. Acidify the digest with TFA to a final concentration of 1% (v/v)
4. Desalt digests over a C_{18} reversed-phase column (see manufacturer's instructions)
5. Dry peptides using a Speed-Vac concentrator

Phosphatase treatment

1. Resuspend the peptides in 40mM ammonium bicarbonate, 1mM $MgCl_2$, pH8.0
2. Add alkaline phosphatase (1 unit/10 μL) and incubate for 4h at 37°C

   Lamda phosphatase is an acceptable alternative to alkaline phosphatase

   Using a phosphopeptide control is essential to ensure that this treatment is effective.
3. Dry peptides using a Speed-Vac concentrator

BEMAD treatment

1. Resuspend the peptides in BEMAD (500μL) solution and adjust the pH to 12.0–12.5 with triethylamine (if necessary)
2. Incubate the reaction for 2.5h at 50°C
3. Stop the reaction by the addition of TFA to a final concentration of 1% (v/v)
4. Clean-up the peptides over a C_{18} reversed-phase column (see manufacturer's instructions)

5. Dry peptides using a Speed-Vac concentrator

Thiol affinity column

1. Swell thiopropyl sepharose resin in degassed Thiol column buffer and wash with ~10 column volumes of Thiol column buffer
2. Resuspend the peptides in Thiol column buffer
3. Bind the peptides to thiol column for at least 1h at room temperature
4. Wash the column with 20mL of Thiol column buffer
5. Elute the peptides three times with Thiol elution buffer (150μL)
6. Acidify the peptides by the addition of TFA to a final concentration of 1% (v/v)
7. Clean-up the peptidesover C_{18} reversed-phase column to remove free DTT
8. Dry down peptides

LC-MS/MS analysis

1. Resuspend peptides in 1% (v/v) acetic acid
2. Load samples onto a nanobore reversed phased column and separate peptides using a linear gradient of acetonitrile. Data should be collected in a manner appropriate for your mass spectrometer.
3. Data analysis software should allow the following mass changes: a differential mass increase of 136.2 daltons to Ser and Thr residues (addition of DTT), 120.2 daltons to Cys residues that may have been derivatized with DTT, 48.0 daltons to performic acid oxidized Cys and Trp residues, and 32.0 daltons to performic acid oxidized Met residues.

   Expected results: *Peptides, such as the control glycopeptide, that were modified by one O-GlcNAc residue should be 136.2 amu greater than the molecular weight of the unmodified peptide. The control phosphopeptide should be detected at either the unmodified molecular weight of the peptide or as the phosphopeptide which should be 79amu greater than the molecular weight of the unmodified peptide.*

BASIC PROTOCOL 8 ENRICHMENT OF O-GLCNAC MODIFIED PEPTIDES BY LECTIN WEAK AFFINITY CHROMATOGRAPHY (LWAC)

As discussed above, one challenge when site-mapping O-GlcNAc modification sites is enriching peptides modified by O-GlcNAc. Unfortunately, most lectins and antibodies directed against O-GlcNAc do not immunoprecipitate O-GlcNAc modified peptides. Vosseller and coworkers have used wheat germ agglutinin in a long column format, known as lectin weak affinity chromatography (LWAC) to enrich O-GlcNAc modified peptides without chemical derivatization (Vosseller et al. 2006). While O-GlcNAc modified peptides do not bind the column, they are significantly retained, and as such can be enriched. Enriched glycopeptides can be site-mapped using traditional techniques, or alternatively by ECD/ETD mass spectrometry.

Materials

Sample Preparation

Glucosamine (Sigma)

Streptozotocin (STZ; Sigma)

PUGNAc (Toronto Research Biochemicals) or Thiamet G Anti-O-GlcNAc antibody, such as clone 110.6 (See above)

Trypsin digestion

100mM ammonium bicarbonate pH 8.0 (Adjust the pH with 1N NaOH)

Ammonium bicarbonate is used as it is volatile

500mM DTT stock solution in 100mM ammonium bicarbonate buffer

500mM Iodoacetamide solution in 100mM ammonium bicarbonate buffer (prepare fresh and store in the dark)

Sequencing grade modified trypsin (Promega, #V511A). Store at −70 °C

Sample desalting

MacroSpin column (capacity 300μg) (The Nest Group, Part #SMM SS18V) 100% acetonitrile

Washing buffer: 0.1% (v/v) formic acid in Milli Q water

Elution buffer: 80% (v/v) acetonitrile, 25mM formic acid, in Milli Q water

Long WGA Column Packing

Equilibration of WGA agarose

WGA coupled to agarose, 10mL (Agarose Wheat Germ Agglutinin, Vector Laboratories, Catalog No. AL-1023)

As above WGA agarose is used as sWGA has a lower affinity for GlcNAc than WGA. Again, it is preferable to enrich O-GlcNAc modified proteins before trypsin digestion, to avoind complications due to peptides modified by prototypical glycans.

WGA buffer: 25mM Tris-HCl pH7.8, 300mM NaCl, 5mM $CaCl_2$, 1mM $MgCl_2$

WGA storage buffer: 25mM Tris-HCl pH7.8, 300mM NaCl, 5mM $CaCl_2$, 1mM $MgCl_2$, 0.05%w/v sodium azide

~40mL empty glass column designed for gravity flow to wash WGA agarose

~20mL empty glass column for chromatography with the frit removed, this should be appropriate for use on an FPLC/HPLC

This column will serve as a reservoir for WGA agarose slurry during column packing

Preparation of the teflon tubing for packing

An opaque 12m (~39 feet) length of teflon tubing (O.D. 1.59m; I.D. 1mm; volume ~10ml)

WGA Isocratic HPLC

AKTA Purifier (Amersham Biosciences) HPLC system, or equivalent HPLC

2x 96-well collection plates, 2ml deep (such as MASTERBOCK®, Greiner)

Sample Clean-up using Zip-Tips C_{18} reversed phase chromatography for MS analysis

ZipTip($C_{18;}$ P10) (Millipore, CAT. No. ZTC18S096)

Reagent A: 0.1% (v/v) formic acid in Milli Q water

Reagent B: 70% (v/v) acetonitrile, 0.1% (v/v) formic acid in MilliQ water

Reuse and Storage of WGA Column

WGA column regeneration buffer: 1M NaCl pH 3 (adjust pH with acetic acid)

Method

Sample Preparation—As wheat germ agglutinin will bind all terminal GlcNAc residues, it is important to fractionate samples (such as a nuclear and cytoplasmic) to enrich for O-GlcNAc modified proteins (found in the nucleus, cytoplasm, and mitochondria) from proteins modified by prototypical glycans (plasma membrane, ER, Golgi). It is ideal to start with ~300μg of enriched O-GlcNAc modified protein.

Tryptic digestion

1. *Adjust the pH to pH7.8–8.0:* Dilute the sample six-fold with 100mM ammonium bicarbonate buffer pH 8.0 (so to 100μL of sample add 500μL of ammonium bicarbonate). Check that the pH is ≥ 7.8.
2. *Reduce any disulfide bonds:* Add DTT stock solution (500mM) to a final concentration of DTT 10mM (so to 600μL of sample add 12μL of 500mM DTT). Vortex briefly, and spin down (pulse). Incubate the sample for 60min at 60°C. Equilibrate to room temperature before proceeding.
3. *Block the cysteines by alkylation:* Add iodoacetamine stock solution (500mM) to the sample to final concentration of 50mM (to 612μL of sample add 61.2μL of 500mM iodoacetamide). Vortex, spin briefly (pulse). Incubate the sample for 60min at room temperature in the dark.
4. *Quench free alkylation reagent:* Add stock DTT solution (500mM) to a final concentration of 10mM (to 673.2μL of sample add 13.4μL of 500mM DTT). Vortex, spin briefly (pulse). Incubate the sample at room temperature for 45min.
5. *Generate tryptic peptides:* Digest proteins by addition of 40:1 (w/w) sequencing grade trypsin (to 300μg of protein add 7.5μg of trypsin). Vortex, spin briefly (pulse) Check the pH, the pH should be ≥ 7.8. Incubate the reaction at 37°C overnight.

Sample desalting—Note: All centrifugation steps should be performed for 1min at 110xg, 800rpm in an Eppendorf microcentrifuge.

1. *Acidify the sample:* add formic acid to final concentration 0.2% (v/v) (for a sample of ~700μL, add 1.6μL 88% formic acid)
2. *Assemble the C_{18} reversed phase spin column:* Remove an end restriction and a cap and place the column in a 2mL microcentrifuge tube
3. *Wet the reversed phase column:* Add 500μL of 100% acetonitrile to the column. Centrifuge. Discard the flow through
4. *Wash the column:* Add 500μL of washing buffer to the column. Centrifuge. Discard the flow through. Remove the collecting tube and dry with a Kimwipe. Place the column into a new 2mL microcentrifuge tube
5. *Load the sample to the column:* Place the sample (maximum 500μL) on the column.

Centrifuge. Discard the flow through. Apply the rest of the sample on the column (if needed).

Centrifuge. Discard the flow through

6. *Wash the column:* Add 250μL of washing buffer on the column. Centrifuge. Place the column into a new 2mL microcentrifuge tube
7. *Elute the peptides:* Add 250μL of elution buffer to the column. Centrifuge. Collect the flow through. Repeat this step twice.
8. Dry the sample completely in a speed vacuum concentrator
9. Dissolve dried sample in 80uL of WGA buffer

Long WGA Column Packing

Equilibration of WGA agarose

1. Pour WGA agarose slurry (~50%, 20mL) into an empty 40mL glass column for gravity flow.
2. Wash 1x 20mL with WGA buffer under gravity flow
3. Transfer equilibrated WGA agarose into an empty 20mL glass column for chromatography (where the FRIT should substituted by an end restriction)

Preparation of the teflon tubing for packing

1. Connect the teflon tubing through the adaptors to HPLC
2. Wash with 70% (v/v) ethanol at 1ml/min, ~30ml
3. Equilibrate Teflon tubing with WGA buffer at 1ml/min, ~30ml
4. Attach on end of the teflon column downstream of the column containing the WGA slurry and the other end of the teflon column to an end restriction (this will restrict the passage of the WGA particles)

Packing the long WGA column

1. Attach the glass column containing the WGA slurry to the HPLC.
2. Start WGA buffer flow (0.15mL/min). The direction of flow should be from the pump to the WGA column and then the Teflon tubing. The buffer will wash the WGA resin into the Teflon tubing and pack the column. Ensure that no air is pumped into the Teflon tubbing.
3. When the packing is done, mount polyetheretherketone unions containing 0.5μm frits at the tubing ends to create a column.

WGA Isocratic HPLC

1. Load the sample dissolved in WGA buffer into a 100μL injection loop.
2. Inject the sample onto the column and run the column in WGA buffer at a flow rate of 0.15 mL/min. Monitor the pressure and UV absorbance. Peptides that do no contain tryptophan, tyrosine or phenylalanine will not absorb at 280nm, 214nm will detect the peptide bond as well as most salts.
3. Collect fractions (1 per minute) using a 96-well collection plate.

4. Pool together fractions, we suggest three fraction per pool. As the glycopeptides should elute last, start from the right shoulder of the major peak, thus creating fractions enriched for O-GlcNAcated peptides to work with.

   To calibrate your column, a glycopeptide control could be separated on the column first, such as the control used in the BEMAD method.

Sample clean-up prior to MS analysis

1. Acidify sample: Add formic acid to combined fractions to final concentration 0.2% (v/v). Check pH, pH should be <4.
2. Wet the reversed phase material: Rinse Zip Tip by pipetting 10μL of reagent B up and down. Repeat three times.
3. Equilibrate zip tip: Rinse Zip Tip by pipetting 10μL of reagent A up and down. Repeat ten times.
4. Load sample: Load sample onto the tip by repetitive pipetting (10 times).
5. Wash Zip Tip: Wash Zip Tip ten times by pipetting up and down with 10μL of reagent A.
6. Elute peptides: Elute peptides with 10μL of reagent B by repetitive pipetting 10 times. Repeat using 5μL of reagent B. Combine with prior eluted fraction.
7. Dry down eluted fractions in a speed vacuum concentrator.
8. Analyze by mass spectrometer of choice, preferably with an ECD or ETD source.

   Expected results*: O-GlcNAc modified peptides should be enriched, eluting from the column in the later fractions. Analysis by mass spectrometry should yield peptides that are* $(204.1950)_n$ *mass units greater than the mass of the unmodified peptide.*

Reuse and Storage of WGA Column

1. Wash the colum ith ~30ml of WGA storage buffer.
2. Seal column with Fit-flow stoppers on both ends of the column and store at +4oC.
3. Use the column in the reverse direction next time.
4. If the column performance decreases, regenerate the column with WGA column regeneration buffer at flow rate 0.15mL/min. Then wash the column with 30mL of WGA buffer at flow rate 0.15mL/min.

BASIC PROTOCOL 9 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, as they may aid in understanding the role of O-GlcNAc in your model of interest. 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; studies showing elevated activity of enzymes in certain tissue/cell lines and tissue fractions; and, finally, studies which have indicated that the enzymes responsible for the addition and removal of O-GlcNAc copurify with kinases and phosphatases.

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; Kreppel et al. 1997). OGT activity is assayed by determining the rate at which [^{3}H]GlcNAc is transferred to an acceptor peptide. A number of peptides have been identified as substrates for OGT in vitro, but a peptide (^{340}PGGSTPVSSANMM^{352}) from the α-subunit of casein kinase II (CKII) is the most efficient in vitro substrate known to date (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). For pure preparations 0.2 to 1μg is typically used per assay, in crude preparations 20 to 50μg, though the latter is precipitated using ammonium sulfate (0% to 30%).

Materials

0.1 mCi/ml UDP-[^{3}H]GlcNAc (20 to 45 Ci/mmol; NEN Life Science Products) in 70% ethanol

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

Nitrogen source

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

10× OGT assay buffer (see recipe)

CKII peptide substrate (^+H_2N-PGGSTPVSSANMM-COO^-): dissolve in H_2O to 10mM and adjust to pH7 if necessary

50 mM formic acid

Methanol (HPLC-grade)

Waters Sep-Pak C_{18} cartridges

Method

1. Dry down an aliquot of UDP-[^{3}H]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 0.02μCi to 0.1μCi/μl.

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

2. Set up assay reactions as follows:

   5 μL of 10× OGT assay buffer

   10 μL of 10 mM CKII peptide substrate

   5 μL of 0.02 to 0.1μCi/μl UDP-[^{3} H]GlcNAc (from 0.1 to 0.5μCi total)

   ≤25 μL of desalted OGT to be analyzed

   H_2O to 50 μL

   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-enzyme” control can be included.

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

3. Incubate at room temperature for 30min.

   An incubation time of 15 to 30min at room temperature is usually sufficient.

4. Stop reaction by adding 450μL of 50mM formic acid.

5. Wet a Waters Sep-Pak C_{18} cartridge with methanol, then wash the cartridge with 5mL H_2O.

   Note, for large numbers of samples we have also used C_{18} resin packed into 96 well plates (commercially available from Phenomonex). The method is similar to that described below.

6. Load the reaction (500μL total) onto the cartridge with a syringe. Wash with 5mL H_2O.

   The CKII peptide binds to the matrix of the C_{18} cartridge. Unincorporated UDP-[^{3}H]GlcNAc is eliminated by the wash.

7. Elute the peptide with 2 to 4mL methanol, directly into a 15mL liquid scintillation counter tube.

8. Add 10mL scintillation fluid and count ^{3}H. Calculate OGT activity according to the following equations.

$$\mu\text{Ci of GlcNAc incorporated} = (\text{dpm in sample} - \text{dpm in blank})/(2.22 \times 10^6\ \text{dpm} \cdot \mu\text{Ci}^{-1})$$
$$\text{mmol of GlcNAc incorporated} = (\mu\text{Ci of GlcNAc incorporated})/(\text{specific activity in } \mu\text{Ci/mmol})$$

   This number should be expressed in terms of mg of OGT or cell extract. If the assay is done at several time points, it can be expressed as mmol/min.

   The activity can be expressed either as dpm ^{3}H incorporation into the peptide, or as μmol ^{3}H incorporation (1 μCi = 2.22×10^6 dpm).

   Expected results*: In ammonium sulfate pellets from tissue extracts we typically obtain 600–6000 counts (above background) from 1μg of tissue in a 30min assay. Use of OGT inhibitors should block incorporation of ^{3}H GlcNAc into the CK2 peptide.*

SUPPORT PROTOCOL 5 DESALTING THE O-GlcNAc TRANSFERASE

OGT activity is sensitive to salt inhibition (IC_{50} = 40 to 50mM NaCl). It is important to desalt the enzyme preparation before assay if high concentration of salt is present.

Materials

Sephadex G-50 slurry (GE Healthcare)

OGT desalting buffer (see recipe)

Protein sample for OGT assay in volume ≤200μL

1-mL tuberculin syringe

1.5-mL tubes, prechilled

1. Pack a column containing exactly 1mL of Sephadex G-50 slurry in a 1-mL tuberculin syringe. Wash the column with 5mL of OGT desalting buffer.

Sephadex G-50 is usually supplied in 20% (v/v) ethanol, if not the resin can be swelled in 20% (v/v) ethanol for several hours.

2. Load protein sample onto column.

   The volume of sample can be up to 200μL.

3. Wash column with desalting buffer so that the total volume of this wash and the protein sample is 350μL. For example, if sample volume is 150μL, add 200μL desalting buffer to the column at this step.

4. Transfer the syringe column to a clean, prechilled 1.5-mL tube. Elute protein with 200μL desalting buffer. Keep on ice.

   This is the desalted sample.

   Alternatively, PD-10 desalting columns (GE Healthcare) can be used if O-GlcNAc transferase is in larger volume (1 to 2.5 mL).

BASIC PROTOCOL 10 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 synthetic substrate, *p*-nitrophenol N-acetylglucosaminide (*p*NP-β-GlcNAc). The cleavage product, *p*NP, has an absorbance peak at 400 nm.

Materials

Partially purified O-GlcNAcase (0.2 to 1 μg) or cell extract sample (20 to 50 μg, precipitated with

30% to 50% ammonium sulfate and desalted).

10× O-GlcNAcase assay buffer (see recipe)

100mM (50×) *p*-nitrophenol N-acetylglucosaminide (*p*NP-GlcNAc) in DMSO

500mM Na_2CO_3

96-well flat bottom plates or 1.5 mL microcentrifuge tubes

Plate reader or spectrophotometer

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 is not active in the assay in the presence of detergent (>0.1% v/v NP-40). Typically, the authors perform the O-GlcNAcase assay in nuclear/cytoplasmic extracts or precipitate O-GlcNAcase from total cell lysates with ammonium sulfate to remove detergent.*

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

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

   1 to 50μL partially purified O-GlcNAcase enzyme or cell extract

10μL 10× O-GlcNAcase assay buffer

2μL 100mM *p*NP-GlcNAc

H_2O to 100μL

The total reaction volume can be scaled up to 500 μL in microcentrifuge tubes. pNP-GlcNAc breaks down chemically. A blank reaction without enzyme should be included to determine the background.

50mM GalNAc 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 5 mM GalNAc.

4. Mix well and cover.

5. Incubate at 37°C for 30min to 4hr.

   Yellow color will develop as pNP-GlcNAc is hydrolyzed by O-GlcNAcase. Reactions should be optimized to keep the absorbance within the linear range of the spectrophotometer. The authors find that 20 to 50 μg of cell extract used in a reaction of 100μL, with a 1 to 2hr incubation time is appropriate.

6. At the end of incubation, add an equal volume of 500mM Na_2CO_3 to each well (100μL or 500μL).

   Na_2CO_3 raises the pH to >pH 9.0, intensifying the yellow color and stopping the reaction, as O-GlcNAcase has little activity at pH 9 to 10.

7. Read the absorbance at 400nm on a plate reader or spectrophotometer.

8. Calculate O-GlcNAcase activity according to the following equation:

$$\text{mM of GlcNAc released} = A_{400} / (17.4 \times 10\,\text{mM}^{-1} \cdot \text{cm}^{-1} \times \text{pathlength})$$

   One unit is the amount of enzyme catalyzing the release of 1 μmol/min of pNP from pNP-GlcNAc.

   The molar extinction coefficient for pNP is $17.4 \times 10\ \text{mM}^{-1} \cdot \text{cm}^{-1}$ at pH 10. The path length for 200μL on a 96-well plate is 0.71 cm.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see **APPENDIX 2E**; for suppliers, see **SUPPLIERSAPPENDIX**.

Biosynthetic labeling medium

Glucose-free culture medium containing:

50 μCi/ml D-[6-^{3}H]glucosamine (22 Ci/mmol; Amersham Pharmacia Biotech)

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, 2×

Dissolve 12.9 g citric acid monohydrate (mol. wt. 210) and 20.6 g disodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 6H_2O$) in 300 ml Milli-Q water. Bring volume to 500 ml.

Galactosyltransferase labeling buffer, 10×

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, 2×

Per reaction:

25 μl 2× 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

O-GlcNAcase assay buffer, 10×

500 mM sodium cacodylate, pH 6.4

500 mM N-acetylgalactosamine (GlcNAc)

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)

10 mM 1-amino-GlcNAc (2-acetamido-1-amino-1,2-dideoxy-β-D-glucopyranose; Sigma)

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) NaN_3

Store up to 1 week at 4°C

Protease inhibitors, 1000×

PIC 1, 1000×:

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

PMSF, 1000×:

0.1 M phenylmethylsulfonyl fluoride in 95% ethanol

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

TBST

10 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)

150 mM NaCl

0.05% (v/v) Tween 20

Store up to 1 month at room temperature

Tris-buffered saline (TBS)

10 mM Tris-Cl, pH 7.5 (*APPENDIX 2E*)

150 mM NaCl

Store up to 1 month at room temperature

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 (1984) first used this enzyme in combination with UDP-[^{3}H]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, βGal1-4βGlcNAc, 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).

In subsequent years, methods such as WGA affinity and western blotting 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 μM), and Thiamet G (1 μM). 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 showing 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 indicate 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 and Cunningham 1994; Shikhman and Cunningham 1994; Shikhman et al. 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 GlcNAc residue, and it is important to perform the described controls such as PNGase F digestion to show 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-Vera and Haltiwanger 1994).

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 unit 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.

Time Considerations

Detection of O-GlcNAc proteins using antibodies (see Basic Protocol 2) and lectins (see Basic Protocol 3) will take approximately 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 ITT 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. 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 to 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.

Enrichment and Site-Mapping using the BEMAD (Basic Protocol 7) and LWAC (Basic Protocol 8) techniques will require at least two weeks. For people who have note used these techniques before, it may be advisable to trial this method on control peptides/proteins.

OGT assays (Basic Protocol 9) are somewhat complicated and typically take two days. Notably, after the OGT assay is stopped that can be stored at −80°C for several weeks before desalting. O-GlcNAcase assays are straightforward and typically take 1 day.

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Table 1

Antbodies and Lectins that Recognize O-GlcNAc and GlcNAc

Name	Antibody Isotype	Recognizes	Commercially Available	Positive Control	Negative Control	Citation
CTD110.6	IgM	O-GlcNAc	Covance, Pierce, SIGMA-Aldrich, Cell Signaling, Santa Cruz Biotechnlogy	BSA-AP-GlcNAc	BSA-AP[†] 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	?	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	O-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)
6D93	IgG	O-GlcNAc	Santa Cruz Biotechnology	BSA-AP- GlcNAc	BSA-AP or Ovalbumin	Santa Cruz
10D8	IgM	O-GlcNAc	Santa Cruz Biotechnology	BSA-AP- GlcNAc	BSA-AP or Ovalbumin	Santa Cruz
HGAC 85	IgG	GlcNAc	Novus Biologicals, Abcam, Pierce, Enzo life sciences, Affinity Bioreagents	BSA-AP- GlcNAc, Ovalbumin	BSA-AP or BSA	(Turner et al. 1990)
HGAC 49	IgM	GlcNAc	No	BSA-AP- GlcNAc, Ovalbumin	BSA-AP or BSA	(Turner et al. 1990)
HGAC 39	IgG	GlcNAc	No	BSA-AP- GlcNAc, Ovalbumin	BSA-AP or BSA	(Turner et al. 1990)

[†]BSA-AP, BSA-aminophenol

[*]succinylation of WGA (sWGA) reduces it's affinity for Sialic Acid. Notably, it also reduces the affinity of sWGA for GlcNAc residues. As such, typically one use WGA for purification and sWGA for immunoblotting.

Table 12.8.1

Reaction Mixtures for Assay of Galactosyltransferase Activity

Sample	Dilution	Vol. (µl) of diluted sample	Vol. (µl) of 200 mM GlcNAc	Vol. (µl)10× Gal labeling buffer	Vol. (µl) 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