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Protein O-mannosylation in Metazoan Organisms

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Abstract

Protein O-mannosylation is a special type of glycosylation that plays prominent roles in metazoans, affecting development and physiology of the nervous system and muscles. A major biological effect of O-mannosylation concentrates on the regulation of α-Dystroglycan, a membrane glycoprotein mediating cell – extracellular matrix interactions. Genetic defects of O-mannosylation result in the loss of ligand binding activity of α-Dystroglycan and causes congenital muscular dystrophies termed dystroglycanopathies. Recent progress in mass spectrometry and *in vitro* analyses has shed new light the mechanism of α-Dystroglycan glycosylation. However, this mechanism is underlain by complex genetic and molecular regulation that remain poorly understood. Protein O-mannosylation is evolutionarily conserved in metazoans, yet this pathway is simplified and more amenable to genetic analyses in invertebrate organisms, indicating that genetically tractable *in vivo* models could facilitate research in this area. This review will describe recent methodological strategies for studying protein O-mannosylation using *in vitro* and *in vivo* approaches.

Introduction

Protein O-mannosylation is an evolutionarily conserved type of glycosylation found in a wide range of metazoans, from insects to humans. Despite the fact that O-mannosyl glycans appear to be present on a number of glycoproteins, until now α-Dystroglycan (α-Dg) represents the only well-studied functional target of this modification (Chiba et al., 1997; Stalnaker et al., 2011b). More recent research in vertebrate and invertebrate species has significantly expanded our knowledge of O-mannosyl glycan structures and glycoproteins bearing this unique type of glycosylation (Fig. 1). The presence of O-mannosylation has been demonstrated for *Drosophila* Dystroglycan and its glycosylation has been analyzed by *in vitro* and *in vivo* approaches (Nakamura et al., 2010b). O-mannose-linked structures have been reported on receptor tyrosine phosphatase β (RPTPβ) that mediates cell signaling and regulates neural cell adhesion and migration (Abbott et al., 2008). Other confirmed targets of this modification include CD24, a mouse GPI-linked cell adhesion molecule that functions in the nervous and immune systems (Bleckmann et al., 2009), neurofascin, a neuronal cell adhesion molecule involved in the nervous system development and neural transmission (Pacharra et al., 2012), and IgG2 light chain antibody recombinantly expressed in Chinese hamster ovary cell culture (Martinez et al., 2007). Previous studies suggested that other O-mannosylated glycoproteins likely exist (Finne et al., 1979; Krusius et al., 1986; Wing et al., 1992; Yuen et al., 1997). This conclusion is consistent with recent glycomic

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analyses that indicated that α-Dg represents just a minor component of the O-mannosylated glycoproteome of mouse brain (Stalnaker et al., 2011a). However, the structure-function relationship of O-mannosylation of proteins other than α-Dg remains unknown. On the other hand, O-mannosyl glycans of α-Dg have been analyzed in a number of studies (Chiba et al., 1997; Harrison et al., 2012; Inamori et al., 2012a; Nakamura et al., 2010b; Stalnaker et al., 2010; Yoshida-Moriguchi et al., 2010), and these glycans have been demonstrated to play crucial roles in the development and physiology of the nervous system and muscles (reviewed in (Muntoni et al., 2011; Nakamura et al., 2010a; Stalnaker et al., 2011b)). Recent research has also implicated O-mannosylation of α-Dg in pathobiological processes such as cancer progression and viral infection (de Bernabe et al., 2009; Kunz et al., 2005; Yoshida-Moriguchi et al., 2010).

Defects in the biosynthesis of O-mannosyl glycans are associated with a group of congenital muscular dystrophies (CMDs), such as Walker Warburg syndrome (WWS), Muscle-Eye-Brain disease (MEB), and Fukuyama congenital muscular dystrophy (FCMD) (Beltran-Valero de Bernabe et al., 2002; Kobayashi et al., 1998; van Reeuwijk et al., 2005; Yoshida et al., 2001). They all show various degrees of hypoglycosylation of α-Dg with concomitant loss of its ligand-binding activity (Fig. 2), and thus these syndromes are termed dystroglycanopathies (see more information about Muscular Dystrophy-Dystroglycanopathy, or MDDG, in OMIM 236670 (OMIM, 2012)). So far, eight genes have been shown to specifically affect the biosynthesis of O-mannosyl glycans of α-Dg and, when mutated, result in some form of MDDG. Two of these genes encode protein O-mannosyltransferases that initiate biosynthesis of O-mannosyl glycans on a protein backbone (POMT1 and POMT2) (Beltran-Valero de Bernabe et al., 2002; van Reeuwijk et al., 2005). Other genes encode glycosyltransferases, or glycosyltransferase-like proteins, that facilitate further elaboration of O-mannosyl glycans. They include β1,2-*N*-acetylglucosaminyltransferase 1 that elongates O-mannose with N-acetylglucosamine (POMGnT1) (Yoshida et al., 2001) and LARGE (aka LARGE1), a bifunctional glycosyltransferase with xylosyltransferase and glucuronyltransferase activities (Inamori et al., 2012a; Longman et al., 2003). LARGE synthesizes a carbohydrate chain of disaccharide repeats attached to O-mannose presumably via an unusual phosphate modification at the mannose 6-position, and this structure is essential for binding between α-Dg and its ligand Laminin (Barresi et al., 2004; Inamori et al., 2012a) (Fig. 2). Similar enzymatic activity has been demonstrated for LARGE2, a paralog of LARGE (Brockington et al., 2005; Fujimura et al., 2005; Grewal et al., 2005; Inamori et al., 2012b). Interestingly, LARGE2 has different pH optima of enzymatic activity and a distinct tissue-specific expression, suggesting that LARGE and LARGE2 may have complementary *in vivo* functions (Inamori et al., 2012b). Fukutin and Fukutin-related protein represent two glycosyltransferase-like proteins with LicD-domains with unknown activities that also affect the biosynthesis of highly glycosylated active form of α-Dg (Brockington et al., 2001b; Kobayashi et al., 1998). These proteins presumably function in the same pathway as LARGE, and the recent mouse Fukutin knockout revealed that the phosphate-linked carbohydrate modification of O-mannose required for Laminin binding is compromised in Fukutin mutants, while the phosphate modification of O-mannose itself remains intact (Beedle et al., 2012). Finally, ISPD (isoprenoid synthase domain containing) and GTDC2 (glycosyltransferase-like domain-containing protein 2) represent the two most recent additions to the group of genes associated with dystroglycanopathies, however the mechanism of their involvement in α-Dg glycosylation is still unknown (Manzini et al., 2012; Willer et al., 2012). In addition to the eight genes discussed above, mutations in dolichol-phosphate mannose synthase genes DPM2 and DPM3 have been implicated in MDDG (Barone et al., 2012; Lefeber et al., 2009). These enzymes are involved in the biosynthesis of dolichol-phosphate mannose (Dol-P-Man), a sugar donor substrate used in O-mannosylation and several other glycosylation

pathways. Thus, DPM2 and DPM3 are expected to have pleiotropic effects on glycosylation that are not limited to protein O-mannosylation pathway.

Metazoan protein O-mannosyltransferases (POMTs) show evolutionary relationship to the multigene family of yeast O-mannosyltransferases (PMTs) that modify numerous secreted and cell wall proteins in fungi (Lommel and Strahl, 2009). Unlike fungi, animal organisms have only two O-mannosyltransferases that evolved to modify a relatively small number of glycoproteins, including Dystroglycan which is also conserved in metazoans. Invertebrate species have two protein O-mannosyltransferases highly homologous to their mammalian counterparts (Ichimiya et al., 2004; Lyalin et al., 2006; Nakamura et al., 2010b). However, invertebrates appear to be missing POMGnT1 and therefore their O-mannosyl glycans have simplified structures (Nakamura et al., 2010a). This was confirmed by mass spectrometry analysis of glycans of *Drosophila* Dg that revealed the presence of non-elongated O-mannose within the mucin-like domain (Nakamura et al., 2010b). Whether invertebrates have a glycan structure analogous to the one generated by LARGE is still unclear, while genetic evidence suggests this possibility since homologues of LARGE and/or FKRP are present in insect genomes (Brockington et al., 2001a; Grewal et al., 2005). Importantly, mutations in *Drosophila* POMTs and Dg result in muscle developmental defects, abnormal synaptic transmission and age-dependent muscle degeneration (Haines et al., 2007; Lyalin et al., 2006; Shcherbata et al., 2007; Wairkar et al., 2008), the phenotypes related to some important clinical findings associated with dystroglycanopathies (discussed in (Nakamura et al., 2010a)). Thus, *Drosophila* represents an attractive model organism for studying evolutionarily conserved mechanisms underlying the function of O-mannosylation in the nervous system and muscles of mammals.

In *Drosophila*, *rotated abdomen (rt)* and *twisted (tw)* genes encode protein O-mannosyltransferase enzymes POMT1 (aka RT) and POMT2 (aka TW), respectively (Ichimiya et al., 2004; Lyalin et al., 2006). It has been demonstrated that POMT1-POMT2 activity towards Dystroglycan is conserved between invertebrates and mammals, since *Drosophila* RT and TW can modify *Drosophila* DG *in vitro* and *in vivo*, and they can also use mammalian α-DG as an *in vitro* substrate (Ichimiya et al., 2004; Nakamura et al., 2010b). POMT1 and POMT2 function as an enzymatic heterocomplex, they co-localize within the ER, can be co-immunoprecipitated, and their co-expression is a prerequisite for protein O-mannosyltransferase activity *in vitro* and *in vivo* (Akasaka-Manya et al., 2006; Ichimiya et al., 2004; Lyalin et al., 2006; Manya et al., 2004; Nakamura et al., 2010b). The substrate specificity of POMT enzymes has been analyzed *in vitro* with polypeptide fragments of mammalian and *Drosophila* Dg recombinantly expressed in bacteria, or using short synthetic peptides corresponding to the mucin-type domain of α-DG (Ichimiya et al., 2004; Manya et al., 2004; Manya et al., 2007; Nakamura et al., 2010b). As a source of O-mannosyltransferase activity, these assays used membrane microsomal fraction prepared from mammalian or *Drosophila* cells. However, the substrate specificity revealed by these *in vitro* experiments is not consistent with the results of *in cellulo* assays that employed transgenic co-expression of POMTs together with their peptide substrates in cultured cells, followed by purification and analyses of O-mannosylated substrates (Breloy et al., 2008). The later approach indicated a potential requirement for some more distant structural determinants upstream of O-mannose attachment sites (Breloy et al., 2008). Mass spectrometry analysis of the whole extracellular domain of *Drosophila* Dg transgenically expressed in flies and modified *in vivo* by RT and TW unveiled some O-mannose-modified sequences that do not conform to the substrate preferences of mammalian POMTs. These experiments also discovered the presence of O-mannose outside of the mucin-type domain and revealed a surprising abundance of O-mannosyl glycans within the mucin-type domain of Dg (Nakamura et al., 2009). Thus, the acceptor specificity of O-mannosyltransferases appears to be complex and regulated by some yet to be identified factors. This specificity is

not determined solely by a consensus sequence at modification sites but also appears to depend on some properties of the acceptor on a larger structural scale (Nakamura et al., 2010a). A similar feature of substrate recognition was shown for yeast PMT enzymes (Hutzler et al., 2007), which suggests that the mechanisms of O-mannosylation may be conserved within a broader family of eukaryotic O-mannosyltransferases. Interestingly, the analysis of *Drosophila* and mammalian substrates revealed that O-mannose can modify the same sites that can be used by mucin-type O-GalNAc modification (Nakamura et al., 2010b; Stalnaker et al., 2010). Since protein O-mannosylation occurs in the ER, while O-GalNAc is attached later, in the Golgi compartment of secretory pathway, this suggests that O-mannosylation could compete with O-GalNAc and therefore regulate the mucin-type glycosylation of protein substrates. This scenario indicates an intriguing possibility that some phenotypes of POMT1/2 mutations may be due to ectopic O-GalNAc modifications of Dg (Tran et al., 2012).

Below we will review the protocols developed for the analysis of O-mannosylation in animal cells using *in vitro* and *in vivo* approaches. We will discuss an *in vitro* assay for protein O-mannosyltransferase activity using purified fragments of Dystroglycan mucin-type domain as an acceptor and microsomal membrane fraction from cells co-expressing POMT1 and POMT2 as a source of enzymatic activity (Basic Protocol 1 with Support Protocols 1.1–3). We will also describe how to express *in vivo* and purify *Drosophila* Dystroglycan, and how to analyze its O-mannosylation by treatment with glycosidases, and using lectin and western blots (Basic and Alternate Protocols 2 with Support Protocols 2.1). In addition, we will review O-linked glycomics strategies that are based on mass spectrometry approaches and used to decipher the structure of O-mannosyl glycans (glycan composition analysis) and their location within polypeptide chains (site-mapping) (Protocols 3–6).

Basic Protocol 1: *In vitro* assay for O-mannosyltransferase activity

The assay can be used to study the enzymatic activity of protein O-mannosyltransferases *in vitro* (Ichimiya et al., 2004; Manya et al., 2008; Manya et al., 2004; Nakamura et al., 2010b). It is useful for testing different proteins acceptors as targets of O-mannosylation (Nakamura et al., 2010b), which can also reveal structural determinants in acceptor recognition (Manya et al., 2007). The *in vitro* O-mannosylation reaction will generate polypeptides with O-linked mannose that can be useful in further experiments, e.g. in the analysis of other enzymes mediating downstream steps in the biosynthesis of O-mannosyl glycan structures (Fig. 1). In the assay reaction, POMT1-POMT2 complex mediates the transfer of Man from sugar donor Dol-P-Man to a peptide acceptor (Endo and Manya, 2006). As an acceptor substrate, non-glycosylated polypeptides corresponding to mucin-type domain of Dg can be used, for example, a Dg fragment recombinantly produced in bacterial cells, eukaryotic cells lacking O-mannosyltransferase activity, or synthesized *in vitro*. As a source of O-mannosyltransferase activity, the assay uses microsomal membrane fraction prepared from cells co-expressing POMT1 and POMT2 (Support Protocol 1.1 and 1.2). Dol-P-Man with radioactively labeled mannose (e.g., [^{3}H]Man) is used as a sugar donor to facilitate the detection of mannose transferred onto the acceptor. Below we include the protocol of protein O-mannosyltransferase assay that can be used with microsomal membrane fraction containing RT and TW (see Support Protocols 1.2–1.3 for expression of RT/TW and microsomal fraction purification), or other POMT1-POMT2.

Materials

Recombinant Dg-GST protein expressed and purified from *E. coli* (see Support Protocol 1.1)

Microsomal membrane fraction with POMT1 & POMT2 expression (see Support Protocols 1.2 and 1.3)

Dolichol phosphate-activated [^{3}H]mannose (Dol-P-[^{3}H]Man, 100 μCi/ml, 60 Ci/mmol, ARC)

Assay Buffer (see Reagents and Solutions)

PBS

Triton X-100

Scintillation counter, scintillation liquid and vials

Protocol Steps

1. Take an aliquot of [^{3}H] Dol-P-Man sufficient for assays to be performed, e.g. 4 pmol per reaction, or 2.5 μl of the manufacturer's stock solution of 100μCi/ml, 60 Ci/mmol Dol-P-[^{3}H]Man in methanol:chlorophorm. Dry under a gentle stream of N_2 at room temperature. Dissolve in the assay buffer (6 μl of assay buffer per 4 pmol of [^{3}H] Dol-P-Man) by brief sonication using an inverted tip filled with ice-cold water.
2. Prepare the assay reactions on ice. For each reaction, mix the following ingredients dissolved in Assay Buffer: 10 μg of protein acceptor (Dg-GST fragment or BSA as a negative control) in 8 μl, 4 pmol [^{3}H] Dol-P-Man dissolved in 6 μl, 80 μg of microsomal membrane fraction in 6 μl.
3. Incubate at 25°C for 1 hour.
4. Stop the assay reaction by adding 200 μl of PBS with 1% Triton X-100. Mix well by flicking the tube.
5. Remove insoluble material from the reaction mixtures by centrifugation at 10,000 g for 10 min at 4°C.
6. During the previous step, prepare GST-affinity beads by rinsing four times in PBS with 0.5% Triton X-100. Prepare 20 μl of beads per each assay reaction.
7. Load the pre-cleared supernatants from step 5 to pre-washed GST-beads, incubate with gentle nutation at 4°C for 1 hour.
8. Wash beads three times with 1 ml of PBS containing 0.5% Triton X-100.
9. Measure the incorporation of [^{3}H] Man in the substrate trapped on GST-affinity beads using a scintillation counter.

Support Protocol 1.1: Expression in *E. coli* and purification of O-mannosyltransferase substrates

E. coli cells do not have protein O-mannosyltransferase activity, hence they can be conveniently used for the recombinant expression of protein substrates without O-mannose modifications. Several protein acceptors have been used in O-mannosyltransferase assays with *Drosophila* RT/TW enzymes; they all represent polypeptide fragments of mucin-type domains of mammalian or *Drosophila* Dg (Ichimiya et al., 2004; Nakamura et al., 2010b). Although currently there is no reliable algorithm to predict the location of O-mannosylation sites within a protein sequence, there is an apparent correlation between the presence of O-mannosylation and increased probability of multiple O-GalNAc sites within the mucin-type domain of *Drosophila* Dg, as predicted by NetOGlyc program (www.cbs.dtu.dk/services/netoglyc (Julenius et al., 2005)). Thus, this prediction can be potentially useful when

designing expression constructs for the production of O-mannosyltransferase substrates based on Dg protein sequence (Nakamura et al., 2010b). So far, the *in vitro* O-mannosylation of other substrates has not been reported. The Dg fragments are expressed for as GST-tagged polypeptides, which facilitates their expression in a soluble form and purification using GST-affinity beads. The GST tag of a protein substrate is also exploited during *in vitro* O-mannosylation assay for the separation of [^{3}H]Man-modified protein acceptor from non-incorporated radioactive sugar.

Materials

E.coli cells trasformed with *pET-41-Dg-GST* construct (e.g. using electroporation protocol (Sharma and Schimke, 1996)).

GST-affinity beads, e.g. glutathione agarose resin from Pierce (Thermo 15160)

Spectrophotometer for measuring bacterial cell density

LB medium for bacterial culture

IPTG

Wash Buffer, Elution Buffer, Dialysis Buffer (see Reagents and Solutions for composition)

Sonicator

0.45 micron low protein-binding syringe filter (Millipore)

Spectra/Por dialysis membrane, MWCO 15,000

Millipore centrifugal concentrators, MWCO 15,000

SDS-PAGE and Coomassie staining reagents

Protocol Steps

1. Incubate E. coli BL21(D3) cells transformed with *pET-41-Dg-GST* expression vector in 200 ml of LB on a shaker with vigorous agitation at 37°C overnight.
2. Induce the expression of Dg-GST with 0.4 mM IPTG when cells reach the density of OD_{600}=0.6.
3. Continue incubation on a shaker at 28°C for 18 hours.
4. Harvest cells by centrifugation at 10,000 g, 4°C for 5 min. Decant the supernatant and resuspend the cell pellet in 25 ml ice-cold Wash Buffer (PBS, 0.1% NP-40, pH 7.5) with freshly added 0.1 % PMSF.
5. Lyse cells by sonication in 50 ml Falcon tubes on ice, applying 6 pulses of 20 sec sonication.
6. Complete cell lysis by incubating cells with gentle nutation for 20 min at +4°C.
7. Pre-clear the cell lysate by centrifugation at 14,000 g, 4°C for 15 min, followed by filtering the supernatant using 0.45 micron low protein-binding filter.
8. During the previous centrifugation step, prepare 200 μl GST-affinity beads by rinsing them four times in Wash Buffer.
9. Add GST-affinity beads to pre-cleared supernatant from step 7 and incubated overnight on a nutator at 4°C.

10. Assemble a mini-column using a pipette tip plugged with glass fiber. Apply the bead slurry from step 8 to the column and let it run through.
11. Wash the beads trapped in the column with 2 ml of Wash Buffer.
12. Wash once with 2 ml of PBS + 0.1% PMSF
13. Elute Dg-GST polypeptides with 0.5 ml of Elution Buffer (100 mM glutathione in 0.5M Tris pH 8.0): load the buffer onto the column, stop the flow, incubate column for 10 min, and collect the flow through. Repeat this elution step 2 times. Add collected fractions together to obtain 1.5 ml total volume of eluted protein in Elution Buffer.
14. Dialyze using Spectra/Por dialysis membrane (Spectrum, MWCO 15,000) in 2 L of Dialysis Buffer (20 mM Tris, 2 mM EDTA, 0.1% PMSF, pH 8) at 4°C for 4 hours. Repeat the dialysis 3 times.
15. Concentrate samples using Millipore centrifugal filters, e.g. with 15 kDa membrane cut-off filters, centrifuge at 12,000 g, 4°C for 20 min.
16. Analyze sample concentration and purity by PAGE and Coomassie staining. Normally, a 1/10 – 1/20 aliquot of the sample is sufficient for the analysis.
17. Store samples 4°C. For a long-term storage, the samples can be quickly frozen using liquid nitrogen or an ethanol-dry ice bath. Store samples at −80°C and avoid repeated freezing and thawing.

Support Protocol 1.2: Expression of *Drosophila* O-mannosyltransferases and Dystroglycan *in vivo*

The purpose of this procedure is to ectopically express proteins of interest (*Drosophila* O-mannosyltransferases RT and TW, or Dg) *in vivo* in flies. *Drosophila* tissue with expressed proteins can be used for the isolation of microsomal membrane fraction with O-mannosyltransferase activity (in case of co-expression of RT and TW), or for the purification of Dg protein for further analysis of its glycosylation (in case of Dg expression). *Drosophila* DG protein and protein O-mannosyltransferase enzymes RT (POMT1) and TW (POMT2) can be expressed *in vivo* using UAS-GAL4 expression system (Brand et al., 1994). To this end, the *pUAST* vector is used to make an expression construct that includes a protein coding sequence of interest, followed by an SV40 termination signal and placed under the control a minimal heat-shock promoter and five upstream-located copies of UAS sequence for binding of GAL4 transcriptional activator (Brand et al., 1994). This *pUAST*-based expression construct is assembled *in vitro* using molecular cloning techniques, verified by sequencing, and then inserted into *Drosophila* genome using embryo injection and P element-mediated transformation (Spradling and Rubin, 1982). Once the stable transgenic *Drosophila* lines carrying the constructs of interest are created using simple genetic manipulations, they are used for *in vivo* expression of corresponding proteins by crossing to a line carrying GAL4 driver with a particular temporal and cell-specific pattern of expression. Large collections of strains with different GAL4 drivers are available from several public *Drosophila* Stock collections (e.g., Bloomington Stock Center <http://flystocks.bio.indiana.edu/> or Kyoto *Drosophila* Genetic Resource Center <http://www.dgrc.kit.ac.jp/en/index.html>).

Materials

Transgenic *Drosophila* strains carrying *UAS*-constructs of interest, e.g. *UAS-RT*, *UAS-TW*, and *UAS-ExDg-FLAG* strains (available upon request from V.M.P.) (Lyalin et al., 2006; Nakamura et al., 2010b).

Materials and reagents for fly rearing, such as fly vials, food, environmental incubator for maintaining fly stocks at 25°C, 40% humidity (e.g., Percival DR36NL) (Roberts, 1998).

Microscope station (e.g., using Nikon SMZ645 microscope) with CO_2 diffusion pads for fly sorting

PBS and 70% ethanol-water solutions

Glass dissection trays (e.g. Corning 7223-34)

Ethanol-dry ice or liquid N_2 bath

Protocol Steps

1. Amplify stable transgenic *Drosophila* strains carrying UAS-based constructs of interest. Depending on the scale of the experiment, flies can be reared in population bottles having 25–30 ml of food and accommodating about 40–60 flies, or in fly vials with 7–10 ml of food and accommodating 10–20 flies.

   Note: When selecting lines for in vivo expression, preferences should be given to the strains that can be propagated as homozygous genotypes. If homozygous-viable strains are not available, a balancer chromosome with a genetic marker suitable for the selection of desired genotypes among F1 generation can be used (e.g., TM6, Tb balancer can be conveniently used to select non-Tb larvae without TM6 chromosome).

2. Using the amplified strains, collect males and virgin females to set up crosses in fresh vials or bottles using equal number of males and females. To ensure that all collected females are virgins, all adult flies are removed from the bottles or vials with parental populations, and then newly hatched flies are collected every 8 hours at 25°C. Reciprocal crosses can be set up for transgenic lines carrying autosomal transgenes and GAL4 drivers. For UAS transgenes or GAL4 drivers located on the X chromosome, it is preferable to use females for the crosses in order to ensure that all progeny will inherit the chromosome of interest.

3. Transfer parents to new vials or bottles with fresh food every 3–5 days to maintain the crossed flies in a healthy environment, maximize the yield and prevent overcrowding of progeny population.

4. Collect the F1 progeny of desired genotype and developmental stage using genetic and morphological markers, e.g. use *Tb* marker to select for flies without a *TM6, Tb* balancer chromosome, or use the size of the larvae and spiracle morphology to discriminate between instar stages.

5. Rinse collected *Drosophila* larvae two times in ice-cold 25% sucrose in dissecting trays to remove residual food, then they rinsed twice in ice-cold PBS. Aspirate PBS from dissecting trays with a Pasteur pipette. Residual PBS is removed by gently blotting with Kimwipe paper, and then larvae are transferred to 1.5 ml eppendorf tubes on ice. When collecting adults, flies are immobilized using CO_2, transferred to dissection trays on ice and rinsed with cold PBS, flowed by a brief rinse with ice-cold 70% ethanol. The ethanol solution is quickly aspirated from the flies using Pasteur pipette, and flies are rinsed one more time with ice-cold PBS. After removing residual PBS with Kimwipe paper, flies are transferred to eppendorf tubes on ice.

6. Immediately proceed with homogenization of collected *Drosophila* to isolate microsomal membrane fractions with protein O-mannosyltransferase activity for *in*

vitro assays (Support Protocol 1.3). An aliquot of tissue lysate can be saved for a western blot to analyze the amount of expressed proteins.

7. For the analysis of *in vivo*-expressed DG proteins, collected *Drosophila* can be immediately homogenized for protein purification (Support protocol 1.4). Alternatively, the samples can be quickly frozen using liquid nitrogen or an ethanol-dry ice bath and stored at −80°C for later experiments.

Support Protocol 1.3: Purification of microsomal fractions with O-mannosyltransferase activity

Microsomal membrane fraction can be purified as a source of protein O-mannosyltransferase activity for *in vitro* assays. Since POMT1 and POMT2 proteins are required to be simultaneously present within the cell in order to form enzymatically active complexes, their co-expression is a prerequisite for isolation of O-mannosyltransferase activity. The microsomal membrane fraction with protein O-mannosyltransferase activity has been purified from cultured mammalian cells with transgenically co-expressed or endogenous POMTs, from wildtype *Drosophila* tissues with endogenous RT and TW proteins, and from *Drosophila* transgenic strains with ectopic co-expression of RT and TW (see Support Protocol 1.2) (Ichimiya et al., 2004; Manya et al., 2008; Manya et al., 2004; Nakamura et al., 2010b). Ectopic co-expression of POMTs provides a way to increase the enzymatic activity of purified microsomal fractions. We include below a protocol for purification of the microsomal membrane fraction with O-mannosyltransferase activity from *Drosophila* larvae.

Materials

Homogenization Buffer, Assay Buffer (see Reagents and Solutions for composition)

Live *Drosophila* 3rd instar larvae (see Support Protocol 1.2)

Sonicator (e.g., Branson Sonifier 150) with microtip

Centrifuges: Eppendorf 5417R for low-speed centrifugation, and Beckman TL100

Ultracentrifuge with TLA 100.3 rotor for high-speed centrifugation

Bradford assay reagent (e.g., Sigma B6916)

Spectrophotometer with cuvettes for Bradford assay

Protocol Steps

Note: Protein O-mannosyltransferase activity of microsomal membrane fraction is not stable in vitro at room temperature, and precautions should be taken to maintain samples at 4°C during all purification steps.

1. Homogenize 3rd instar larvae in Homogenization Buffer using a glass Dounce homogenizer on ice. Use 10–15 μl of Homogenization Buffer per one larva. Approximately 40 larvae are sufficient for one experiment.
2. Sonicate on ice for 20 sec in an eppendorf tube at power level 2 using Branson Sonifier 150 with microtip. Repeat four times with 40 sec intermittent incubation on ice to prevent overheating of the sample.
3. Incubate with gentle nutation at 4°C for 20 min to complete tissue lysis.

4. Pre-clear the lysate by removing tissue debris with centrifugation at 800 g, 4°C for 10 min. Discard pellet, transfer supernatant to centrifuge tubes for high-speed centrifugation (13×15 mm tubes for Beckman TLA100.3 rotor)
5. Centrifuge at 100,000 g, 4°C in Beckman TL100 Ultracentrifuge using TLA 100.3 rotor for 1 hour.
6. Discard supernatant, resuspend the pellet representing membrane microsomal fraction in 20–40 μl of ice-cold Assay Buffer.
7. Estimate total protein concentration in the membrane microsomal fraction using Bradford assay (Sigma B6916). Use the microsomal fraction in *in vitro* assays as a source of protein O-mannosyltransferase activity within few hours.

Basic Protocol 2: Analysis of *Drosophila* Dystroglycan O-mannosylation using lectin blots

Mammalian α-Dg is a highly glycosylated protein bearing a variety of complex carbohydrate structures, including of N- and O-linked modifications (Chiba et al., 1997; Inamori et al., 2012a; Nakamura et al., 2010a; Yoshida-Moriguchi et al., 2010). Thus, a direct analysis of α-Dg glycosylation by lectins is usually not very informative. On the other hand, *Drosophila* Dg has fewer glycans, and their structures are less complicated (Nakamura et al., 2010a). *Drosophila* Dg or its extracellular domain, ExDg, can be expressed *in vivo* as a FLAG-tagged protein, purified, and its glycans can be analyzed by lectins (Nakamura et al., 2010b). In order to analyze O-linked structures attached to a protein, N-glycans can be removed prior to this analysis by PNGase F treatment to avoid their potential interactions with lectins. The specificity of lectin staining should be confirmed by a separate control experiment including incubation with the lectin in the presence of inhibiting sugar (e.g., using 0.2 M of Methyl α-D-Mannopyranoside, GalNAc or Gal for ConA, VVA or PNA, respectively). Ideally, all steps of the control and the main experiments should be carried out in parallel. The analysis of *Drosophila* Dg glycosylation can be performed for different mutant backgrounds, which can shed light on the involvement of other genes in the regulation of Dg glycosylation and function.

Materials

Purified ExDg-FLAG protein (see Support Protocol 2.1)

PNGase F glycosidase supplied with 10x denaturing, 10x G7 buffers (NEB P0704S)

Reagents and instrumentation for SDS-PAGE and western blot (e.g., from Bio-Rad)

BSA Fraction V (Roche)

TBST buffer (see Reagents and Solutions for composition)

Biotinylated lectins (e.g., ConA, VVA and PNA, Vector Laboratories)

Inhibiting sugars for sugar competition controls (e.g., α-methyl mannoside, GalNAc or Gal for ConA, VVA, PNA, respectively)

Instrumentation for chemiluminescent western blot analyses (e.g., ChemiDoc XRS system with Quantity One software, Bio-Rad).

Protocol steps

1. Remove N-linked glycans by incubating ExDg-FLAG protein purified on FLAG-affinity beads (see Support Protocol 2.1) with the PNGase F glycosidase. Use 500 units of PNGase F in a volume of 30μl of 1x G7 buffer per 2–3 μl of FLAG beads

(corresponding to ExDg purified from 3–4 larvae). Incubate at 37°C with gentle agitation for 1 hour. As a control, set up in parallel a mock incubation reaction without adding the enzyme.

2. Release purified ExDg-FLAG from FLAG-affinity beads by incubating beads in SDS-PAGE lading buffer at 95°C for 5 min.
3. Cool briefly on ice and separate samples on 5% SDS-PAGE gel. Use the amount of sample corresponding to approximately 1–2 larvae per one lane.
4. Transfer gel-separated samples to nitrocellulose membrane.
5. Block the membrane with 2% BSA (Fraction V, Roche) in TBST (10mM Tris-HCl pH8.0, 150mM NaCl, 0.05% Tween 20) with gentle agitation at room temperature for 30 min.
6. Incubate the membrane with 2.5μg/ml of a biotinylated lectin at room temperature for 1 hour. For instance, use Concanavalin A for α-mannose-containing structures, VVA for α-GalNAc (the Tn antigen), and PNA for Galβ1,3GalNAc (the T-antigen) (Vector Laboratories).
7. Carry out the detection of lectin binding using Vectastain ABC kit with chemiluminescent detection (Vector).
8. Quantify blots using an instrument for western blot analysis (e.g., ChemiDoc XRS system, Bio-Rad).

Alternate Protocol 2: Analysis of *Drosophila* Dystroglycan O-mannosylation using glycosidase treatment and western blots

In addition to lectin blots (Basic Protocol 2), treatments by glycosidases with known specificities can also reveal the structure of glycans attached to ExDg. Glycosidase incubation time varies significantly for different glycosidases. Some glycosidase are highly active towards their substrates, and treatments with them require only ~1-hour incubation (e.g., PNGase F treatment, step 1 of this Protocol and Basic Protocol 2). The removal of O-linked mannose with Jack bean α-mannosidase can take up to 5–8 hours because the activity of this enzyme towards O-mannose is relatively low (step 3a of this Protocol). The removal of a specific sugar structure by glycosidase treatment leads to a change in molecular mass of the glycoprotein and thus can be detected by SDS-PAGE and western blot (e.g., (Nakamura et al., 2010b)). The analysis is facilitated by the presence of FLAG tag that provides a sensitive way of ExDg detection using western blot.

Materials

Purified ExDg-FLAG protein (see Support Protocol 2.1)

Glycosidase with a specific activity (e.g., α-mannosidase from Jack beans, Sigma)

Reagents and instrumentation for SDS-PAGE and western blot (e.g., from Bio-Rad)

BSA Fraction V (Roche)

TBST Buffer (see Reagents and Solutions for composition)

Mouse anti-FLAG M2 antibody (Sigma)

Anti-mouse HRP-conjugated secondary antibody (e.g., from Jackson ImmunoResearch Labs, catalog #11-035-003)

Pierce SuperSignal WestPico Chemiluminescent Substrate kit (Pierce).

Instrumentation for chemiluminescent western blot analyses (e.g., ChemiDoc XRS system with Quantity One software, Bio-Rad).

Protocol Steps

1 Perform PNGase F –mediated release of N-linked glycans as described in step 1 of Basic Protocol 2.

2 Rinse beads with 50μl of 1x G2 buffer (50mM sodium citrate pH4.5, NEB): add G2 buffer, mix beads by flicking the tube several times, separate beads by a brief centrifugation (800 g, 20 sec). Repeat twice.

3a Treat beads with 0.4 units of α-mannosidase from Jack beans (Sigma) in 30μl of 1x G2 buffer for 3 hr at 37°C.

3b As a control, set up a mock incubation without α-mannosidase.

4 Stop the reactions by adding 40μl of 2x SDS loading buffer.

5 Analyze the samples by western blot using standard protocol. Use anti-FLAG M2 as primary antibody at 1:4,000 dilution. Carry out detection using HRP-conjugated anti-mouse secondary antibody and chemiluminescent detection with Super Signal West Pico Chemiluminescent Substrate kit (Thermo Scientific).

6 Quantify blots using an instrument for western blot analysis (e.g., ChemiDoc XRS system with Quantity One software, Bio-Rad).

Support Protocol 2.1: Purification of *in vivo*-expressed *Drosophila* Dystroglycan using affinity beads

Drosophila model system offers a convenient possibility of *in vivo* expression of transgenic constructs in various cells and at different development stages using UAS-GAL4 ectopic expression system (Brand et al., 1994). Extracellular part of Dg can be expressed as a FLAG-tagged protein (ExDg-FLAG) while retaining its activity *in vivo* (Nakamura et al., 2010b). This secreted version of Dg can be purified using FLAG-affinity beads for the analysis of glycosylation by lectin or western blots (Protocol 2 and Alternate Protocol 2). While approximately 20 *Drosophila* larvae or pupae can be sufficient for a small-scale purification, the purification protocol can be scaled up for larger experiments.

Materials

Collected *Drosophila* with transgenically expressed ExDg-FLAG (see Support Protocol 1.2)

Glass Dounce homogenizer

Anti-FLAG M2 Affinity Gel (Sigma A2220)

Lysis Buffer (see Reagents and Solutions for composition)

Centrifuge Eppendorf 5417R

Protocol Steps

1. Homogenize collected *Drosophila* with transgenically expressed ExDg-FLAG (see Support Protocol 1.2) using a glass Dounce homogenizer on ice. Use 10–15 μl of Lysis Buffer per one larva or pupa.

2. Incubate with nutation at 4°C for 20 min to complete tissue lysis.

3. Remove insoluble material by centrifugation at 18,000 g, 4°C for 20 min.

4. During the previous centrifugation step, prepare FLAG affinity beads by rinsing them four times in Lysis Buffer.

5. Add the supernatant from the centrifugation step 3 to the FLAG beads. Use 10 μl of beads per volume of cell lysate corresponding to 20 larvae. Incubate on a nutator at 4°C for 2–4 hours.

   Separate the beads from supernatant by low-speed brief centrifugation (800 g, 20 sec). Remove supernatant, resuspend beads in fresh ice-cold Lysis Buffer. Use 20–50 volumes of Lysis Buffer per one volume of beads. Incubate on a nutator at 4°C for 5 min. Repeat this centrifugation-washing step 3 more times. Purified ExDg bound to beads can be directly used in later assays. The purified sample can be stored at 4°C in the Lysis Buffer with Triton X-100 concentration decreased to 0.05% (150 mM NaCl, 50 mM Tris pH 7.4, 0.05% Triton X-100) for several weeks.

Basic Protocol 3: O-linked Glycomics (release, permethylation)

Mass spectrometry-based analyses of released and permethylated glycans have been previously described and allow for an in-depth characterization of the O-glycome from a variety of sources (Aoki et al., 2008; Haslam et al., 2006; Jang-Lee et al., 2007; Wada et al., 2010). The method relies on organic extraction and precipitation of all proteins from a biological source followed by release of the O-linked glycans via reductive beta-elimination. The resulting free glycans are then permethylated and analyzed via mass spectrometry. Analysis via direct infusion of permethylated glycans into a tandem mass spectrometer for MS^n analysis is a powerful approach for detailed structural analysis but other approaches including MALDI-TOF and LC-MS/MS techniques can also be performed (Anumula and Taylor, 1992; Haslam et al., 2006; Jiao et al., 2011).

Materials

Intact isolated proteins (or proteolytic peptides).

Sodium Hydroxide

Sodium borohydride

Glacial acetic acid (AcOH)

Anhydrous dimethyl sulfoxide (dry DMSO)

Anhydrous methanol (dry MeOH)

Prepared base (see Support Protocol 3.2)

50% w/w sodium hydroxide solution

Iodomethane (MeI)

Nanopure water

Methylene chloride (DCM)

Screwtop tubes with, teflon lined caps, glass syringes, pasteur pipettes, and pipette bulbs

Centrifuge

Concentrator – attached to N_2 gas to dry down sample

Nitrogen tank and regulator

Protocol Steps

Elimination

1 Weight out 38 mg (per one sample) of sodium borohydride ($NaBH_4$) in a glass tube, add 500 μL of 50 mM NaOH resulted in ~ 2 M of $NaBH_4$, and vortex.

2 Add 500 μL of 50 mM NaOH to intact isolated proteins (or proteolytic peptides).

3 Add 500 μL of 2 M of $NaBH_4$ in the sample tube resulted in 1 mL of 1 M $NaBH_4$.

4 Vortex and sonicate quickly the sample tube.

5 Incubate this for 16 – 18 hrs at 45 °C.

6 Remove the sample from the heat and allow it to cool to room temperature.

7 Neutralize the sample by adding 10 % AcOH dropwise with vortexing until bubbling stops (be careful to minimize sample loss by bubbling).

8 Clean up the sample using cation exchange column (see Support Protocol 3.1).

9 Move the column onto a screwtop tube and load the sample in the column.

10 Elute the sample into a glass screwtop tube using 5 – 6 mL of 5 % AcOH.

11 Dry sample in SpeedVac overnight.

12 Remove borate by methanol:glacial acetic acid (9:1) mixture.

13 Add 1.5 mL of MeOH/AcOH mixture in the sample tube with a glass pipette and transfer the sample into the screwtop tube.

14 Dry the sample down with dry N_2 at 45 °C.

15 Repeat steps 13 and 14 twice.

When the sample is dry, sample can be permethylated.

Permethylation

16 Add 200 μL dry DMSO to the sample using a glass pipette or syringe.

17 Vortex to dissolve sample.

18 Add 300 μL of the prepared base (see Support Protocol 3.2) to the sample using a glass pipette or syringe. **Comment**: Prepare the base immediately prior to permethylation

19 Add 150 μL of iodomethane using a glass syringe (rinsed with dry DMSO) to the sample and then immediately cap the sample.

20 Vortex the sample vigorously for 5 min at maximum speed.

21 (Optional) Sonicate the sample for maximum 10 minutes or vortex the sample vigorously for another 5 min at maximum speed.

22 Add approximately 2 mL (1 pipette full) of nanopure water and mix well. The sample should turn cloudy as an indicator of a successful permethylation.

Note: If the sample does not turn cloudy, iodomethane may have evaporated before permethylation is completely done. May repeat permethylation.

23 Add approx. 2 mL of DCM (1 pipette full) using a glass pipette and vortex vigorously to extract the permethylated glycans.

24 Centrifuge and remove the aqueous (top) layer.

25 Rinse the DCM layer with approx. 2 mL of water (1 pipette full), vortex vigorously then centrifuge briefly, and remove the aqueous top layer.

26 Repeat step 10 two - four times (total rinsing would be 3–5 times).

27 After the final rinsing, transfer the DCM layer into another tube being careful not include any water.

28 Dry off the DCM using N_2. Sample is ready for mass spectrometry analysis.

Support Protocol 3.1: Preparing cation exchange resin stock and packing chromatography column

In order to clean up the glycans for further analysis after release, cation exchange chromatography is performed as described.

Materials

Methanol

Chromatography column

Cation exchange resin

1M HCl

5% Acetic Acid

1. Add ~15 mL of resin to glass tube, add sufficient amount of 100% methanol to wash resin. Allow resin to incubate at room temperature. Remove methanol. Repeat this step 3 times.
2. Incubate resin overnight at room temperature in methanol.
3. Pour resin and methanol into 20mL chromatography column.
4. Rinse resin 3x with methanol, with 1M HCl until bubbles are gone, and 5% AcOH pushing through with air.
5. Keep the resin in glass tube with methanol at 4 °C and use this as a stock.
6. Pack the resin (1 mL bed volume) from the stock in the column.
7. Wash resin with methanol (x2), 1M HCl, and 5%AcOH forcing through with air until dry. Repeat this step twice.
8. Wash resin with 5%AcOH, start with air, and allow to pass through by gravity.

Support Protocol 3.2: Base preparation for permethylation

Preparation of fresh anhydrous base is crucial for complete permethylation and should be used the same day as prepared.

Materials

Glass screw cap tubes

50% w/w NaOH solution

Anhydrous methanol

Anhydrous DMSO

Glass Pipettes

Centrifuge for 10 cm × 13 cm tubes capable of 2,000 rpm

1. In a glass screw-cap tube (10 cm × 13 mm), combine 100 μL of 50% w/w sodium hydroxide solution (NaOH) using plastic pipits and 200 μL of anhydrous methanol (dry MeOH) using a glass pipette or syringe.
2. Vortex the mixture.
3. Add approx. 4 mL (2 pipits full) of anhydrous dimethyl sulfoxide (dry DMSO) using a glass pipette and vortex.
4. Centrifuge the tube (quick spin at 2,000 rpm at room temperature) and then remove DMSO, salts, and white residue from pellet.
5. Perform steps 3 and 4, total 3 through 5 five times to remove all water and white residues from the pellet.
6. Finally, add 1mL of DMSO and break down gel gently.

Basic Protocol 4: Preparing O-glycosylated peptides for MS analyses

Given the high degree of glycosylation than can occur on many O-glycoproteins, proteolytic digestion for shotgun proteomic analysis can yield poor results (Kobata, 1979; Nakamura et al., 2010b; Stalnaker et al., 2010). There is not an equivalent enzyme to N-glycanase (PNGaseF or PNGaseA) for O-glycoproteins. Thus, pretreatment of samples with a cocktail of glycosidases is often required to maximize proteolytic digestion. Commercial kits are available from multiple vendors and commonly include the enzymes PNGase F, galactosidase, N-acetylglucosaminidase, and sialidase. Note that many of these kits also contain O-glycanase that can remove core 1 structures. For site-mapping of core 1 and related structures, this enzyme should be omitted from the treatment, whereas, if optimal protein coverage is sought, the enzyme may be included. Mapping sites of O-glycoslyation on glycopeptides can be challenging given the lability of the glycosidic linkage to collision-induced dissociation frequently used for tandem mass spectrometry. However, beta-elimination can be used to remove the O-glycan resulting in a dehydro-amino acid (Greis et al., 1996). This resulting alpha,beta-unsaturated carbonyl is subject to conjugate (Michael) addition with a nucleophile that allows for a unique mass tag to be placed on the previously modified site of glycosylation. Nucleophiles that have been used for these methods include ammonia, dithiothreitol, and biotinylated primary amines (Rademaker et al., 1998; Vosseller et al., 2005; Wells et al., 2002).

Materials

Enriched glycoprotein sample

Cocktail of glycosidase enzymes (e.g. Enzymatic CarboRelease Kit, QA-Bio KE-DG01)

Trypsin (sequencing grade)

8M Urea in 40 mM ammonium bicarbonate, pH 8.1

27.5 mM iodoacetamide

Triethylamine

Sodium hydroxide

Ethanol

Trifluoroacetic acid

Nucleophile (dithiolthreitol or biotin pentylamine)

C18 Spin Columns (The Nest Group)

Protocol Steps

1. Treat glycoproteins with a cocktail of glycosidase enzymes as described by manufacturer (e.g., QA-Bio KE-DG01 or)
2. Add equal volume of 8M urea in 40 mM ammonium bicarbonate, pH 8.1, to sample.
3. Add dithiolthreitol to 5 mM final concentration and incubate at 37°C for 1 hour.
4. Add equal volume of 27.5 mM iodoacetamide to sample and incubate in dark for 45 minutes with quick vortexing every 15 minutes.
5. Dilute sample to final urea concentration of 0.8 M with 40 mM ammonium bicarbonate.
6. Digest with sequencing grade trypsin (typically 1:25 w/w of starting protein) overnight at 37°C.
7. Add Trifluoroacetic acid to 0.5% final concentration.
8. Purify with C18 reverse phase spin columns (Nest Group) using manufacturer's protocol.
9. Dry desalted, eluted tryptic peptides in a SpeedVac system to complete dryness.
10. Resuspend desalted proteolytic glycopeptides in 1.5% triethylamine/0.15% NaOH/ 10% ethanol in an eppendorf tube.
11. Add nucleophile in excess (typically to 10 mM final concentration) and incubate sample at 50°C for 2 hours.
12. Add an equal volume of 2% trifluoracetic acid to quench the reaction.
13. Desalt sample using C18 spin columns according to manufacturer's protocol (e.g. Nest Group, http://www.nestgrp.com/).

Basic Protocol 5: Glycopeptide Analysis via Neutral-loss triggered MS^n approaches

Given that the glycosidic linkages are labile, the main peak often observed for a glycopeptide in a tandem mass spectrometer using collision-induced dissociation is the intact peptide with the neutral loss of the peptide. However, this can be used to one's advantage in an ion-trap-based tandem mass spectrometer that is capable of trapping MS^2 peaks and generating further fragmentation events (MS^n). An instrument can be programmed to identify a major fragment in MS^2 that corresponds to a particular loss (for examples 162 or 204 Da for Hexose or HexNAc, respectively). If such a neutral loss peak is observed, the instrument can isolate and fragment this species further to generate sequence information. Such approaches have been used routinely for phosphorylation (resulting in a dehydroamino acid scar where the phosphate once was) and more recently for O-glycopeptides (producing Ser/Thr residues without changes in mass where the glycan once

was) (Jiang et al., 2008; Stalnaker et al., 2010). Given that O-glycopeptides result in “true” neutral loss peaks and do not leave behind a scar, the neutral loss approach is very sensitive at identifying glycopeptides but often times cannot reliably identify the exact residue modified when the peptide contains multiple hydroxyl-containing amino acids. This method benefits from no special care beyond the typical proteolytic digestion and desalting performed before analysis by LC-coupled tandem mass spectrometry. Only the method settings for the particular instrument used need be modified and analysis is restricted to those instruments capable of generating MS^3 spectra.

Materials

No special materials required

Instrumentation

LTQ or an LTQ-Orbitrap hybrid instrument (ThermoFisher) is routinely used for these analyses, however multiple other mass spectrometers can be used as well.

Protocol

Tryptic peptides are analyzed by typical proteomic analysis with changes in the instrument method files. Pseudo-neutral loss-MS^3 is chosen for analysis looking for a neutral loss peak as one of the 3 (5, for higher coverage but more false-positives) major fragments in MS^2 that then triggers a MS^3 event. A neutral loss table is generated that includes the m/z for +1, +2, +3, and +4 for Hexose, HexNAc, Deoxyhexose, and Sialic Acid (Neu5Ac, may also include Neu5Gc if looking at non-human samples). For example for hexose, the neutral loss table would include 162.1, 81.05, 54.03, and 40.53.

Basic Protocol 6: Glycopeptide Analysis via HCD-triggered ETD approaches

The current generation of hybrid mass spectrometers allows creative combinations of fragmentation energy to yield valuable information about an analyte. One such application is the use of higher-energy C-trap dissociation (HCD) of glycopeptides to yield abundant sugar oxonium ions (204.1 for example for HexNAc). HCD-analysis is rapid and sensitive and can be used to trigger another fragmentation event upon the presence of sugar oxonium ions in the mass spectrometer. In this case, electron transfer dissociation (ETD) of the precursor mass is used if HCD analysis detects a sugar oxonium ion (Zhao et al., 2011). Many post-translational modifications, including glycosylation, are quite stable in ETD allowing good backbone fragmentation of the peptide for sequence assignment and maintenance of the glycan on the modified amino acid for accurate site-mapping and glycan compositional analysis (Mikesh et al., 2006). In the particular format described above, analysis is restricted to a linear-ion trap/orbitrap mass spectrometer equipped with ETD capability. However, the method theoretically would work on any instrument capable of generating and scanning for oxonium ions generated by collisions that was also equipped to generate electron dissociations.

Materials

No special materials required

Instrumentation

Reverse-phase nano-high-performance liquid chromatography (HPLC) system (LC Packings, Dionex, etc.) connected to an LTQ-OrbitrapXL with ETD capability (ThermoFisher, or similar instrument).

Protocol

Tryptic peptides are analyzed by typical proteomic analysis with changes in the instrument method files. MS^2 is acquired by HCD fragmentation of the top 5 most abundant ions from the full MS spectra. Importantly, the m/z range needs to be set to include the range from 125–300 m/z to capture single charged sugar oxonium ions. The instrument uses an oxonium ion list for Hexose, HexNAc, Deoxyhexose, and sialic acid (Neu5Ac, Neu5Gc may also be considered if looking at non-human samples). If an ion of sufficient intensity (differs depending on instrument) is present in the MS^2 spectra within the tolerance of the instrument for a sugar oxonium ion (204.1 m/z for HexNAc for example), then the precursor ion is selected for ETD fragmentation.

Reagents and Solutions

Homogenization Buffer: 20 mM Tris (pH 8), 10 mM EDTA, 0.5% n-octyl-β-thioglucoside, 1mM PMSF (100 mM stock), 3 μg/ml pepstatin A, 1 μg/ml leupeptin, 1mM benzamidine HCl, 250 mM sucrose, 1 mM mercaptoethanol.

Assay Buffer: 20 mM Tris (pH 8), 10 mM EDTA, 0.5% n-octyl-β-thioglucoside, 2 mM mercaptoethanol.

Wash Buffer: PBS (phosphate-buffered saline), 0.1% NP-40, pH 7.5

Elution Buffer: 0.5M Tris, 100 mM glutathione, pH 8.0

Dialysis Buffer: 20 mM Tris, 2 mM EDTA, 0.1% PMSF, pH 8

TBST Buffer: 150 mM NaCl, 10 mM Tris pH 8.0, 0.05% Tween 20

G7 Buffer: 50 mM Sodium Phosphate pH 7.5, 1%NP-40

G2 Buffer: 50 mM Sodium Citrate pH 4.5

Lysis Buffer: 150mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100

Commentary

Background Information

O-linked mannosyl glycans were first found on a mammalian brain glycoprotein about thirty years ago, yet it was not until twenty five years later when the protein O-mannosyltransferase activity was reconstituted *in vitro* (Finne et al., 1979; Krusius et al., 1986; Manya et al., 2004). The first unambiguously identified glycoprotein bearing O-mannosyl glycans *in vivo* was α-dystroglycan, however, it was challenging to demonstrate *in vitro* that mammalian POMT1 and POMT2 can modify α-Dg with O-linked mannose (Chiba et al., 1997; Manya et al., 2004). The initial evidence of the enzymatic activity of these enzymes came from the work of Endo and coworkers who demonstrated that the microsomal fraction of HEK293T cells transfected with pcDNA3.1-based constructs expressing human POMT1 and POMT2 could modify a peptide acceptor with O-linked mannose *in vitro* (Manya et al., 2004). HEK293T cells showed only minimal endogenous O-mannosyltransferase activity, and the reconstitution of high level of this activity required co-transfection of both POMT constructs. In these experiments, O-mannosyltransferase activity was purified *in vitro* in the form of a microsomal membrane fraction. The isolated activity was found to be unstable above 25°C, and also required a special detergent, octyl-thioglucoside, for optimal function (Manya et al., 2004). As a sugar donor, protein O-mannosyltransferases use dolichol phosphate-activated mannose (Dol-P-Man) to modify Ser/Thr residues of protein acceptors. To demonstrate the activity of POMTs, Endo and co-workers used a radioactive sugar donor, Dol-P-[^{3}H]Man, and a fragment of mouse α-DG as a protein acceptor. This fragment encompassed the amino acid sequence 313-483 of α-DG

including mucin-type domain with multiple putative O-GalNAc attachment sites, based on the prediction by NetOGlyc program at (www.cbs.dtu.dk/services/netoglyc (Julenius et al., 2005)). Similar assays have been performed with microsomal fractions containing RT and TW while using as an acceptor the fragment of mammalian α-DG (Ichimiya et al., 2004), or mucin-type domain regions of *Drosophila* Dg-C and Dg-A isoforms (Nakamura et al., 2010b).

Critical Parameters and Troubleshooting

In vitro assay for O-mannosyltransferase activity—The assay appears to be sensitive to impurities that may arise from incomplete removal of solvent from Dol-P-[^{3}H]Man (e.g., methanol:chlorophorm), or from some contaminants in protein acceptor preparations. Thus, careful preparation of both, donor and acceptor substrates for the assay can ensure their purity and improve the assay. Additionally, Dol-P-[^{3}H]Man donor is not very stable at room temperature, and it should be used immediately once reconstituted in Assay Buffer, or quickly frozen and kept at −80°C. Moreover, Dol-P-[H]Man can be absorbed by some plastic tubes and lost during reconstitution or the assay. Therefore, it is recommended to check the concentration of the donor sugar after reconstitution in Assay Buffer using a scintillation counter. Bacterial expression of some Dg-based substrates can be inefficient due to a problem with protein folding that leads to protein aggregation in inclusion bodies. This problem can be solved by decreasing temperature during protein expression to ≤25°C. Note that these conditions may require longer time of incubation after the induction of protein expression.

Expression of Drosophila O-mannosyltransferases and Dystroglycan in vivo —Crosses between *UAS* and *GAL4* genotypes for ectopic expression can sometimes result in synthetic lethality (lethality caused by some combination of alleles that have little or no apparent disadvantage in other genetic backgrounds (Dobzhansky, 1946)) originating from a particular combination of several transgenic insertions. This lethality may not necessarily result from the overexpression of transgenic constructs, but rather be a consequence of mutations associated with transgene insertions that are combined together in the same genotype. In order to solve this problem, one can obtain other insertions of the constructs at the different genomic loci not causing synthetic lethality. Another potential problem can be associated with using a combination of a *UAS* construct and a *GAL4* driver that may result in sick organisms due to exceedingly high level of expression causing toxicity. This situation can be revealed by a low number of progeny from a *UAS* x *GAL4* cross, or dead pupae (or larvae) phenotype. To ensure the quality of tissue samples, it is important to avoid this situation and start with a different *GAL4* and/or *UAS* transgene.

Purification of microsomal fractions with O-mannosyltransferase activity—The protein O-mannosyltransferase activity of microsomal membrane fraction is rather unstable, especially at temperatures above 15–20°C. Thus, it is important to carry out the protocol promptly, while keeping samples at ice-cold temperature throughout purification. Once the microsomal fraction is isolated, it should be used in assays right away for best results.

Analysis of Drosophila Dystroglycan O-mannosylation using lectin blots—Lectin blots can be prone to a high background and non-specific bands. To troubleshoot these problems, one can decrease lectin and/or ABC reagent concentration during incubation with blots. Additionally, it's imperative to perform control incubation in the presence of inhibiting sugar. Finally, the specificity of the staining can be confirmed by treating the sample with a glycosidase that is expected to remove the structure recognized by the lectin. In this case, lectin blot analysis should confirm that specific bands disappear upon this treatment.

Analysis of Drosophila Dystroglycan O-mannosylation using glycosidase treatment and western blots—Some glycosidase isolates can be contaminated with a protease activity that could destroy glycoprotein during glycosidase treatment. Thus, it is important to compare the amount of glycoprotein before and after the treatment (e.g., by western blot). A different lot or source of glycosidase can be used to troubleshoot this problem. Additionally, some glycan chains can be more resistant to glycosidases due to their location, overall composition and presence of additional modifications. If incomplete digestion or glycosidase resistance is suspected, an alternative way to confirm the structure should be used, such as a different glycosidase with similar or overlapping activity, lectin blot analysis, or mass spectrometry approach.

Analysis of O-glycosylated peptides via beta-elimination—One must take caution in the mapping of O-glycoslyation sites with this method since other post-translational modifications, such as phosphorylation, are susceptible to beta-elimination. One possible strategy to address this shortcoming is to treat a portion of the sample with a phosphatase or a cocktail of glycosidases before the beta-elimination (Vosseller et al., 2005; Wells et al., 2002).

Anticipated Results

***In vitro* assay for protein O-mannosyltransferase activity—**The assay can be useful for comaparing protein O-mannosyltransferase activity toward various protein acceptors, in different types of cells or in organisms of different genotypes. For example, this approach has been used to analyze this activity at its endogenous level, as well as upon transgenic overexpression of POMT1 and POMT2, in HEK293 cell cultures (Manya et al., 2004), in wildtype, mutant or transgenic *Drosophila* (Ichimiya et al., 2004; Nakamura et al., 2010b), to compare activity of mutant POMT enzymes (Manya et al., 2009), and to compare protein O-mannosyltransferase activity toward different Dg-derived peptide and protein substrates (Manya et al., 2007; Nakamura et al., 2010b). As an example of anticipated results for protein O-mannosyltransferase assays, Figure 3A illustrates the analysis of endogeneous RT-TW in Drosophila larvae with or without knockdown of *rt* and *tw*, demonstrating that both *POMT* genes are required for protein O-mannosylation (Ichimiya et al., 2004). A typical result of expression and purification of O-mannosyltransferase substrates from bacterial cells (Support Protocol 1.1) is shown in Figure 3B as a Coomassie staining of purified mucin-type domain of *Drosophila* Dg-A (Nakamura et al., 2010b).

Analysis of *Drosophila* Dystroglycan O-mannosylation using lectin blots—*In vivo* expression of *Drosophila* Dystroglycan protein can be induced at relatively high levels in larvae or pupae using *act-GAL4* or *hs-GAL4* drivers (Nakamura et al., 2010b). A purification experiment using about twenty larvae expressing *UAS-ExDg-FLAG* under control of *act-GAL4* is expected to generate the amount of ExDg-FLAG protein sufficient for several western and lectin blot analyses. As little amount of material as 0.8 larvae/lane of SDS-PAGE can suffice to reliably detect ExDG-FLAG by western blot (Fig. 4A). Combining glycosidase treatments with western and lectin blot analyses can provide important information on the presence of certain glycan structures on purified protein. Figures 4B shows an example of such analysis performed with ExDg purified from different genetic backgrounds.

O-linked glycomics—Direct glycomics experiments as outlined can provide much more detailed results as to the exact O-glycans present in a sample and direct glycoproteomics analysis can define sites of attachment. As an example of direct glycopeptide analysis, see Figure 5. Using collision-induced dissociation, one can see that the major fragment of a singly O-mannose modified peptide is the neutral loss of the hexose (M.W. 162, loss of 81

m/z due to doubly charged state of the peptide) (Nakamura et al., 2010b). This type of neutral loss signature indicates the presence of a hexose modified peptide and can be used to trigger the instrument to perform an electron-transfer dissociation fragmentation in which the modification is not as labile that facilitates exact site mapping (Zhao et al., 2011).

Time Consideration

In vitro assay for O-mannosyltransferase activity (Basic Protocol 1) requires a couple of hours to complete and analyze the results. Expressing in bacterial cells and purification of protein acceptor substrates usually takes 2.5–3 days, starting from cell transformations and finishing with the analysis of purified proteins by gel staining. Expression of *Drosophila* O-mannosyltransferases and Dystroglycan *in vivo* requires two-three weeks, starting from setting up crosses till collecting progeny of genotypes of interest. This time is approximate and can vary significantly depending on the vigor of genotypes used in the experiments and the scale of the experiment. For large-scale expression experiments and when working with difficult genotypes, this time can be as along as a couple of months. Purification of microsomal fractions with O- mannosyltransferase activity takes 2–3 hours. A typical lectin blot procedure takes 1.5–2 days. Glycosidase treatment followed by western blot analysis normally requires 2–3 days. Isolation of glycoproteins, release and permethylation takes 2–3 days, and analysis by mass spectrometry takes 1–2 hours. Data interpretation can take up to a week depending on expertise and availability of software to assist. For analysis of glycopeptides, generation of peptides takes one day and desalting and LC-MS/MS analysis takes 1–2 hours. Data interpretation can take up to two days depending on expertise and availability of software to assist.

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Abbreviations

Ser	serine
Thr	theronine
α-Dg	α-Dystroglycan (protein)
Gal	galactose
GlcNAc	*N*-Acetylglucosamine
GalNAc	*N*-Acetylgalactosamine
Man	mannose
PMT	Protein O-mannosyltransferase
POMT1(2)	Protein O-mannosyltransferase 1(2)
rt	*rotated abdomen* (gene)
tw	*twisted* (gene)
Dol-P-Man	dolichol-phosphate-activated mannose
POMGnT1	

FKRP	*Fukutin-related protein (gene)*
ECM	Extracellular Matrix
WWS	Walker Warburg Syndrome
MEB	Muscle-Eye-Brain disease
FCMD	Fukuyama congenital muscular dystrophy
CMD	congenital muscular dystrophy
MDDG	Muscular Dystrophy-Dystroglycanopathy
DGC	dystrophin-associated glycoprotein complex
NOS	nitric oxide synthase

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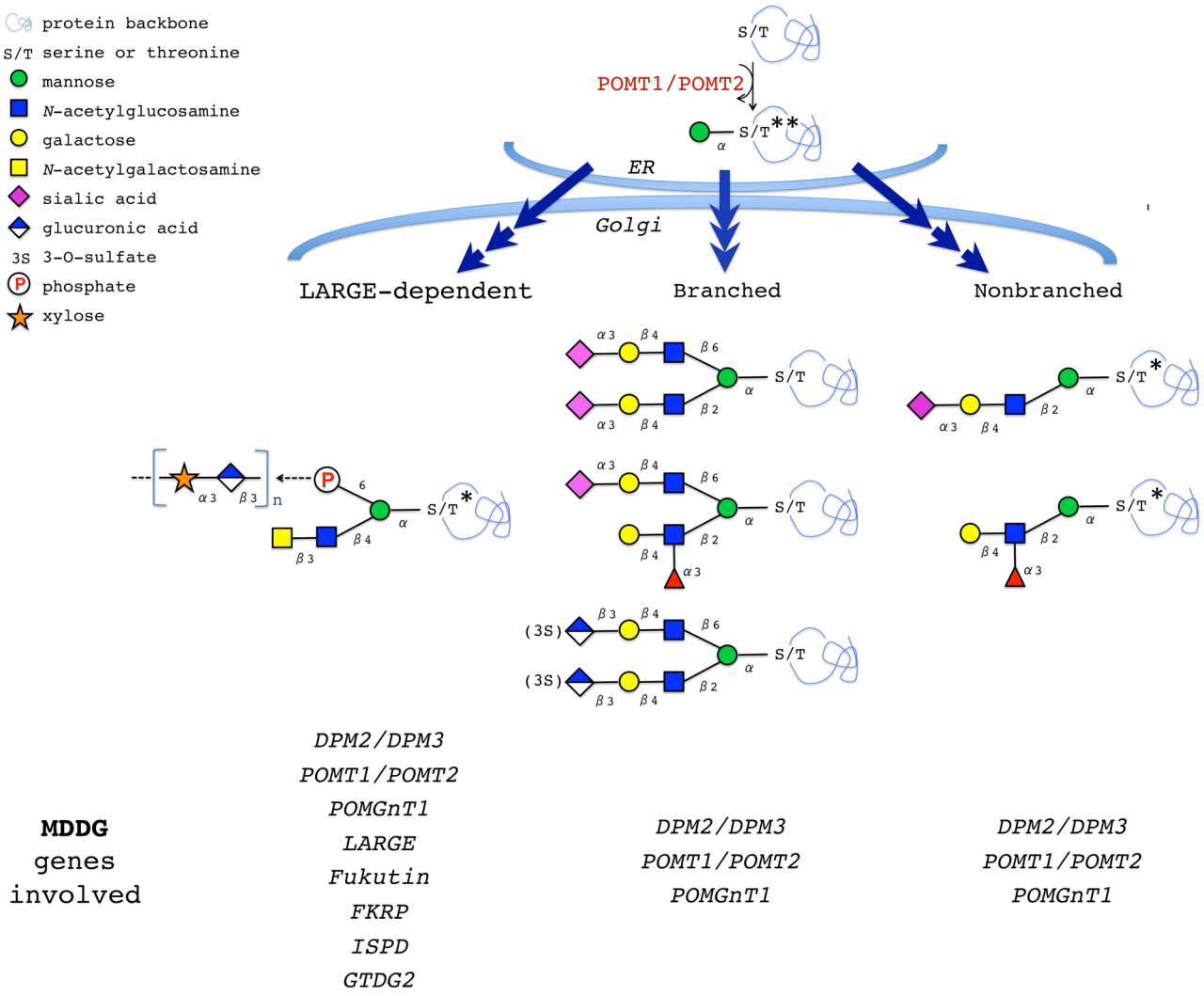

Figure 1. O-mannosylated glycans

Biosynthesis of O-mannosyl glycans is initiated in the ER by a complex of two protein O-mannosyltransferases, POMT1/POMT2. The O-mannose-linked structures undergo further maturation in the Golgi. LARGE-dependent structures are thought to be responsible for Laminin-binding activity of α–Dg. MDDG-associated genes affecting (or predicted to affect) different structures are indicated. *, structures identified on mammalian α-Dystroglycan. **, the structure found on *Drosophila* Dystroglycan. Only mature structures are shown, however, O-mannosyl glycans with incomplete maturation have been also detected on glycoproteins (Stalnaker et al., 2011b).

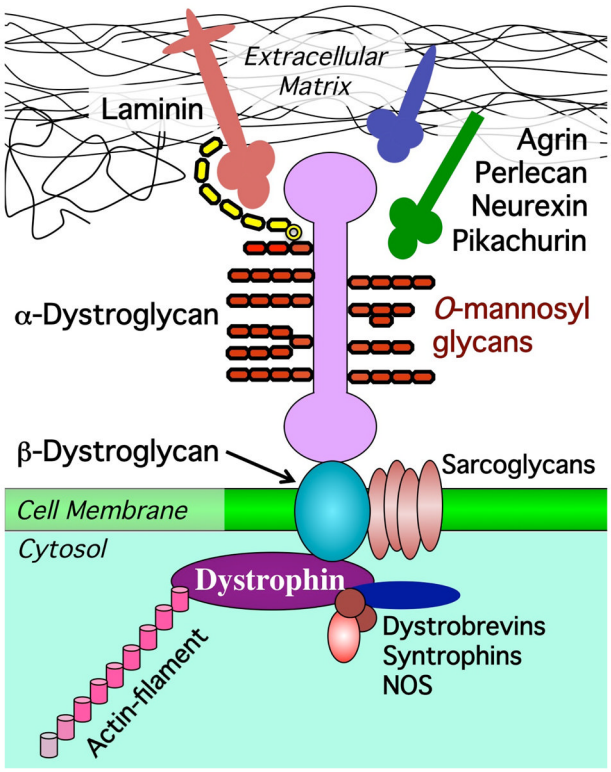

Figure 2. O-mannosyl glycans mediate interactions between α–Dg and extracellular matrix

Dystroglycan interacts with basal lamina by binding ECM ligands (Laminin, Neurexin, Agrin, Perlecan, and Pikachurin). β-Dystroglycan is a transmembrane protein that connects extracellular (α-Dystroglycan) and intracellular (Dystrophin) components of the Dystrophin-associated glycoprotein complex (DGC). Dystrophin links the DGC to actin cytoskeleton. Other DGC-associated proteins include sarcoglycans, dystrobrevins, syntrophin, and NOS (nitric oxide synthase). Note that in addition to O-mannosylation, α–Dg has also O-linked mucin-type and N-linked glycans (not shown here). LARGE-dependent carbohydrate chain responsible for Laminin binding is shown in yellow.

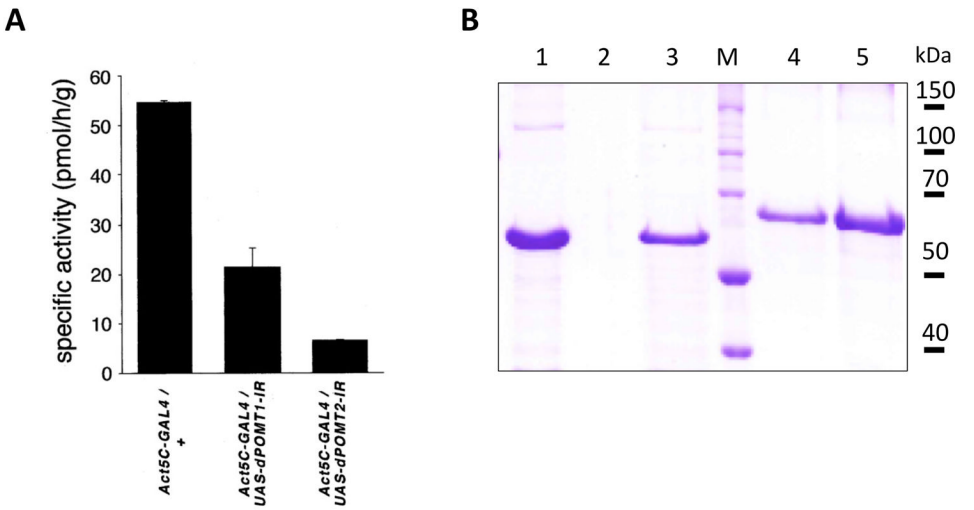

Figure 3. Examples of protein O-mannosyltransferase assays (A) and a purified protein substrate for O-mannosylation (B)

A, The activity of microsomal fraction isolated from *Drosophila* larvae was assayed using a fragment of mucin-type domain of mouse α-DG protein. The activity was assayed for 3 genotypes: *Act5C-GAL4/+* (having wild-type level of rt and tw expression), *Act5C-GAL4/ UAS-dPOMT1-IR* (RNAi-mediated knockdown of *rt*) and *Act5C-GAL4/UAS-dPOMT2-IR* (RNAi-mediated knockdown of *tw*). Figure adapted, with permission, from (Ichimiya et al., 2004). **B**, Coomassie staining of SDS-PAGE gel with purified fragment of *Drosophila* DG-A-GST protein expressed in *E.coli* cells (Nakamura et al., 2010b). Lane 1, DG-A-GST released from 10 μl of GST beads; Lane 2, blank; Lane 3, 1 μl of purified DG-A-GST eluted from after dialysis and concentration (estimated amount ~1.5 μg); M, protein molecular mass standards; Lane 4–5, BSA control samples for protein amount quantification, 1 and 2 μg, respectively.

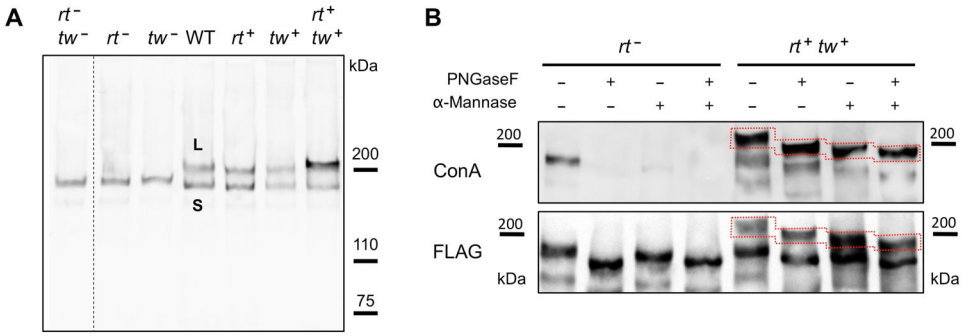

Figure 4. Western and lectin blot analyses of O-mannosylated forms of *Drosophila* ExDg-FLAG expressed *in vivo*

A, Western blot detection of ExDG expressed in *rt-tw* double mutants (rt^{-} tw^{-}), *rt* mutants (rt^{-}), *tw* mutants (tw^{-}), wildtype background (WT), and backgrounds with ubiquitous ectopic expression of RT (rt^{+}), TW (tw^{+}), or RT-TW co-expression (rt^{+} tw^{+}). L band represents a highly O-mannosylated glycoform, while S band corresponds to a glycoform without significant O-mannosylation. **B**, Analysis of ExDg glycosylation by glycosidase treatments. The top panel shows Con A reactivity of purified ExDG after treatments with PNGaseF and α-mannosidase. The S glycoform purified from *rt* mutant background (left side) loses its Con A reactivity either after the removal of N-linked glycans by PNGaseF or after treatment with α-mannosidase removing α-linked mannose residues, suggesting the absence of O-mannose modifications and efficient removal of oligomannose structures either by trimming N-linked branches with α-mannosidase or by complete elimination of N-linked glycans with PNGaseF. The L glycoform purified from RT-TW co-expression background (right side) retains Con A reactivity after treatment with PNGaseF, α-mannosidase, or both glycosidases, suggesting that L glycoform is O-mannosylated, and that α-mannosidase does not remove O-mannose completely. The bottom panel shows anti-FLAG western blot control corresponding to the lectin blot shown in the top panel. Dashed outline shows the region of the L glycoform on the blots. Figure adapted with permission from (Nakamura et al., 2010b).

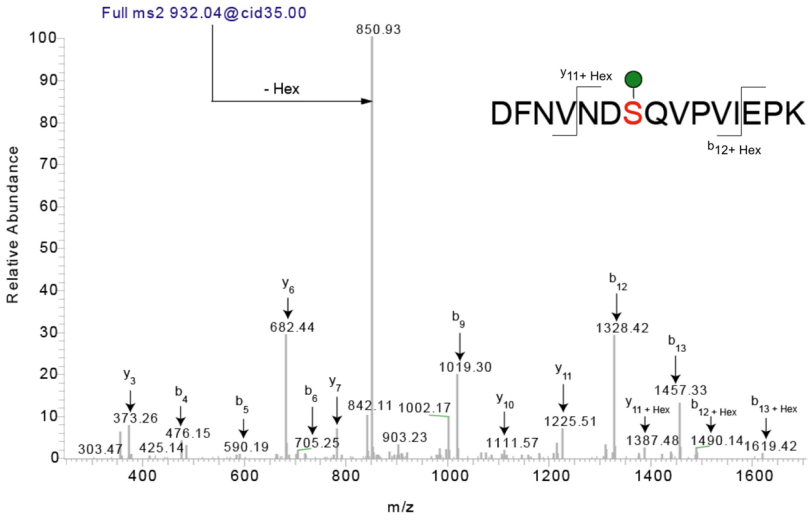

Figure 5. Identification of O-linked mannose sites using mass spectrometry

Collision induced MS^2 fragmentation of a singly O-mannosylated peptide derived from *Drosophila* Dystroglycan with m/z of 932.04 results in a predominant neutral loss ion at 850.93. This loss represents a loss of a hexose from the doubly charged peptide. In this example, there are sufficient fragments (b and y) to assign the peptide confidently and there is only one possible site of attachment. For peptides with multiple potential sites, the neutral loss observed upon collision induced fragmentation could be used to trigger an electron transfer dissociation fragmentation for confident site mapping. Figure adapted with permission from (Nakamura et al., 2010b).