Chapter 2

Proteoglycan: Site Mapping and Site-Directed Mutagenesis

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Abstract

Identification of proteoglycan chain modification sites cannot yet be reliably predicted from primary amino acid sequence data. A number of studies have shown that serine is the predominant amino acid that is modified and it is frequently flanked by a C-terminal glycine and proximal N-terminal acidic amino acids; however, not all simple Ser-Gly motifs constitute a modification site. Here we present a rapid method for cloning small, defined segments of putative proteoglycan attachment sites and expressing them as a mini-reporter protein in an insect tissue culture system that is expandable to high throughput analysis. Reporter proteins with attached proteoglycans can be readily discerned from their unmodified form, by a simple gel-shift assay and Western blot detection for an epitope tag engineered into the reporter. Unmodified proteins are generated as a reference standard by treating cells with dsRNA to knock down the endogenous polypeptide xylose transferase, which is responsible for initiating proteoglycan site attachment. Examination of proteoglycan attachment by different metazoan organisms can be studied in the same cell line by cotransfecting a polypeptide xylose transferase expression plasmid and reporter construct from human, mouse, frog, or worm, for example. Reporter proteins engineer with point mutations can be rapidly generated with this system to pinpoint the exact residue that is glycosylated, to verify the mapping data.

Key words: Proteoglycans, Mutagenesis, RNAi, Glycosyltransferase, Site mapping

1. Introduction

Synthesis of the proteoglycans begins with the attachment of xylose to a serine side chain, followed by the stepwise addition of monosaccharides to build the tetrasaccharide linker: GlcA-β1,3-Gal-β1, 3-Gal-β1,4-Xyl-1β-*O*-serine-(protein). This tetrasaccharide is the substrate for attachment and polymerization of chondroitin and heparan sulfate chains. The precise amino acid that is first modified with a xylose monosaccharide is defined by the substrate specificity of the polypeptide xylose transferase. Numerous studies have shown prevalence for proteoglycan attachment at a serine-glycine motif (1, 2);

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however, many studies have shown that not all "SG" motifs are glycosylated (3, 4) and that some modification sites diverge from the SG "rule" (5). Experimental mapping data reported in the literature are difficult to work though, as many sites are qualitatively mapped in a way that does not rank weak proteoglycan modification sites separately from dominant modification sites. Previously, we reported a rapid method for experimentally mapping these sites, by cloning small 12–15 amino acid segments of putative proteoglycan attachment sites and expressing them as part of a minireporter protein in a tissue culture system (3). Predominant proteoglycan attachment sites modify a serine but not a threonine, while mucin glycosylation modifies both serine and threonine, with a slight preference for the latter (6). Glycine residues are present in both proteoglycans and mucins, but are nearly always present C-terminal and next to the serine attachment position of a proteoglycan. While proline activates a mucin glycosylation (7), it inhibits proteoglycan attachment (3) (as in N-glycosylation) (8). One acidic amino acid is required at a proximal position (-4, -3, or -2, N-terminal to the serine) or a pair of acidic residues is required at distal N-terminal positions -6 and -5 from the proteoglycan modification site, whereas negative residues tend to inhibit mucin-type glycosylation. While these general rules can help to reduce the list of potential modified positions in a protein, the interplay of charge density and position and role of proline, glycine, and other amino acids is complicated, as in the case of mucin glycosylation sites (9), emphasizing the importance of experimental mapping data, including a measure of the proteoglycan site's occupancy rate, to obtain a definitive answer.

2. Materials

2.1. Molecular Biologicals, Expression Plasmids, and Oligonucleotides

1. BsaI restriction enzyme (New England Biolabs) for cloning all inserts. This enzyme allows for production of unique 4 bp customized sticky ends from the sequence of the pLC-S2-SP-BsaI plasmid (see Fig. 1c).
2. Polynucleotide kinase (New England Biolabs) and 10 mM rATP (Roche) for phosphorylating synthetic oligonucleotides, prior to cloning.
3. TriReagent and polyacrylamide carrier (Molecular Research Center) for isolation of RNA.
4. Super Script III (Invitrogen) for first-strand cDNA synthesis, to produce a template for PCR amplification of glycosyltransferases.
5. A high fidelity polymerase chain reaction (PCR) kit for amplification of cDNAs for cloning/expression and for production of templates for dsRNA synthesis.

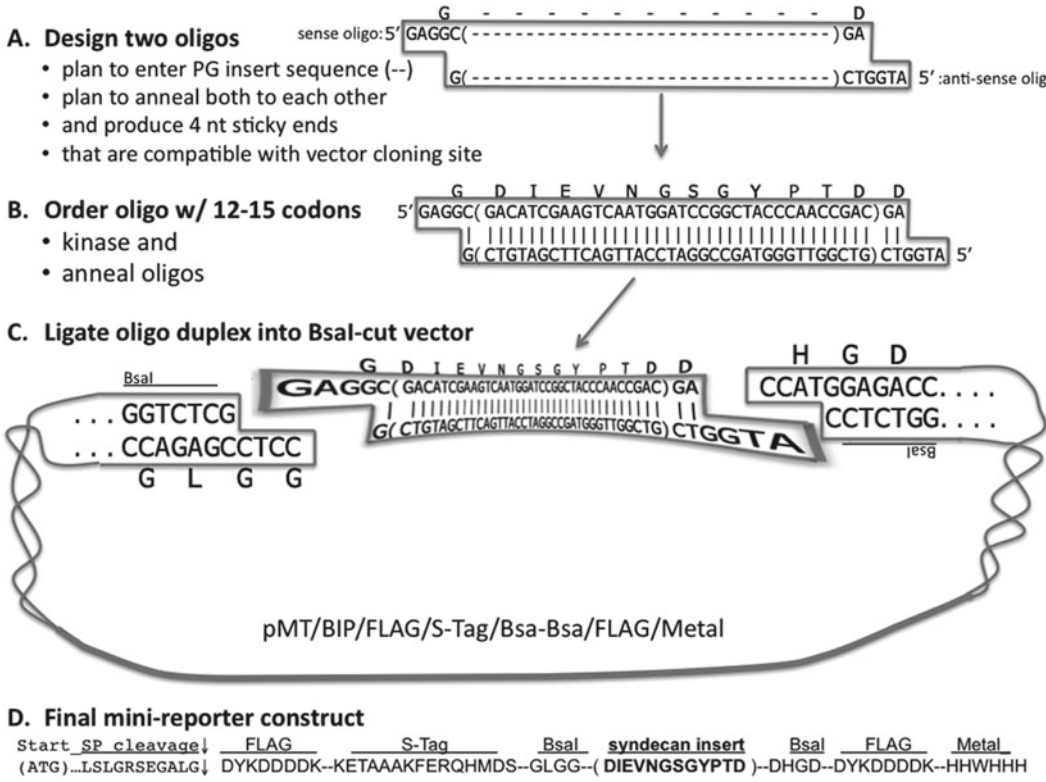

Fig. 1. Rapid cloning of defined proteoglycan modification sites into a minireporter protein expression construct. (**a**) A cDNA insert is created by designing a pair of complementary oligonucleotides with overhanging, "sticky" ends for ligation into a plasmid vector. (**b**) An example of annealed sense and antisense oligonucleotides that encode for a syndecan proteoglycan modification site with 12 amino acid codons (between the parenthesis, and labeled above the DNA in single-letter amino acid code). A BamHI restriction site (GGATCC) is in the insert coding region and used for restriction mapping/screening of expression clones. (**c**) Complementary cohesive ends in the vector are produced by digestion of the vector with BsaI, for directional cloning of the insert. The inserted cDNA needs to be in-frame with two glycine codons upstream in the vector and with the His–Gly–Asp codons in the downstream vector sequence. (**d**) The configuration of the insert (*in parenthesis*) relative to the BIP signal peptide cleavage site, purification/detection tags, and amino acid sequences that flank the proteoglycan insert. *Dashed lines* represent variable length spacer sequences between the specific epitope tags.

6. T7 RNA polymerase (Megascript Kit, Ambion) for production of double-stranded RNA from a PCR product that is flanked with T7 promoters on both ends.
7. Klenow (Large fragment of DNA polymerase I) (New England Biolabs) for creating 4-bp sticky ends at the end of PCR products, when used in the presence of dGTP and the PCR primers described in this study (see Fig. 2).
8. pMT/V5-His (Invitrogen): A plasmid for cloning and expression of full-length glycosyltransferases in S2 cells, like the pLC-S2-FL-CE-pXTase plasmid for the *C. elegans* pXTase rescue plasmid.
9. pMT/BIP/V5-His (Invitrogen): A parent plasmid for construction of a reporter with FLAG, S-Tag, and BsaI sites (see pLC-S2-SP-BsaI in ref. (3)).

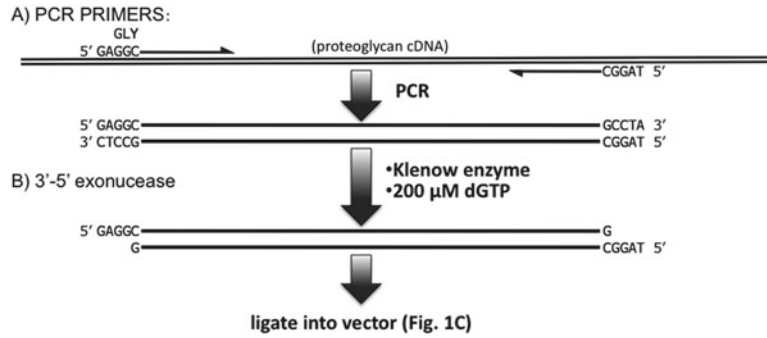

Fig. 2. Cloning of proteoglycan cDNAs without the use of restriction enzymes. (**a**) PCR primer pairs need to be designed carefully to maintain the correct reading frame and to allow for production of sticky ends for cloning. (**b**) Treatment of PCR products with Klenow and 200 µM dGTP will produce cohesive ends for ligation to vector in Fig. 1c. This PCR-amplified cDNA will need to be in the identical reading frame as defined in Fig. 1c.

10. Oligonucleotides are requirements for producing minireporter peptide inserts (see Fig. 1). Sense and antisense oligos will be annealed to each other, such that overhanging ends will be compatible cohesive ends for the cloning site. For short oligonucleotides (<50 nt), standard oligo desalting is sufficient purity. Inserts longer than 15 amino acid codons can be ordered if the oligonucleotides are purified by polyacrylamide electrophoresis. The sense oligo must begin with 5′ GAGGC, prior to the cDNA sequence and end with a GA, while the antisense oligo must begin with 5′ ATGGTC and end with a 3′ "G" (see Fig. 1 and Note 1 for sequence requirements).
11. Requirements for large proteoglycan or full-length cDNA inserts (see Fig. 2). Sense strand PCR primers need to begin with GAGGC immediately before the first codon of the proteoglycan insert; therefore, a glycine codon (GGC) will precede the insert. The antisense oligo must begin with the sequence ATGGC at its 5′end, such that the downstream 3′end of the PCR product ends with GCCAT-3′ (on the sense strand), so that a histidine codon (CAT) is in frame with the proteoglycan cDNA.
12. QuikChange Mutagenesis Kit (Stratagene) includes Turbo-PFU DNA polymerase and Dpn-1 for removal of the parent template after PCR.

2.2. Cell Culture

1. *Drosophila* S2 Cells (Invitrogen) (see Note 2).
2. Schneider's *Drosophila* Medium (SDM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen).
3. Fugene-6 (Roche Applied Science) will be diluted in serum-free SDM.
4. 100 mM $CuSO_4$ (Sigma) filter sterilize and plan to dilute 1:166 to a final concentration of 600 µM $CuSO_4$.

2.3. Sample Preparation

1. Anti-FLAG M2 antibody agarose (Sigma)—do not confuse with the FLAG M1 antibody which has a slightly different sequence requirement for binding.
2. IP Wash Buffer (20 mM Tris, 200 mM NaCl, 5% glycerol, pH 7.4).
3. Glycosidases from the Glycoprotein Deglycosylation Kit; Calbiochem:
   - *N*-glycanase (500 mU of peptide *N*-glycanase F).
   - *O*-glycanase (0.125 mU of endo-α-*N*-acetylgalactosaminidase).
   - Sialidase (0.5 mU of α 2–3,6,8,9-neuraminidase).
4. Amylase (Sigma): control for deglycosylation of N-linked protein. The amylase protein is about 50% unmodified and 50% *N*-glycosylated and thus runs as a dimer on SDS-PAGE.

2.4. Western Blotting

1. SDS-PAGE on Bis-Tris 12% SDS-PAGE with MOPS running buffer (NuPAGE; Invitrogen).
2. Nitrocellulose membranes (this is more sensitive than PVDF membranes).
3. TBS (Tris-buffered Saline): 20 mM Tris, 100 mM NaCl, pH 7.6.
4. TBS-T: TBS with 0.05% Tween-20.
5. S-Protein HRP-conjugate (horseradish peroxidase) (Novagen): use at a 1:5,000 dilution.
6. SuperSignal West Femto Maximum Sensitivity substrate (Pierce) for detection of HPR.

3. Methods

Mapping the position of a proteoglycan site is accomplished by mutating a suspected amino acid that defines the modification site and then conducting Western blot analysis of the wild-type and mutated protein. If a single amino acid point mutation results in the loss of an attached carbohydrate chain, then there will be a mobility shift in the electrophoretic gel run. Carbohydrate chains may be attached to single or multiple serine residues of proteoglycans; therefore, mapping experiments are designed either on full-length proteins, when there is high confidence of a single proteoglycan attachment site, or on isolated glycosylation sites in a minireporter protein containing a short segment of 10–30 amino acids. The latter recombinant minireporter system is useful and lends to more rapid analysis and interpretation, when there are many candidate proteoglycan modification sites in a large protein.

The expression of glycosylated reporter proteins requires careful consideration with regard to the repertoire of the endogenous

glycosyltransferase machinery that is expressed in the cell culture system. Generally, all four glycosyltransferases that attach and build the tetrasaccharide linker (GlcAβ1-3Galβ1-3Galβ1-4Xyl-) to a protein serine side chain are ubiquitously expressed in all cell types. However, the glycosyltransferases that express the terminal sugars chondroitin and heparan may not be expressed in all cell lines and this will impact the use of anti-carbohydrate antibodies for mapping glycosylation sites. Should there be any question about the ability to probe for specific terminal sugars, then it is desirable to map the glycosylation site in a minireporter system, as the addition of the tetrasaccharade linker to a small reporter protein/peptide is sufficient for mobility shift in SDS-PAGE, which eliminates the need to use expensive anti-carbohydrate antibodies.

It is also possible to probe the proteoglycan sequence requirements in a species-specific manner, using a single cell culture system, if the endogenous initiating glycosyltransferase is knocked down by RNA interference and an exogenous polypeptide xylose transferase is coexpressed with the reporter protein in the RNAi-treated culture cells. These molecular tools to knock down and to reintroduce glycosylation activity are also critical for setting up controls and interpreting the gel mobility shift and Western blot assay data (see Fig. 3).

3.1. Molecular Cloning of Single Modification Sites, Large Proteoglycans, and Mutants

1. *Expression plasmid*. Both full-length cDNAs or small DNA segments that encode for single proteoglycan modification sites can be introduced into the same type of expression plasmid with a directional, sticky-end cloning strategy, using sites generated by the restriction enzyme BsaI in the expression plasmid pLC-S2-SP-BsaI (3). Cleavage of this plasmid with BsaI creates upstream and downstream sites for directional cloning, as depicted in Fig. 1c, for insertion of a cDNA fragment generated by either oligoduplexes or PCR products. Prior to the ligation of insert, the cleaved plasmid is treated with calf intestinal phosphatase to remove the 5′ phosphates of the cloning sites and prevent vector self-ligation.
2. *Proteoglycan minigene reporter*. Oligonucleotide duplexes that encode for a single proteoglycan modification site are designed with upstream and downstream overhanging ends, which are used for ligation into the vector (see Fig. 1a, b). It is essential that these oligonucleotides be treated with T4 polynucleotide kinase and 1 mM rATP prior to ligation into the dephosphorylated vector. Ligation reactions use 10–40 ng of vector with 0.4 ng of the oligonucleotide duplex that was previously annealed by heating to 90°C for 1 min and cooling gradually in a temperature gradient to room temperature over 30 min in a thermal cycler instrument. All oligonucleotide inserts contain a restriction site that is used for screening for positive expression

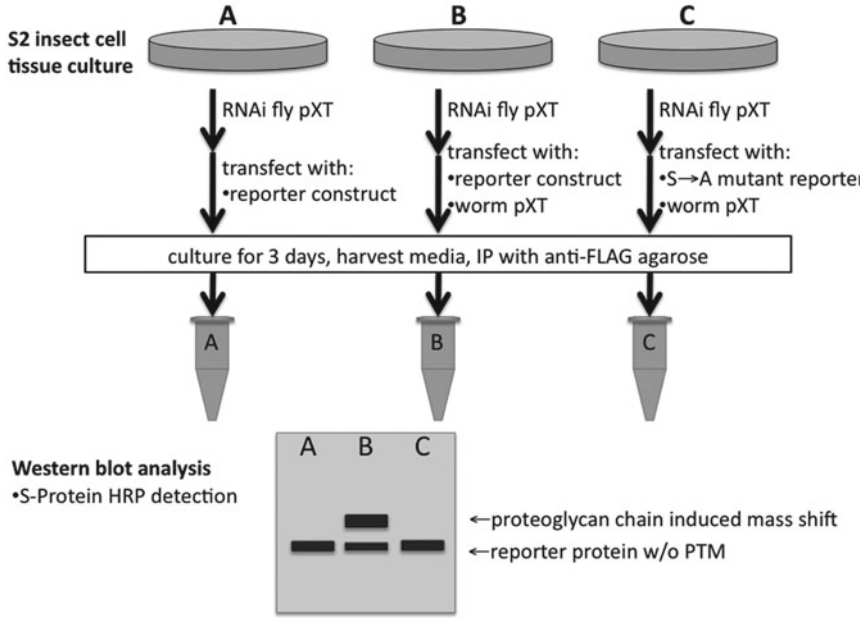

Fig. 3. Expression of reporter glycoproteins in insect cells. All cells are RNAi-treated to knock down endogenous insect cell polypeptide xylose transferase (pXT) activity, producing cell background that is devoid of glycosyltransferase activity that initiates proteoglycan attachment. Reporter proteins are immuno-precipitated and examined by SDS-PAGE and Western blot analysis. A hypothetical western blot of three samples (*A*, *B*, *C*) demonstrates how a proteoglycan is detected and definitely mapped. (*A*) Transfection with a wild-type reporter construct in the absence of pXT activity will produce an unmodified protein, which serves as a reference maker for this reporter. (*B*) Cotransfection of a *C. elegans* pXT expression plasmid with the report protein construct will reconstitute pXT activity and allow for glycosylation at the proteoglycan attachment site, producing a larger MW reporter that can be observed at a slow mobility by SDS-PAGE. (*C*) Confirmation of the glycosylation site is obtained by coexpressing the pXT with a reporter construct, harboring a point mutation at the suspected proteoglycan modification site. In this insect cell model system, both the pXT expression construct and proteoglycan reporter can be derived from any metazoan organism, to examine species-specific glycosylation. See Wang et al. (3) for many examples of reporter gel-shift assays.

constructs, which are always sequenced to confirm reading frame and insert sequence integrity. This cloning scheme is very efficient, allowing for the production of minireporters in batches of 10 or more unique reporter constructs at the same time.

3. *Large proteglycan expression constructs.* To enable insertion of a proteoglycan cDNA into the cloning site above, full-length secreted regions of the proteoglycan need to be PCR-amplified with an upstream primer that starts with the sequence 5′GAGGC contiguous with frame 1 of the cDNA. The downstream antisense primer needs to begin with the sequence 5′ATGGC. PCR products of the proteoglycan cDNA with these engineered ends are cleaned up with a phenol extraction and then ethanol-precipitated by addition of 50 μL of 7.5 M ammonium acetate and 330 μL of 100% ethanol to a 100-μL DNA sample. 5′ protruding sticky ends with 4 nt overhangs for cloning

the cDNA are generated by using the 3′ to 5′ exonuclease activity of Klenow fragment of DNA polymerase I and a final concentration of 0.2 mM dGTP, to stop 3′ to 5′ processing at the G, the fifth nucleotide. This Klenow-treated DNA fragment is finally gel-isolated and ligated into the plasmid.

4. *Mutagenesis of minireporter genes.* An alanine substitution of select serine positions can unambiguously eliminate the cell's ability to attach a carbohydrate chain at the mutated position. Sequences that flank serine can impact the degree to which a serine residue is posttranslationally modified with a glycosaminoglycan side chain, and this will modulate the occupancy rate of proteoglycan modification sites in nature. In addition, it is also possible to design mutations in the flanking sequence to probe how or whether the flanking sequence defines what terminal sugars (heparin, chondroitin) are attached. Another class of mutations that are introduced into the insert are silent mutations for screening of DNA constructs. We usually direct one point mutation in the insert, so that a restriction enzyme cleavage site is created or destroyed. This enables rapid restriction mapping of mutant reporter constructs and selection of positive clones. All positive clones are then submitted for DNA sequencing.
5. *Mutagenesis of large cDNA inserts.* PCR is used in an oligonucleotide-directed mutagenesis method, based from the QuikChange Mutagenesis Kit (Stratagene) to alter the sequence of 25 ng of the target plasmid. Sense and antisense oligonucleotides (about 40 nt long, with >12 nt long segments that flank the directed base change) at 0.3 μM final concentration are used with 200 μM dNTPs, 2.5 U of Turbo PFU in a 25-μL reaction. First, five thermal cycle reactions are conducted at 95°C for 30 s, 45°C for 30 s, and 68°C for 8 min. Next, 13 cycles are immediately conducted at 95°C for 30 s and 68°C for 8 min. After the thermal cycling reaction is complete and cooled, the amplified samples are treated with 3.5 U of DpnI, which cleaves the methylated parental DNA strand and leaves the newly synthesized strands intact. After a 1-h digestion at 37°C, the sample is ethanol-precipitated by adding 12.5 μL of 7.5 M ammonium acetate and 60 μL of 100% ethanol, placing the tube on ice for 10 min and centrifuging at 4°C for 30 min. The pellet is washed by addition of 50 μL of ice-cold 70% ethanol, immediate centrifugation for 2 min, and finally removal of the supernatant. After drying under vacuum in a microcentrifuge for 2 min, the mutated DNA pellet is resuspended in 2.5 μL of water and all of it is used in a single transformation of chemical competent *E. coli*. Individual colonies are screened (four per construct) for the restriction enzyme ablated or created by mutagenesis (see Subheadings 3 and 3.1, step 4) and one restriction enzyme positive clone is selected for DNA sequence analysis for each mutant construct.

3.2. Expression of Recombinant Proteoglycans in Tissue Culture

1. Expression of the peptide reporters in insect cells—*Drosophila* Schneider (S2) cells was cultured in SDM medium (Gibco) supplemented with 10% (v/v) FBS at 27°C. Cells were passaged every 3–4 days. One million S2 cells were added to fresh media in each 35-mm dish for transfections, incubated for 1 day, and then treated with a DNA-FuGENE-6 mixture. Briefly, 3 μL of FuGENE-6 (Roche Applied Science) transfection reagent was diluted into 97 μL of serum-free SDM medium and incubated at room temperature for 5 min. Then 1 μg of reporter plasmid DNA was added with mixing to the diluted FuGENE-6, and this mixture was incubated for 20 min at room temperature. Then the DNA-FuGENE-6 mixture was added in a drop-wise manner to cells. Vigorous agitation/swirling was used to mix the cells and transfection reagent. After a 1-day incubation period, cells are induced to express the reporter protein by the addition of $CuSO_4$ to a final concentration of 600 μM. After three additional days in cell culture, the culture medium is harvested for the secreted reporter, clarified by centrifugation at 2,000 rpm in a SH3000 rotor (Sorval) for 10 min, aliquoted to three tubes, and stored at −80°C.
2. Inhibiting the expression of endogenous polypeptide xylose transferase-RNA interference was used to eliminate the endogenous initiating enzyme. This is performed for two goals: (a) to produce a control reporter lacking an attached proteoglycan sugar chain, which is important in the gel mobility shift assay for establishing the electrophoretic position of the negative control (unmodified) peptide, and (b) to generate an endogenous glycosyltransferase knockdown coupled with an induced expression of a polypeptide xylose transferase from a different species or isoform. RNA interference in S2 cells is easily achieved by exposure of cells to dsRNA, prior to the transfection step. Briefly, on the day of transfection, cells are first washed once with serum-free SDM media, resuspended with 1 mL serum-free SDM, treated with 15 μg of fly pXT dsRNA (>800 bp in length) template in 1 mL SDM media for 2 h, and then immediately transfected with plasmid, without washing the cells, using the procedure described above.
3. For coexpression of worm (or any other) polypeptide xylosyltransferase with the reporter proteins, 0.05–0.1 μg of pLC-S2-FL-CE-pXTase plasmid, harboring a species- or isoform-specific polypeptide xylose transferase cDNA, is cotransfected at the same time with 0.95 μg of the reporter plasmid construct. Theoretically, a glycosyltransferase cDNA from any metazoan (worm, insect, frog, mouse, human, etc.) can be cotransfected with a reporter plasmid to examine how proteoglycan modification sites or machinery is evolutionarily conserved.

3.3. Sample Preparation for the Identification of Proteoglycan Modification Sites

1. *Immuno-precipitation of proteoglycan reporter proteins.* 25 μL (packed resin volume) of anti-FLAG M2 agarose is added directly to 1 mL of clarified cell culture medium. The affinity resin is rocked at 4°C overnight and washed twice in IP Wash Buffer. Reporter proteins bound to beads are treated on-bead, with 0.125 mU of *O*-glycanase, 0.5 mU of sialidase, and 500 mU of *N*-glycanase in a final volume of 48 μL for 5 h at 37°C, according to the manufacturer's specifications. For SDS-PAGE analysis, 16 μL of 4xNuPage sample loading buffer is added to the glycosidase-treated samples.
2. *Controls for glycosidase treatment.* To demonstrate that the glycosylhydrolases are removing N- and O-linked sugar chains, control *N*-glycanase digestions are run using human salivary amylase, which migrates as a doublet of *N*-glycosylated and nonglycosylated isoforms. An *O*-glycanase control protein requires a mucin-type O-glycosylated domain, rich in serine/threonine/proline that is not larger than 80 kDa, so that an electrophoretic mobility shift is easily visible after deglycosylation treatment.

3.4. Gel-Shift Assays for Proteoglycan Attachment

1. Detection of glycosylated reporter proteins—in this cell culture system, we have never observed a minireporter protein that is 100% posttranslationally modified with carbohydrate chains. There is always a nominal percentage of protein that is secreted in its unmodified state, and this unmodified protein migrates faster on SDS-PAGE, by virtue of its lower molecular mass. Western blot analysis is used to determine the percent of glycosylation of the reporter protein, by calculating the ratio of the glycosylated protein (higher MW) over the unmodified (lower MW) isoform in the same lane (see Fig. 3b).
2. Important controls for Western blot analysis of glycoproteins—to verify that the unmodified form of a protein doublet is the lower MW protein species, the reporter construct is transfected in a parallel set of cells with and without RNAi-treatment to knock down the glycosylation machinery, to demonstrate the mobility of the same peptide sequence in the absence of glycosylation (see Fig. 3a). Parallel expression of a serine to alanine point mutant will definitively identify the modification site and is especially useful if multiple serine residues are present in the reporter.
3. Western blot analysis must be of very high quality and very sensitive. Because recombinant proteins are immuno-precipitated with anti-FLAG M2 antibody agarose, blots are probed for a second tag in the reporter protein, the S-Tag, or they are probed with anti-carbohydrate antibodies (see Note 3). High-quality nitrocellulose membranes are blocked in 0.5% casein in Tris-buffered Saline with 0.05% Tween for 2 h at room temperature. S-protein conjugated to horseradish

peroxidase is added to this blocked membrane at a 1:5,000 dilution for an overnight incubation. On the next morning, the membranes are washed 5 times with TBS-T and 1 time with TBS, before HRP detection, using the Super Signal West Femto Maximum Sensitivity substrate. All gels are run in triplicate to obtain an average densitometry scan of the fraction of the protein in the glycosylated state.

4. Notes

1. We avoid designing peptide minireporter proteins that contain cysteine residues, as they tend to multimerize in SDS-PAGE, even in the presence of reducing agents. Multimerized reporters are difficult to interpret in the Western blot analysis.
2. We favor the use of *Drosophila* S2 insect cells as our tissue culture system, as they do not require a CO_2 incubator, are very sensitive to RNA interference (using dsRNA), transfect well, and can be scaled up to large 1 L suspension culture with stable selection for transfected cells. Theoretically, cells from other insects (sf9, sf21, Hi-5) could also be used if they are available, but RNAi knockdowns will need to be tailored to that organism's glycosyltransferase cDNAs.
3. *Probing for proteoglycan sugars directly.* Such probing can be performed on duplicate Western blots, using anti-chondroitin or anti-heparin stub antibodies after chondroitin ABC or heparin lyase treatment of the anti-FLAG M2 antibody immunoprecipitates (see Subheadings 3 and 3.3, step 1). However, not all cell lines produce these terminal sugars with high efficiency, so this could be problematic for detection. Also, probing directly for terminal sugars is usually considered as a qualitative experiment, as it lacks an internal control for quantification. Therefore, Western blotting for terminal sugars may be used for confirmation of proteoglycan modification sites, but ratios of unmodified and glycosylated reports are determined by probing with the epitope tags in the fusion protein.

References

1. Brunner, A., Kolarich, D., Voglmeir, J., Paschinger, K., and Wilson, I. B. (2006) Comparative characterisation of recombinant invertebrate and vertebrate peptide O-Xylosyltransferases. *Glycoconj J.* **23**, 543–554.
2. Dong, S., G.J. Cole, and Halfter, W. (2003) Expression of collagen XVIII and localization of its glycosaminoglycan attachment sites. *J Biol Chem.* **278**, 1700–1707.
3. Wang, H., Julenius, K., Hryhorenko, J., and Hagen, F. K. (2007) Systematic Analysis of proteoglycan modification sites in Caenorhabditis elegans by scanning mutagenesis. *J Biol Chem.* **282**, 14586–14597.
4. Brinkmann, T., Weilke C. and Kleesiek, K. (1997) Recognition of acceptor proteins by UDP-D-xylose proteoglycan core protein beta-D-xylosyltransferase. *J Biol Chem.* **272**, 11171–11175.

5. McCormick, D., van der Rest, M., Goodship, J., Lozano, G., Ninomiya, Y., and Olsen, B. R. (1987) Structure of the glycosaminoglycan domain in the type IX collagen-proteoglycan. *Proc Natl Acad Sci USA*. **84**, 4044–4048.
6. Hagen, F.K., Van Wuyckhuyse B. and Tabak, L.A. (1993) Purification, cloning, and expression of a bovine UDP-GalNAc: polypeptide N-acetyl-galactosaminyltransferase. *J Biol Chem*. **268**, 18960–18965.
7. Julenius, K., Mølgaard, A., Gupta, R., and Brunak, S. (2005) Prediction, conservation analysis, and structural characterization of mammalian mucin-type O-glycosylation sites. *Glycobiology* **15**, 153–164.
8. Shakin-Eshleman, S.H., Spitalnik, S.L., and Kasturi, L. (1996) The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. *J Biol Chem*. **271**, 6363–6366.
9. Nehrke, K., Ten Hagen, K. G., Hagen, F. K., and Tabak, L. A. (1997) Charge distribution of flanking amino acids inhibits O-glycosylation of several single-site acceptors in vivo. *Glycobiology* **7**, 1053–1060.

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