Decrease of core 2 *O*-glycans on synovial lubricin in osteoarthritis reduces galectin-3 mediated crosslinking

Received for publication, February 3, 2020, and in revised form, September 11, 2020 Published, Papers in Press, September 14, 2020, DOI 10.1074/jbc.RA120.012882

Sarah A. Flowers[1,‡], Kristina A. Thomsson[1,‡], Liaqat Ali[1,‡], Shan Huang[1,‡], Yolanda Mthembu[2,‡], Suresh C. Regmi[3,‡], Jan Holgersson[2], Tannin A. Schmidt[4], Ola Rolfson[5], Lena I. Björkman[6], Martina Sundqvist[6], Anna Karlsson-Bengtsson[6,7], Gregory D. Jay[8], Thomas Eisler[9], Roman Krawetz[10,11], and Niclas G. Karlsson[1,‡,*]

From the Departments of [1]Medical Biochemistry and Cell Biology and [2]Laboratory Medicine, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, the [3]Faculty of Kinesiology, University of Calgary, Calgary, Alberta, Canada, the [4]Biomedical Engineering Department, University of Connecticut Health Center, Farmington, Connecticut, USA, the Departments of [5]Orthopaedics, Institute of Clinical Sciences and [6]Rheumatology and Inflammation Research, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, the [7]Chalmers University of Technology, Gothenburg, Sweden, the [8]Department of Emergency Medicine, Warren Alpert Medical School and Division of Biomedical Engineering, School of Engineering, Brown University, Providence, Rhode Island, USA, the [9]Department of Clinical Sciences, Danderyd Hospital, Karolinska Institute, Stockholm, Sweden, the [10]Department of Cell Biology and Anatomy, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada, and the [11]McCaig Institute for Bone and Joint Health, University of Calgary, Calgary, Alberta, Canada

Edited by Gerald W. Hart

The synovial fluid glycoprotein lubricin (also known as proteoglycan 4) is a mucin-type *O*-linked glycosylated biological lubricant implicated to be involved in osteoarthritis (OA) development. Lubricin's ability to reduce friction is related to its glycosylation consisting of sialylated and unsialylated Tn-antigens and core 1 and core 2 structures. The glycans on lubricin have also been suggested to be involved in crosslinking and stabilization of the lubricating superficial layer of cartilage by mediating interaction between lubricin and galectin-3. However, with the spectrum of glycans being found on lubricin, the glycan candidates involved in this interaction were unknown. Here, we confirm that the core 2 *O*-linked glycans mediate this lubricin–galectin-3 interaction, shown by surface plasmon resonance data indicating that recombinant lubricin (rhPRG4) devoid of core 2 structures did not bind to recombinant galectin-3. Conversely, transfection of Chinese hamster ovary cells with the core 2 GlcNAc transferase acting on a mucin-type *O*-glycoprotein displayed increased galectin-3 binding. Both the level of galectin-3 and the galectin-3 interactions with synovial lubricin were found to be decreased in late-stage OA patients, coinciding with an increase in unsialylated core 1 *O*-glycans (T-antigens) and Tn-antigens. These data suggest a defect in crosslinking of surface-active molecules in OA and provide novel insights into OA molecular pathology.

Lubricin is a large glycoprotein that is found in synovial fluid (SF). Lubricin is produced by articular chondrocytes and synoviocytes in the cartilage surface layer and secreted into the SF. In healthy joints, lubricin molecules are bound to the surface of articular cartilage, are chondroprotective, and provide lubrication at very low friction under high stress (1, 2). The lubricating properties of lubricin are dependent, in part, on dense *O*-glycosylation (3).

Lubricin, encoded by the *Prg4* gene, has been associated with pathological conditions in the joint tissue, with mutations in *Prg4* associated with camptodactyly-arthropathy-coxa vara-pericarditis syndrome (4), a rare, arthritis-like autosomal recessive disorder causing joint abnormalities. The lubrication properties of lubricin also suggest that the protein is associated with arthritic diseases, because it has been proposed that defective lubrication aggravates joint degradation (5). Osteoarthritis (OA) is the most common arthritic disease, with high prevalence in elderly people, and involves cartilage degradation in the articular joints, leading to pain and restricted motion (6). Boundary lubrication of OA SF deficient in lubricin has been shown to be lowered in *in vitro* experiments (7).

Different isoforms of lubricin are found throughout the body. In addition to presence in SF and the boundary articular cartilage, lubricin has also been detected in low amounts in menisci, blood, urine, and tendons (8–12). A possible immunological role of lubricin has been suggested because it has been shown to be involved in sepsis in a mouse model (13), being the most up-regulated protein detected in hepatic tissue. Lubricin is also associated with the plasma membrane of human neutrophils (14), and a lubricating and protective function of lubricin has been reported in ocular surfaces (15, 16).

The full-length isoform found in SF consists of 1,404 amino acids (aa), equivalent to 150 kDa, but is ~300 kDa when fully *O*-glycosylated. The protein contains a central mucin domain consisting of 59 imperfect repeats of the aa sequence EPAPTTPK, where the threonine residues are potential sites for diverse and dense *O*-glycosylation. Despite its gene name, it is not a typical proteoglycan and has merely a single proposed glycosaminoglycan site that may or may not be present/occupied (17). The mucin domain is flanked by two somatomedin B-like domains on the N-terminal side and a hemopexin domain on the C-terminal side, domains which have been proposed to interact with

This article contains supporting information.
‡These authors contributed equally to this work.
*For correspondence: Niclas G. Karlsson, niclas.karlsson@medkem.gu.se.

extracellular matrix proteins such as cartilage oligomeric matrix protein (COMP), collagen II, and fibronectin (18, 19). The glycans on the lubricin mucin domain are the main contributors to its molecular weight and are responsible for the lubricating function of the molecule, attracting water to the superficial layer, and providing repulsive negative charges to the cartilage superficial layer (20).

Recombinant human lubricin (rhPRG4) has been evaluated for a large number of potential clinical applications as a friction-reducing lubricant on biological surfaces (16). Forms of rhPRG4 have been investigated for use as an agent to prevent abdominal adhesion, a treatment of bladder impermeability, a lubricant supplement in contact lens hydrogels, and a therapeutic in patients suffering from dry eye disease (21–24). In a rodent model, addition of lubricin has been shown to delay the progression of OA (25). Intra-articular injections of rhPRG4 in guinea pigs (26) and Yucatan minipigs (26) with medial meniscal destabilization, a model system for posttraumatic OA, exhibited decreased cartilage damage and inflammation. Lubricin may therefore be a potential candidate for treatment of OA.

Galectins are glycan-binding proteins with many proposed intra- and extracellular functions within cell regulation, the immune system, and cancer progression (27). They bind a large range of β-galactosides present on both *N*- and *O*-linked glycoproteins, with binding affinity highly dependent on branching and terminal residues (28). Galectin-3 has been shown to be associated with chondrocytes and has been proposed to be involved in OA pathogenesis (29, 30). It is unique among the galectins because it is the only galectin that comprises a C-terminal carbohydrate recognition domain linked to a nonlectin N-terminal collagen-like domain, making it possible for galectin-3 protein subunits to oligomerize (31, 32). In the joint, galectin-3 and lubricin have been proposed to generate a complex, stable lubricating network on the articular cartilage (33). Limited studies have addressed galectin-3 binding to *O*-glycans. Galectin-3 displays only a weak binding affinity to the T-antigen (Galβ1-3GalNAcα1-) on synthetic MUC1 glycopeptides (34, 35). The T-antigen is also highly abundant on *O*-glycans on lubricin and has been proposed to be responsible for the interaction between galectin-3 and lubricin (33).

Because carbohydrates play such a crucial role in the function of lubricin, we decided to use state-of-the-art glycomic and glycoproteomic analysis techniques to compare the different glycoforms of synovial lubricin from OA patients and control individuals and to compare these with rhPRG4 expressed in CHO cells. We confirmed the presence of galectin-3 in SF and explored differences in the binding of galectin-3 to the various *O*-glycoforms. Our results enable us to correlate pathological glycosylation changes in OA with a functionally defective superficial layer and provide a pathway for recombinant lubricin glyco-design for optimal galectin-3 binding.

Results

Decreased core 2 and sialylation on SF lubricin from OA patients

We wanted to determine whether changes in *O*-linked glycosylation of synovial lubricin were associated with OA pathology. Detailed quantitative analysis of the released *O*-glycans was performed. Lubricin isolated from SF of controls (n = 7) was compared with lubricin isolated from late-stage OA patients (n = 7). *O*-linked oligosaccharides from lubricin in age- and sex-matched OA patients' and controls' SF were released by reductive β-elimination and analyzed with quantitative multiple reaction monitoring (MRM) on a QTRAP 6500 triple quadrupole-linear ion trap hybrid mass spectrometer as has been described in detail (36). The MRM method was developed to semi-quantify a range of 22 core 1, 2, and 3 glycans on native lubricin from both controls and OA patients (36). We refer to semi-quantification here because without the appropriate standards, a technical difficulty of *O*-glycan research, absolute quantification is not attained. This is because of differences in ionization and stability between glycans, particularly between charged and neutral glycans. MRM is able to mitigate some of this because all glycan structures, even the sialic acid isomers, are optimized for the conditions that allow for their best quantification (36). Nevertheless, the most apt use of this approach is to compare the same glycan or type of glycans between samples.

Although the abundances of these glycans were found to differ between the two sample types, the glycan repertoire was the same (Fig. 1, *A* and *B* and Table S1). A significant increase in the unmodified core 1 structure (T-antigen) on lubricin isolated from OA patients (40.41% $\pm$ 3.43% (mean $\pm$ S.E.) of all glycans, p = 0.0274) was detected compared with normal controls (29.90% $\pm$ 3.10%). This increase in glycan truncation was accompanied by a significant decrease in a range of lower abundant core 2 structures, predominantly sialylated and sulfated structures. There was an overall reduction in core 2 structures in OA samples (4.67% $\pm$ 0.98%, p = 0.0288) compared with control samples (9.11% $\pm$ 1.50%) and an increase in core 1 structures (OA, 94.85% $\pm$ 1.05% and control, 90.32% $\pm$ 1.47%; p = 0.0275). Altogether, this resulted in a trend toward a reduction in charged structures, primarily because of a reduction in core 2 structures, in the OA samples (57.16% $\pm$ 3.03%, p = 0.558) compared with the controls (66.51% $\pm$ 3.21%). This indicated that changes in *O*-glycosylation, specifically the loss of core 2 structures that are predominately charged, is associated with OA.

CHO-expressed rhPRG4 has mainly sialylated core 1 O-glycans

Given the potential of rhPRG4 as a biopharmaceutical lubricant, where the function relies on the *O*-linked glycosylation, we analyzed the released glycans from CHO-expressed rhPRG4 to identify the presence of core 1 and core 2 glycans and sialylation and compare it to *O*-glycosylation of the native human SF lubricin.

The composition of *O*-glycans released from rhPRG4 was analyzed using the same MRM lubricin method as for native human lubricin. These analyses were performed on two different batches of rhPRG4, which revealed a composition of solely core 1 structures (Fig. 1, *C* and *D*), the same core 1 structures observed on human synovial lubricin (Fig. 1, *A* and *B*). The most abundant oligosaccharide was the sialylated core 1

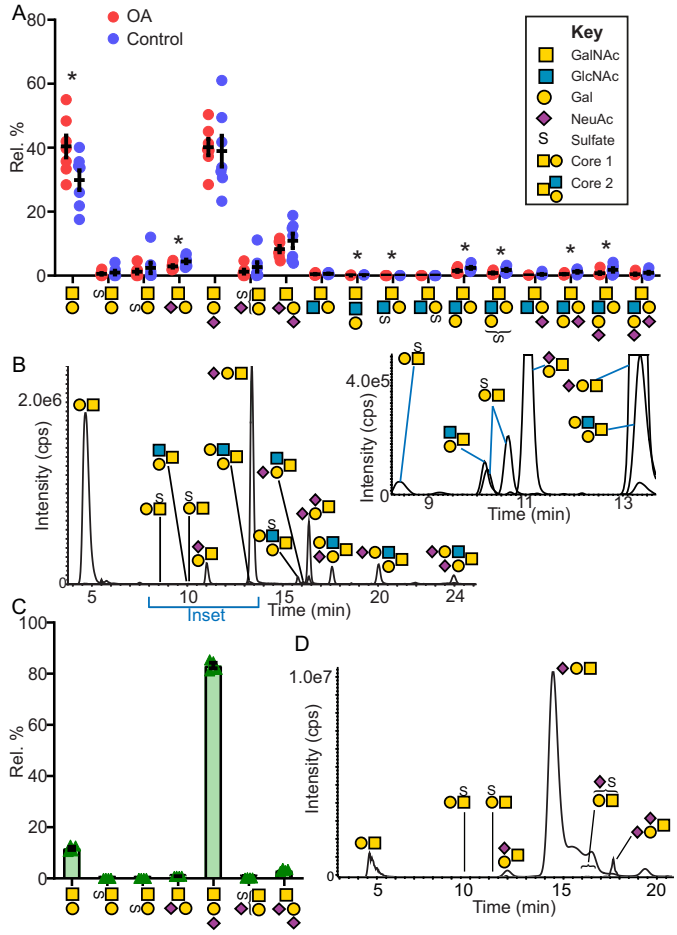

Figure 1. MRM analyses of *O*-glycans from SF and recombinant lubricin. *A*, relative quantification by MRM of released *O*-glycans from synovial lubricin purified from control and OA individuals. Relative quantification comparison of OA patients ($n = 7$, *red*) and control individuals ($n = 7$, *blue*) released glycans from SF lubricin showing core 1 and core 2 *O*-glycans, with the most dominant structures being the unmodified and monosialylated core 1. Graph shows individual data points, average, and S.E. *$p \leq 0.05$. Statistical analyses, two-tailed Student's *t* tests, were carried out to compare the normal and OA synovial lubricin glycan relative abundances. *Symbol key* is according to the Symbol Nomenclature for Glycans. *B*, example of extracted ion chromatogram of *O*-glycans from lubricin from a control individual. Inset is zoomed in area of low intensity MRM transitions from 8.5–13.5 min. *C*, relative quantification by MRM of released *O*-glycans from rhPRG4 showing only core 1 structures and an abundance of the linear monosialylated structure. Data are from two batches, each repeated in duplicate. Graph shows individual data points, average, and S.E. *D*, example of extracted ion chromatogram of rPRG4. For key of symbols, see (*A*).

NeuAcα2-3Galβ1-3GalNAc structure, which made up 85% of all glycans. A low proportion of neutral *O*-glycans (12% of all glycans) and a higher amount of charged glycans (88% of all glycans) were found in this rhPRG4. The glycosylation pattern of rhPRG4 is similar to what has been reported for CHO cell *O*-glycosylation with an abundance of core 1 structures, particularly the linear monosialylated NeuAcα2-3Galβ1-3GalNAc structure (37).

Glycoproteomics reveal location of Tn-antigens, sialyl-Tn, and core 1 structures both within and outside the rhPRG4 mucin domain

We next performed a glycoproteomics study of rhPRG4 (with and without partial deglycosylation) to identify glycosylated aa sites and compared the results with glycosites previously reported for native human lubricin from pooled SF from OA and rheumatoid arthritis (RA) patients (38). In all, we detected 318 glycopeptides from rhPRG4 covering 172 glycosylated Ser/Thr sites, the monosaccharide compositions consistent with core 1 type *O*-glycans GalNAcα1- (Tn-antigen), Galβ1-3GalNAcα1- (T-antigen), NeuAcα2-3Galβ1-3GalNAcα1- (sialyl-T), and NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcα1- (disialylated T). The results are summarized in Fig. 2 and in Table S2. Glycopeptide coverage in rhPRG4 was found to be similar to native human lubricin, with the majority of the glycopeptides originating from the Ser/Thr-rich mucin domain (aa 232–1056, Fig. 2*A*). Site specificity overlapped well with native lubricin and within the mucin domain (Fig. 2, *B* and *C* and Fig. S1). 83 sites were detected in rhPRG4, which had not previously been identified in native lubricin (Fig. 2*B*). The majority of the glycopeptides contained 1–3 glycans, although up to five glycosylation sites were detected on one peptide (TTPETTTAAPK, aa sequence position 926–936). Glycopeptides generated after partial deglycosylation revealed additional site-specific information, enabling the detection of larger glycopeptides in the size range of 10–20 aa:s. In all, 31 additional glycosylated Ser/Thr were identified after partial deglycosylation (Table S2). Approximately 77% (132 of the 172) of the glycosylated Ser/Thr were found in peptides originating from the mucin-like repeat region (aa 348–855, Fig. 2*A*) of rhPRG4. The most abundant aa sequence in this region was the EPAPTTPK, repeated 17 times in the molecule. 13 EPAPTTPK-derived glycopeptides were detected and glycosylated on either or both Thr residues (Table S2). We found 19 glycosylation sites in the N-terminal region (aa 125–347) and 26 in the C-terminal region (aa 1057–1404). Most of these sites (16 and 25 sites from the N- and C-terminal regions, respectively) were found in the nonrepeat regions in the STP-rich domain flanking the mucin domain 348–1057 (Fig. S1). The most intense glycopeptides identified in rhPRG4 correspond well with those identified from pooled SF from patients with RA and OA. The main difference was that in regions with clustered Thr and to some extent also Ser, we usually found that more sites were positively identified in rhPRG4 compared with native lubricin.

Glycoproteomics of SF lubricin from OA patients

Because previous glycopeptide analysis was performed on pooled SF samples (38), we pursued more sensitive MS analyses using higher-energy collisional dissociation (HCD) MS and MS/MS, addressing the characterization of SF lubricin glycopeptides from two individual OA patients. This approach revealed 171 and 158 lubricin-derived glycopeptides from the two patients, respectively, and the full glycopeptide list is found in Table S2. Glycopeptide coverage did largely match what was found in the samples derived from rhPRG4 and our previous study of pooled native lubricin from OA and RA patients (Fig. 2*A* and Table S2), suggesting that many glycosylation sites coincide.

To compare SF lubricin and rhPRG4 glycopeptide analyses, the peptide backbone sequence VLAKPTPK (aa 327–334,

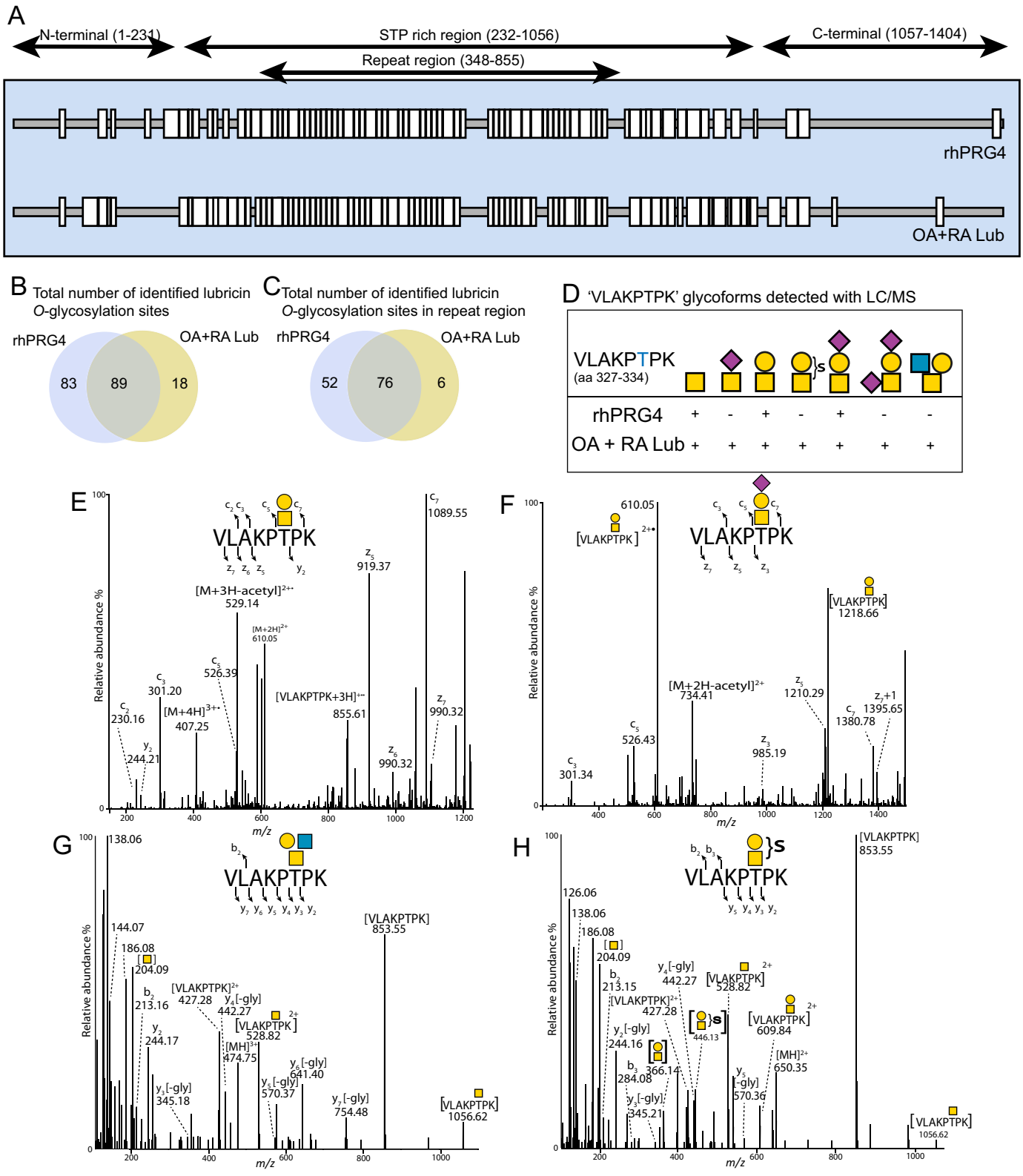

Figure 2. *O*-linked glycosite map of rhPRG4 compared with synovial lubricin. *A*, glycopeptides from recombinant (rhPRG4) (this report) and SF lubricin pooled from five patients with OA and RA (this report and Ali et al.) were analyzed with LC-MS with CID and ETD fragmentation before and after partial deglycosylation with sialidase and *O*-glycanase. *B*, total number of *O*-glycosylation sites confirmed by ETD in rhPRG4 and from previous analysis of SF lubricin from pooled RA and OA patients on whole lubricin and (*C*) in the repeat region (aa 348–855) (*D*). Inserted table shows the glycovariant of the glycopeptide VLAKPTPK found in SF lubricin and in rhPRG4. *E–H*, examples MS/MS of the glycopeptides variants of VLAKPTPK from lubricin identified from rhPRG4 (ETD) and from an OA patient (HCD); *m/z* at 406.90 (3+) from rhPRG4 interpreted as a T-antigen (Galβ1-3GalNAcα1-) (*E*); *m/z* at 503.93 (3+) from rhPRG4 interpreted as sialyl-T (NeuAcα2-3Galβ1-3GalNAcα1-) (*F*); *m/z* 474.59 (3+) interpreted as a core 2 glycan (Galβ1-3(GlcNAcβ1–6)GalNAcα1-) from an OA patient (G); *m/z* 649.83 (2+) interpreted as a sulfated core 1 glycan (HSO_3 + Galβ1-3GalNAcα1-) from an OA patient (*H*). For key of symbols, see Fig. 1*A*.

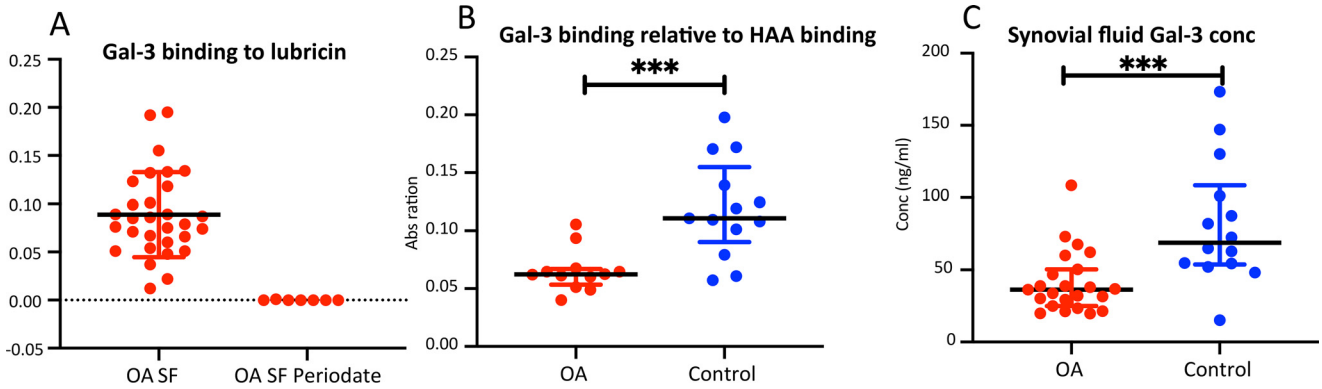

Figure 3. Lubricin *O*-glycans and Galectin-3. *A*, recombinant galectin-3 binding to OA lubricin. Galectin-3 binding to lubricin from SF of late-stage OA patients ($n = 30$) was measured using a sandwich lectin ELISA assay (see "Experimental procedures"). Treatment of OA SF ($n = 8$) with periodic acid shows that the binding to galectin-3 was glycosylation dependent. *B*, increase of extended structures binding to galectin-3 relative to unextended GalNAcα1-Ser/Thr (measured by HAA lectin) present on lubricin from late-stage OA patients ($n = 12$, *red*) and control SF ($n = 13$, *blue*). Ratio of the absorbances at 450 nm were calculated for each sample. Samples were measured in duplicates. *C*, endogenous galectin-3 in SF. Galectin-3 concentrations in SF were measured in samples from 23 late-stage OA patients (*red*) and 14 controls (*blue*). Data in this figure is represented as median with interquartile range, and significant difference was calculated by nonparametric Mann-Whitney test with $^{***}p < 0.001$.

Fig. 2*D*) will be used as an example. Seven VLAKPTPK glycopeptides were identified in each of the two sample types. This peptide backbone sequence contains only one potential *O*-glycosylation site (T). In addition to simple core 1 structures identified in rhPRG4 (Fig. 2, *E* and *F*), we also detected more exotic type *O*–linked glycans attached to native lubricin. Examples of two of these spectra are shown in Fig. 2, *G* and *H*. Both spectra were dominated by a major fragment ion corresponding to the peptide backbone without its glycosylation (m/z 853.55). The core 2 glycopeptide variant of this glycopeptide was identified containing two *N*-acetylhexosamine (HexNAc) and one hexose (Hex), consistent with a (Galβ1-3(GlcNAcβ1–6)GalNAcα1-) core 2 sequence. Low molecular oxonium ions at m/z 138, 144, 168, 186, and 204 originated from fragmentation of monosaccharide residues and were diagnostic for glycan containing peptides. The other example from SF lubricin is a VLAKPTPK glycopeptide with a T-antigen with an associated sulfate residue (Fig. 2*H*). We have previously shown that sulfated core 1 *O*-glycans exist on SF lubricin both as 3-linked and 6-linked sulfates to the nonreducing end Gal residue but also to the 6-position of the reducing end GalNAc residue (39). The diagnostic glycan oxonium ions at m/z 204, 366, and 446 were glycan fragment ions consisting of HexNAc, HexNAc-Hex, and HexNAc-Hex + sulfate, respectively. This peptide example illustrates that sulfoglycopeptides can successfully be identified among other glycopeptides in positive ion mode without targeted approaches that have been suggested (40).

One *N*-glycosylated site was found on lubricin from both recombinant and OA patient–derived lubricin at Asn-1159. The two dominant glycoforms found in both samples were of high mannose–type $Hex_5HexNAc_2$ and $Hex_6HexNAc_2$, respectively. Annotated HCD and collision-induced fragmentation (CID) spectra of these are displayed in Fig. S2 and Fig. S3.

Lubricin glycosylation identified using lectin ELISA

We investigated the use of lectins to study lubricin glycosylation based on the knowledge generated by glycomics and glycoproteomics. With glycomics, singly and doubly sialylated core 1 and core 2 structures were readily detected. Detection of Tn-antigens on lubricin was based on glycoproteomic analysis. We selected peanut agglutinin (PNA) (Galβ1-3GalNAcα1-), galectin-3 (Galβ-1GlcNAcβ1-), Sambucus nigra agglutinin (SNA) (NeuAcα2-6GalNAcα1-), and Helix aspersa agglutinin (HAA) (GalNAcα1-) based on detected lubricin oligosaccharides. We used them in a sandwich ELISA using anti-lubricin 9G3 antibody. Additional lectins included Aleuria aurantia lectin (AAL) (Fucα1-3/6) and L-selectin (sialyl 6-sulfo Lewis X) because it has been shown that lubricin contains both fucosylated and sulfated structures (14). Our lectin sandwich ELISA was used to probe the glycosylation of lubricin from OA patients' and controls' SF (Fig. S4). We found that lectins, corresponding to less-abundant structures detected by MS glycomics, varied the most. For galectin-3, AAL, and L-selectin, the percents coefficient of variation (CV) were found to be 147% (OA patients)/141% (controls) and 78% (OA patients)/120% (controls), respectively. For SNA and PNA, the percents CV varied less: 36% (OA patients)/74% (controls) and 25% (OA patients)/40% (controls), respectively. HAA lectin binding showed the least variation, with percents CV of 37% and 34% in controls and OA patients, respectively. No significant differences were detected in the ELISA response between different glycoforms of SF lubricin ($n = 6$ controls, $n = 8$ OA patients; Fig. S4).

The combined conclusion from MS glycomics, glycoproteomics, and lectin sandwich ELISA is that Tn-antigen, T-antigen, and T-antigen's sialylated version are by far the dominating structures on lubricin, and that pathologic OA changes manifest as lowered level of core-2 structures and increased level of unsialylated Tn- and T-antigens.

Galectin-3 binding to lubricin and endogenous galectin-3 in SF

We further investigated the ability of lubricin to bind galectin-3. SF from 30 OA patients was probed with recombinant galectin-3 using the lectin ELISA assay. We found that the level of galectin-3 active lubricin present in SF varied ~1–2 orders of magnitude between these individuals (Fig. 3*A*).

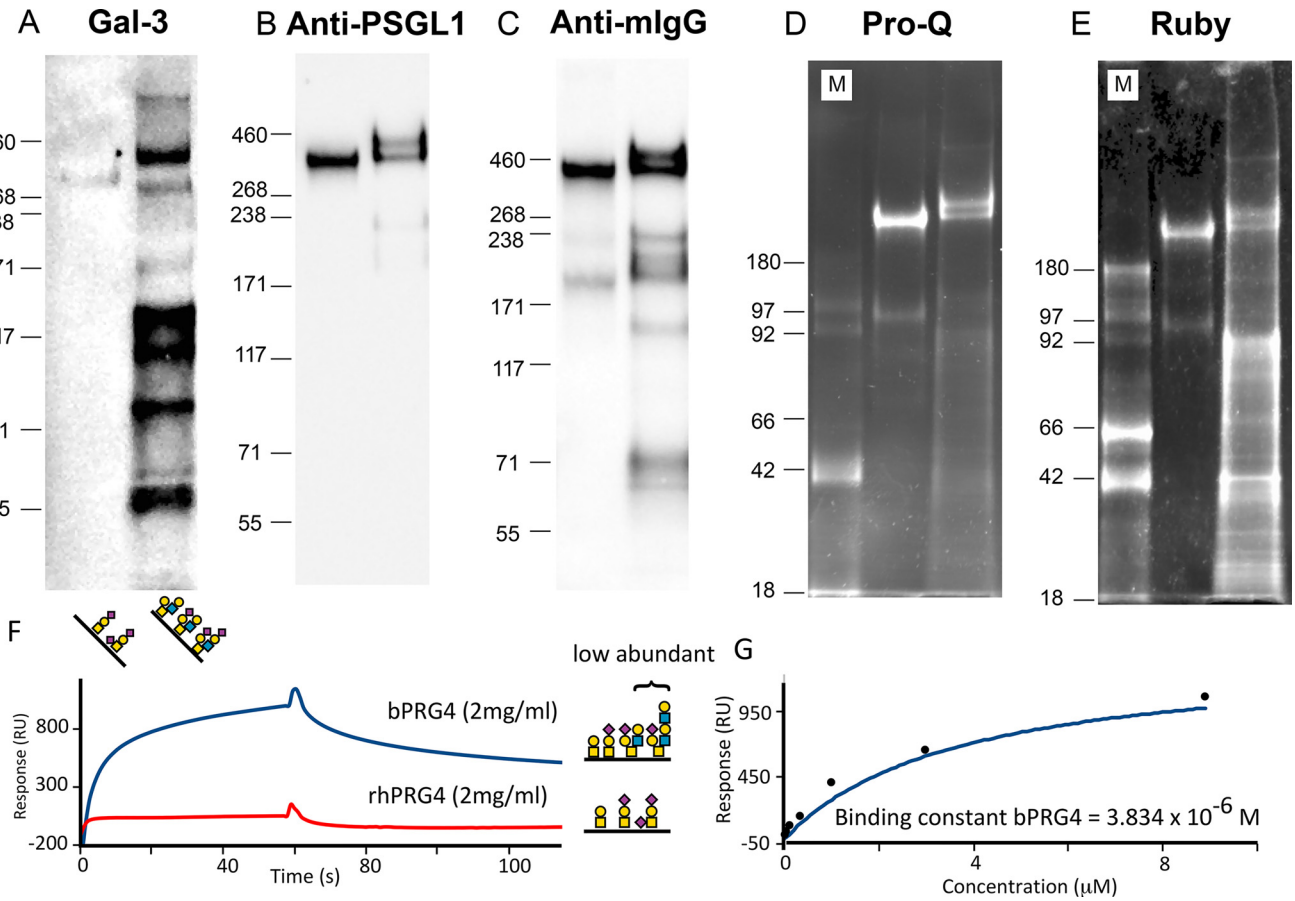

Figure 4. Galectin-3 binding to core 2 glycans. To display the difference of galectin-3 binding between core 1 and core 2, 1 μg of PSGL-1/mIgG2b (with core 1 glycans) (*lane 1*) and PSGL-1/mIgG2b + GCNT1 transferase (with core 2 glycans) (*lane* 2), immunoprecipitated from CHO tissue culture, were loaded, separated, blotted, and subsequently stained with biotinylated galectin-3 (*panel A*). Below the lanes, representative oligosaccharide cartoons show dominant *O*-glycans found on the two PSGL-1/mIgG2b glycoforms as described elsewhere. PSGL-1/mIgG2b monomers were detected in the area between 268–460 kDa, showing intense staining of the PSGL-1/mIgG2b expressed in CHO cells transfected with the GCNT1 transferase but less staining with the PSGL-1/mIgG2b with core 1 glycans. The same samples (50 ng each) were also separated, blotted, and stained by anti-PSGL1 (*panel B*) and anti-mouse IgG (*mIgG*) (*panel C*) antibodies in separate experiments, confirming similar concentration of the two constructs. The figure represents a typical result performed under optimized conditions from a selection of three repeats. For control, samples (1 μg each) loaded in the same order were separated and gels were stained for sugars using Pro-Q Emerald (*panel D*) and for proteins using Ruby (*panel E*). The gels include a molecular weight marker lane (*M*) identifying the PSGL-1/mIgG2b proteins with a molecular mass >180 kDa. *F*, SPR experiment showing binding of bovine lubricin (bPRG4) to recombinant galectin-3, whereas rhPRG4 did not bind. Inserted are cartoons showing prominent *O*-glycans on the two proteins, with symbol key displayed in Fig. 1*A*. *G*, calculation of binding constant of bPRG4/galectin-3 from SPR based on molecular mass of bPRG4 estimated to 220 kDa.

Periodate treatment, which destroys the glycans, abolished the binding, proving that the interaction was purely dependent on glycosylation (Fig. 3*A*). Because our glycoproteomic analysis showed that the Tn-antigen was present on lubricin, we investigated whether there was a difference between OA patients and controls in how much of this biosynthetic precursor was further processed into core 2. The relative proportion of galectin-3 binding (core 2) against the binding to the HAA lectin (Tn-antigen) showed that the glycosylation of lubricin from late-stage OA (n = 12) compared with the controls (n = 13) had changed. The relative binding to galectin-3 compared with HAA decreased by almost a factor of 2 in OA patients ($p <$ 0.001, Fig. 3*B*). These data corroborate the shortening of glycans in OA observed by glycomics where core 2 glycans were decreased relative to T-antigen (Fig. 1). Here we show by using HAA that core 2 glycans, measured by galectin-3, were decreased relative to the truncated Tn-antigen (Fig. 3*B*). Together this suggests that lubricin carried fewer core 2 glycans in OA on a molecular basis.

Because galectin-3 has been reported to be present in SF (41), we speculated that the difference in binding of galectin-3 to lubricin may have functional consequences. Hence, we went on to also establish the level of SF galectin-3 in our sample set. The data generated showed a significant decrease (50%) in the total amount of endogenous galectin-3 in SF of the late-stage OA (n = 23) compared with controls (n = 14) (Fig. 3*C*). Our data suggest that both the level of galectin-3 and its ability to bind SF lubricin are altered as a consequence of OA.

Galectin-3 and core 2 glycosylation

A potential functional impact of the altered OA glycosylation identified was investigated further. Lubricin glycans are essential for several critical properties of lubricin involving lubrication and binding properties and have been suggested to bind to galectin-3 to improve lubrication of the joint via the abundant T-antigen epitopes (Galβ1-3GalNAc1α-) (33). However, we observed decreased lubricin binding to galectin-3 in OA in

parallel with an increase of this glycan on OA lubricin, which led us to test the hypothesis that the lubricin–galectin-3 interaction may be mediated by epitopes presented on extended core 2 glycans.

To address whether core 2 glycans compared with the simpler core 1 glycans were responsible for the galectin-3 binding, we used recombinant glycotechnology. We used a CHO cell line stably transfected by the human core 2 GlcNAcβ1–6 glycosyltransferase shown to convert the native CHO cell core 1 glycosylation into sialylated core 2 with over 90% efficiency (42). A reporter *O*-linked mucin-type glycoprotein (PSGL-1/mIgG2b) expressed in these cells was shown to be able to bind recombinant galectin-3 (Fig. 4, *A–E*). Without transfection of the glycosyltransferase, only low binding to PSGL-1/mIgG2b was observed (Fig. 4*A*, *first lane*). This demonstrated that core 2 type oligosaccharides are key for recognition by galectin-3.

Further support for this was provided by surface plasmon resonance (SPR). The core 1 recombinant version of lubricin CHO-rhPRG4 glycosylation was not able to bind galectin-3, whereas the positive control of lubricin isolated from bovine chondrocyte cell culture (bPRG4) with both core 1 and core 2 glycosylation (Table S3) displayed significant interaction with galectin-3 with a K_D calculated to 3.8 μM (Fig. 4, *F* and *G*). The data together indicate that the avidity of the multimeric galectin-3 relies on numerous epitopes within lubricin and its mucin domain. The PSGL-1/mIgG2b *O*-linked oligosaccharides generated after transfection of CHO cells using human core 2 GlcNAcβ1–6 glycosyltransferase terminate with sialic acid after adding a LacNAc unit to the natively expressed core 1 structures (42). This is also the type of core 2 structure found on SF lubricin (36).

Discussion

Altered glycosylation and lubricin level in OA

The data from lectin ELISA suggested that the glycosylation in late-stage OA had changed compared with controls (Fig. 3*B*). However, using only two lectins (HAA and galectin-3) to display this change makes it difficult to pinpoint the overall nature of this glycosylation change. For this, the MRM semi-quantification of *O*-glycans from SF lubricin gave more information, demonstrating a 10% increase of the truncated unsialylated core 1 structures (Galβ1-3GalNAcα1-) in late-stage OA, accompanied by a decrease of low-abundance core 2 glycans (Fig. 1*A*). This MRM result and the data from the lectin ELISA suggest that the Tn-antigen (GalNAcα1-) is also increased at the expense of larger structures including both core 1 and core 2. However, because the lectin ELISA is limited in its ability to quantify the level of lubricin in the sample, the absolute levels of the various Tn-antigen, core 1, and/or core 2 structures in lubricin are difficult to appreciate. The fact that glycosylation of lubricin is changing makes the quantification of lubricin difficult. Current methods for determining lubricin concentration are often based on antibodies targeting the changing mucin domain and/or lectins for detection or capturing (43). Hence, the glycosylation change of lubricin may have contributed to the confusion in the literature, where the level of SF lubricin has been shown to be increased, decreased, or unaltered in OA (43).

Functional implications of lubricin glycosylation

It has been demonstrated previously that the high number of negatively charged sialic acid residues located within the center of the lubricin molecule with positively charged lysine- and arginine-rich N- and C- termini creates the amphoteric nature of the lubricin glycoprotein (38). The glycosylation, along with the organization of sialic acid within the molecule, has been associated with the effective lubrication property of lubricin (38, 44). This organization creates a central hydration shell and sparingly glycosylated N- and C- termini available to bind to other cartilage proteins including COMP and collagen II. An effective boundary lubrication model with firm adherence to the articular cartilage surface of glyco-mediated lubrication can be proposed (18, 19, 38). The reduction in charged residues in OA observed in this report may affect the overall charge and potentially the lubricating properties of the lubricin molecule in OA.

Our data on SF lubricin *O*-glycan truncation in OA also bear implications on binding to galectin-3. Previous studies have indicated that galectin-3 exhibits only weak affinity to *O*-glycoproteins and *O*-glycopeptides expressing single core 1 *O*-glycans (Galβ1-3GalNAcα1- or NeuAcα2-3Galβ1-3GalNAcα1-) (35, 45). Instead, internal LacNAc glycans on extended oligosaccharides have been described to be the high-affinity ligands for galectin-3 (46). Here, we suggest that interaction between galectin-3 and native lubricin is mediated by short single LacNAc extended core 2 structures. Indeed, expression of the core 2β-1,6-*N*-acetylglucosaminyltransferase (C2GnT) responsible for biosynthesis of core 2 *O*-glycans has been shown to enable binding of galectin-3 to bladder tumor cells (47).

rhPRG4 has been shown to have similar lubrication properties as bPRG4 *in vitro* (48). Hence, the proposed galectin-3 binding to lubricin (33) is likely to only fine-tune the boundary-lubricating glycosurface of the joint.

In regard to galectin-3 levels in SF, it has also been shown that patients with RA have increased SF galectin-3 (41, 49, 50), whereas the literature is less conclusive about the levels in OA. Our data indicate that compared with normal SF, the galectin-3 levels are diminished in late-stage OA. Our hypothesis suggests that an altered ability of OA lubricin to bind galectin-3 together with decreased galectin-3 levels in OA may contribute to destabilization of boundary lubrication and contribute to joint degradation (Fig. 5). However, this notion needs to be reconciled with results from a large animal OA model where CHO cell–derived rhPRG4 was shown to retard cartilage degeneration (26). Our data show that glycomic and glycoproteomic analyses provide the ability to unravel the multifaceted enigmatic OA etiology and pathology. Of note, this is even without taking into account that cartilage harbors the largest volume of glycans (keratan sulfate and chondroitin sulfate) in the body.

Glycomap of native lubricin

A previous map of native lubricin was performed on tryptic and Lys-C–digested *O*-glycopeptides of intact and partially deglycosylated glycopeptides from a pooled sample (38). Here,

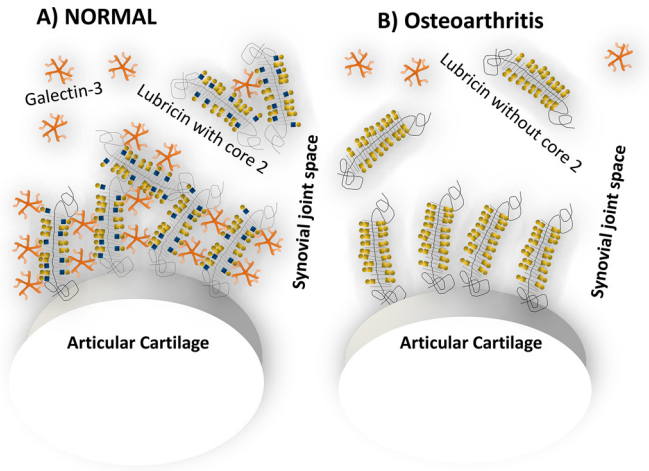

Figure 5. Proposed model for the organization of lubricin and galectin-3 in the superficial articular layer in normal joint and in OA. *A*, in the normal joint, synovial lubricin mostly secreted by synoviocytes and chondrocytes adheres to the cartilage surface and contains a significant amount of LacNAc epitopes (represented as *linked blue square and yellow circle*) that allows galectin-3 interaction and crosslinking of lubricin molecules. *B*, in OA, lubricin with changed glycosylation because of an altered glycosylation machinery in synoviocytes and/or chondrocytes is still capable of adhering to the cartilage and provide boundary lubrication, but the decreased amount of galectin-3 and its decreased interaction with lubricin with lower amount of LacNAc destabilize the glycosylated articular surface.

we included sites identified on native lubricin from individual patients using a fast-scanning Orbitrap mass spectrometer to make the glycosite map more complete. Because of fast scanning and sensitivity, low-abundance glycoforms could be selected for MS/MS without any prefractionation and multiple digestion strategies. We identified 171 and 158 glycopeptides from the two lubricin tryptic digests from OA patients, many of which had not been detected before. This sensitive approach allowed the first detection of a sulfated *O*-glycopeptide from lubricin (Fig. 2) and evidence for an *N*-glycosylation site on lubricin (Fig. S1 and Fig. S2). In our previous study of pooled lubricin from both OA and RA patients, we reported 185 *O*-glycopeptides, yet after excluding those glycopeptides that were enzymatically deglycosylated, this number dropped down to 122. However, partial deglycosylation may still need to be carried out to identify sites within the mucin domain. For example, the glycopeptides with aa sequences EPAPTTTK, KPAPTTPK, and SAPTTPK, which are found repeated eight, four, and four times in the protein, respectively, were either not detected or poorly represented in the analyses of individual OA patients but were found in both rhPRG4 and in our previous study after deglycosylation. In this paper we identified eight sites in native lubricin in addition to already recorded sites (38).

The rhPRG4 molecule

The analyses here showed the detailed glycomic and glycoproteomic analyses of the heavily *O*-glycosylated CHO expressed protein lubricin giving glycosite-specific information and detailed *O*-glycan identification and quantification. Obtained glycopeptides from rhPRG4 digested with trypsin or Lys-C were analyzed with LC–MS/MS and CID/electron-transfer dissociation (ETD) fragmentation before and after partial deglycosylation. In total, 172 glycosylation sites (Ser/Thr) were identified compared with the previously reported 107 for native lubricin obtained with the same approach (38). The majority of the glycosylation sites of rhPRG4 were found on peptides that were repeated between 2–17 times in lubricin mucin domain. The dominant glycans found corresponded to simple core 1 type *O*-glycosylation, whereas native lubricin also contained core 2. The difference in glycosylation sites is probably a reflection of differences in specificity and level of GalNAcα1-polypeptide transferases. Human synovial cells are dominated by T1-, T2-, T5-, and T15-GalNAc transferase (38), whereas CHO cell glycosylation predominantly expresses the hamster equivalent of human T2-transferase (51). We may also attribute differences in detected glycosylation sites to the increased glycosylation complexity of native lubricin compared with rhPRG4. In addition, the decreased complexity of glycans in rhPRG4 would cause decreased glycopeptide diversity leading to increased molar amounts and detectability of individual glycopeptides. In addition, the differences in the level of glycosylation could also contribute to differences in efficiency in the digestion of the mucin domain.

Our glycoproteomic data shows that glycans on rhPRG4 are positioned in the same central domain as in synovial lubricin. Lubricin (both native and recombinant) contains one of the largest mucin domains where glycosylation sites have been mapped. In synovial lubricin, 37% of Ser/Thr residues were glycosylated in the STP-rich region aa 232–1056, whereas in rhPRG4, 60% of Ser/Thr residues were found to be glycosylated. However, we need to state that the site localization relies on positive identification, so absence of detection does not mean that remaining sites are not glycosylated.

Differences in glycosylation between native lubricin and rhPRG4 invoke a question about the impact of glycosylation on the N- and C- termini involved in interactions. Lubricin has, for example, been shown to bind noncovalently to COMP within aa 105–202 and covalently by specific disulfide bonds between aa 64–86 (19). Glycosylation sites were identified just adjacent to or within these regions (Table S2).

The *O*-glycomic data shows that rhPRG4 glycosylation is dominated by the linear monosialylated core 1 structure, similar to native synovial lubricin. rhPRG4, as such, contains the main molecular functions attributed to native lubricin. Although there is a reduction in disialylated structures, there is an overall increase in sialylation, giving the rhPRG4 molecule a more negatively charged central domain similar to SF lubricin from control individuals rather than lubricin found in OA patients (Fig. 1). However, rhPRG4 does have a more limited range of glycans with no core 2 or extended core 1 structures present. These structures may be involved in other glycan-glycan interactions with the ECM, interacting with galectin-3, or involved in the immune system (14). Overall, it is clear that both the type and the specific pattern of glycosylation are quality attributes to the lubrication and binding function of this very heavily glycosylated protein. The data indicate that glyco-design could help to further improve functionality of rhPRG4. Active modulation of *O*-linked glycosylation for biologics is an unexplored area. As of yet, it is unknown whether these changes of lubricin in SF are deleterious to the function of the molecule *in vivo*. It is also unknown whether

alterations in lubricin glycosylation are a biological response to OA conditions, where the change in negative charges is an attempt to alter the lubricating function of the expressed lubricin. This could be because of a metabolic response to simplify and maintain expression of a less energetically expensive molecule in the face of exogenous stress or to potentiate downstream signaling through altered galectin-3 release. Given the complexity of timing and severity of effects on biological functions within the OA joint, it can be summarized that there are important alterations in the amount and type of glycosylation in a critical lubricating system and that functional and inflammatory aspects of the disease are likely regulated by the attached sugars, all of which warrant additional research and focus for heretofore unexplored pathogenic mechanisms in a very serious disease.

Experimental procedures

Samples, human tissues, and cells

rhPRG4 expressed from CHO cells was purified as described elsewhere (2, 52). bPRG4 was purified using CsCl gradient and anion exchange chromatography, and purity was confirmed with SDS-PAGE/Western blotting and MS as described elsewhere (53). Recombinant galectin-3 used in all experiments unless stated elsewise was provided by Professor Haakon Leffler (Lund University, Sweden). SF samples from OA patients that were scheduled for total knee replacement (n = 2, 76 and 84 years old) for the glycopeptide analyses were collected during therapeutic joint aspiration at Sahlgrenska University Hospital (Gothenburg, Sweden). SF samples for *O*-glycomic analyses were collected from patients with symptomatic chronic knee OA requiring aspiration (OA patients n = 7, average age 56 years, range 34–72 years). The patients for *O*-glycomics were diagnosed as having knee OA by two sports medicine physicians following a review of the patients' symptoms, a physical examination, and plain-film radiography. Control samples (n = 7, average age 56 years, range 31–72 years old) were collected postmortem within 4 h. The samples were collected from the knee by aspiration, clarified (3,000 × g for 30 min at 4 °C), and stored at −80 °C. SF samples for galectin-3 studies were obtained from late-stage OA patients prior to total knee replacement, mean age 69 years (n = 30, range 58–78 years old) at Sahlgrenska University Hospital and at Danderyd Hospital (Stockholm, Sweden). Control SF samples were collected as described above, mean age 65 years (n = 18, range 47–76 years old). All samples were collected after written consent from patients and controls, and the procedure was approved by the Conjoint Health Research Ethics Board of the University of Calgary (REB15–0880) or by the Regional Ethical Review Board in Gothenburg (ethical application 172-15); the study abided by the Declaration of Helsinki principles.

Release and LC-MRM analysis of oligosaccharides from rhPRG4 and synovial lubricin

Lubricin from SF of OA patients (n = 7) and controls (n = 7) were isolated via a multistage process. The same amount of SF (500 μl) was used for each sample to allow for consistent MRM analyses on the resultant released glycans. The acidic SF proteins were isolated from SF samples by diethylaminoethyl chromatography as previously described (54). The samples were reduced and alkylated and separated by SDS-PAGE using NuPAGE 3–8% Tris acetate gels to allow isolation of the lubricin. The gels were transferred to PVDF membrane by semi-dry transfer blotting, stained with Alcian blue, and destained with methanol (55). The lubricin band was excised from each sample individually and the *O*-linked oligosaccharides were released by reductive β-elimination (50 mM sodium hydroxide and 0.50 M sodium borohydride) overnight, followed by cleanup on cation exchange columns (AG50WX8, Bio-Rad) in C18-ZipTips (Millipore) (56). Oligosaccharides with reducing end (alditols) were separated on porous graphitized carbon columns (5-μm particles, Hypercarb, Thermo Fisher Scientific) prepared in-house, with the dimensions of 10 cm (length) and 250 μm (inner diameter). The gradient, after 5 min of 98% solvent A (10 mM ammonium bicarbonate), increased with solvent B (80% acetonitrile in 10 mM ammonium bicarbonate) from 2–45% in 41 min, then to 95% solvent B in 4 min. Solvent B (95%) was held for 5 min before re-equilibration at 98% A for 35 min. The flow rate was kept at a constant 10 μl/min using an Ekspert MicroLC 200 HPLC system (Eksigent, AB Sciex, Framingham, MA, USA).

The oligosaccharides were analyzed on a QTRAP 6500 ESI-triple quadrupole-linear ion trap hybrid mass spectrometer (AB Sciex) in negative-ion, high-mass mode. A turbo V ion source was used with a 25-μm electrode. An ion spray voltage of 4,200 was used. Collision energy, declustering potential, and collision cell exit potential were optimized for each transition. The final method included transitions to quantify and identify all 24 identified structures on lubricin. All transition information is published in detail (36).

Relative quantitation and statistical analyses of MRM data

MultiQuant 3.0 (Sciex) was used for MRM peak integration. Area under the curve was determined for all structures and combined for total glycan abundance. Each glycan was then represented as a relative percentage of the total glycan abundance. Statistical analyses, two-tailed t tests, were carried out to compare the normal and OA synovial lubricin glycan relative abundances. GraphPad Prism 5.0 for windows (GraphPad Software) was used to carry out statistical analyses and to draw graphs.

Release and analysis of oligosaccharides from bPRG4

Oligosaccharides from bPRG4 (60 μg) were released in 1.0 M $NaBH_4$/0.10 M NaOH at 50 °C (100 μl) overnight, followed by neutralization with concentrated acetic acid, and desalted with 150 μl of cation exchange media AG50WX8 in a 100-mg C18 Strata SPE column (Phenomenex). The glycans were analyzed with LC-MS and MS/MS using porous graphitized carbon chromatography as described above, connected to an LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific) as described elsewhere (14). Structures were assigned based on MS/MS fragmentation by comparison with fragment spectra available in UniCarb-DB (57) and -DR (58) and by manual interpretation aided by using GlycoWorkbench software (59, 60). Assignment of structural data based on MS interpretation was recorded according to reporting guidelines (MIRAGE) for glycomics sample preparation (61) and MS (58, 62).

Purification, digestion, and LC-MS analysis of glycopeptides from rhPRG4

Aliquots of purified proteins from rhPRG4 were treated with or without glycosidases (sialidase A and/or *O*-glycanase), followed by SDS-PAGE (3–8% Tris acetate NuPAGE gels) (Invitrogen, Stockholm, Sweden) and tryptic digestion, or digested in-solution with either trypsin or Lys-C as described previously (38). The peptides were desalted with C18 stagetips (63) followed by glycopeptide enrichment using HILIC tips prepared in-house using cottonwool (38, 64) or commercially available columns (Thermo Fisher Scientific). For LC-MS analyses, C18 columns were prepared in-house and used with formic acid in acetonitrile gradients. The glycopeptides were analyzed on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with CID and ETD fragmentation as described (38).

Purification, digestion, and LC-MS analysis of glycopeptides from SF lubricin from two OA patients

SF lubricin from patients with OA was purified as described elsewhere (54). Briefly, SF (0.5 ml) was subjected to anion exchange chromatography and the acidic protein fraction was collected and precipitated in ethanol. The acidic proteins were reduced in 10 mM DTT (70 °C, 1 h) and alkylated in 50 mM iodoacetamide for 30 min at room temperature in the dark. Salts were removed and buffer was exchanged to 50 mM ammonium bicarbonate (Sigma-Aldrich) using 30-kDa cutoff filters (Merck Millipore), followed by lyophilization. The reduced and alkylated protein-containing gel bands were digested with trypsin, and the peptides were extracted and desalted with StageTip C18 columns (63). The peptides were separated using in-house packed C18 columns at 200 nl/min using a 120-min gradient of 5–40% buffer B (A: 0.1% formic acid, B: 0.1% formic acid, 80% acetonitrile). The column was connected to an Easy-nLC 1000 system (Thermo Fisher Scientific), a nano-electrospray ion source, and a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). For full-scan MS, the instrument was scanned at m/z 350–2000, resolution 70,000 (at m/z 200), AGC target 1×10^6, max injection time 120 ms, and dynamic exclusion was auto or 20 s. The 12 most intense peaks (charge states 2, 3, and 4) were selected for fragmentation. For MS/MS, resolution was set to 17,500 (at m/z 200), AGC target to 5×10^5, max injection time 256 ms, and normalized collision energy = 30 with stepped normalized collision energy of 25%.

Assignment of lubricin glycopeptides

Peptide searches were performed using Mascot version 2.3.02 against the Swissprot database version 2017-06. Glycopeptide searches were carried out using both manual and software-assisted interpretation using Byonics (version 2.6.46, Protein Metrics, San Carlos, CA) against human lubricin (Uniprot entry Q92954). For Orbitrap XL data (recombinant lubricin), parameters were set as follows: precursor tolerance 10 ppm; fragment tolerance 0.5 ppm; enzyme: Lys-C or trypsin, cleavage before proline included; maximum two missed cleavages; fixed carbamidomethyl modification of cysteines, variable modification: oxidized methionine. For the glycopeptide searches in Byonics, settings were maximum two glycan modifications per peptide; glycan compositions: HexNAc(1), HexNAc(1)Hex(1), HexNAc(1)Hex(1)Sulf(1), HexNAc(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1), HexNAc(1)Hex(1)NeuAc(1)Sulf(1), and HexNAc(1)Hex(1)NeuAc(2). The glycans were all defined as "common." For Q Exactive data (native lubricin from OA patients), parameters were the same except fragment ion tolerance was set to 10 ppm. In Byonics, the data set was searched against seven additional glycans of core 2 type: HexNAc(2)Hex(1), HexNAc(2)Hex(1)Sulf(1), HexNAc(2)Hex(2)Sulf(1), HexNAc(2)Hex(1)NeuAc(1), HexNAc(2)Hex(2)NeuAc(1), HexNAc(2)Hex(2)NeuAc(2), and HexNAc(2)Hex(2). Sulfated glycans were defined as "rare," the remaining as common. Glycopeptide spectra with a score higher than 200 were considered and validated manually. For positive identification, at least five b/y ions originating from the peptide backbone sequence were required, and all major peaks in the spectra were assigned.

Production of secreted PSGL-1/mIgG2b fusion protein

Mucin-type fusion protein P-selectin glycoprotein ligand-1/mouse immunoglobulin G2b (PSGL-1/mIgG2b) and human core 2 β1,6-*N*-acetylglucosaminyltransferase 1 (C2GnT-1) plasmids carrying puromycin and geneticin (G418 sulfate) drug resistance genes, respectively, were constructed as described previously (65, 66). PSGL-1/mIgG2b derived from the CP-55 clone containing only core 1 glycans was generated and purified as previously described (67). The core 2 clone was from a previous publication generated by transfecting CHO-K1 cells with cDNA encoding PSGL-1/mIgG2b. This clone was shown to convert standard CHO-cell core 1 glycosylation into core 2 with more than 90% efficiency (42). The core 2 clone was cultured in serum free ProCHO-4 medium (Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), as well as selection drugs.

Quantification of PSGL-1/mIgG2b by ELISA

A sandwich ELISA method was used for the quantitative measurement of PSGL-1/mIgG2b concentrations in supernatants. The 96-well ELISA plates (Costar 3590; Corning) were coated with an affinity-purified polyclonal goat anti-mouse IgG Fc-specific antibody (Sigma-Aldrich) at a concentration of 10 μg/ml in 50 mM carbonate buffer, pH 9.6, at 4 °C overnight. The plates were blocked with 1% (w/v) BSA in PBS to minimize nonspecific binding. This buffer was also used as an antibody dilution buffer. The plates were sealed and incubated at room temperature for 2 h. Supernatants were incubated in the 96-well plate in triplicate for 2 h before incubating with a peroxidase-conjugated anti-mouse IgG Fc-specific antibody (Sigma-Aldrich) diluted 1:2,000 for 2 h at room temperature. PBS containing 0.05% (v/v) Tween-20 (Sigma-Aldrich) and 0.15 M sodium chloride was used as the wash buffer between incubations. A bound peroxidase-conjugated antibody was visualized with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma-Aldrich) as a substrate. The color reaction was stopped by the addition of 2.0 M H_2SO_4, and the optical density of the samples was read at a wavelength of 450 nm in a microplate reader

(Synergy 2, BioTek). The PSGL-1/mIgG2b concentration was estimated using a dilution series of purified mIgG2b (AbD Serotec, Oxford, UK) in blocking buffer as a standard.

PSGL-1 constructs analyzed by SDS-PAGE/Western blotting

For SDS-PAGE/Western blotting, PSGL-1/mIgG_{2b} was purified from clarified supernatants on goat anti-mIgG agarose beads by rotating at 4 °C overnight. The beads with captured fusion proteins were washed three times in PBS, resuspended in 4× SDS sample buffer without reducing agent (Invitrogen), and incubated at 95 °C for 5 min for protein denaturation. The samples were analyzed by SDS–PAGE using 3–8% Tris acetate gradient gels and Tris acetate SDS running buffer (Invitrogen). Precision protein standard (Hi-Mark, Invitrogen) was applied as reference for protein molecular weight determination. Separated proteins were blotted using iBlot (Invitrogen) onto nitrocellulose membranes. The membranes were blocked with 1× carbo-free blocking solution (Vector laboratories), which was also used for dilution of antibodies and lectins. A mouse anti-human CD162 was used to detect the N-terminal part of PSGL-1 (1:1,000 dilution; BD Pharmingen, San Diego, CA, USA) together with an HRP-conjugated polyclonal goat anti-mouse IgG (Fab-specific, 1:10,000 dilution; Sigma-Aldrich) used as secondary antibody. Biotinylated galectin-3 (diluted 1:100) was used to detect galectin-3 together with streptavidin (diluted 1:10,000; Sigma-Aldrich). Bound antibody and lectins were visualized by chemiluminescence using the ECL kit according to the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). The presence of glycosylated and nonglycosylated proteins was assessed by staining of the gels using a glycoprotein staining kit and the Ruby stain (Thermo Fisher Scientific), respectively. Candy Cane markers (Thermo Fisher Scientific) were used for molecular weight reference. PSGL-1 constructs from transient transfection of C2GnT-1 of CHO cells (68) were also subjected to the procedure of SDS-PAGE, blotting, and staining as described above.

Lectin ELISA of lubricin from SF

SF Lubricin was probed using an in-house sandwich lectin ELISA method. 96-well Nunc-Immunomaxisorp plates (Thermo Fisher Scientific) were coated with 1 μg/ml monoclonal anti-lubricin antibody clone 9G3 against rhPRG4 mucin domain (Thermo Fisher Scientific) in PBS at 4 °C overnight. After blocking with 3% BSA in Tris-buffered saline (TBS)-Tween (TBS, pH 7.4, + 0.05% Tween-20), the same volume of SF samples (duplicate of each sample) that were pre-diluted 1 in 50 in assay buffer (1% BSA in TBS-Tween) were loaded (100 μl) and incubated for 90 min. The captured lubricin was incubated with 2 μg/ml biotinylated HAA (Sigma-Aldrich), PNA (Vector laboratories), L-selectin (Sigma-Aldrich), AAL (Vector laboratories), SNA (Vector laboratories), or biotinylated galectin-3 for 1 h, followed by a 1-h incubation with 0.2 μg/ml HRP-conjugated streptavidin (Vector Laboratories). The plate was washed three times with TBS-Tween between each incubation procedure and five times before the staining step. After staining with 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific), absorbance was read at 450 nm. The ratios of the average absorbance of galectin-3 and HAA staining for each individual sample were calculated to estimate the level of extension of GalNAcα1-Ser/Thr (HAA) into galectin-3 reactive glycans on lubricin. To test the glycodependency of galectin-3 binding to lubricin, 6 μl of SF was incubated with 40 μl of 1% periodic acid for 1 h on shaker, and the system was then neutralized with 40 μl TBS for 20 min. The samples were diluted in assay buffer and subject to protocol as described above. Statistical evaluation of lectin sandwich ELISA was conducted as described in Fig. 3 and Fig. S4. Relative differences between OA and control of galectin-3 binding were compared using the lectin ELISA absorbance ratio of galectin-3 *versus* HAA.

Endogenous synovial galectin-3

Endogenous galectin-3 levels in OA and control SF were measured by the galectin-3 test ELISA kit (BG Medicine) according to the manufacturer's instructions. OA samples were measured in duplicates and control samples were measured as singlets because of limited amounts of material. Statistical evaluation of ELISAs were performed by GraphPad Prism 8 (GraphPad Software). Statistically significant differences were calculated by nonparametric *t* test using the Mann-Whitney test.

SPR

Binding of rhPRG4 and bPRG4 (53) to galectin-3 was assessed using a Biacore × 100 SPR instrument (GE Healthcare). Galectin-3 (R&D Systems) was covalently attached to the CM5 chip by running 50 mg/ml galectin-3 solution in 20 mM sodium acetate solution at pH 5, giving a response unit of 3,500. Injection of lubricin was performed as described (69). Lactose was used as a regeneration buffer after lubricin injections (70).

Data availability

Raw MS data and assigned glycomic structures are available for synovial lubricin at GPST000100 and for rhPRG4 at GPST000099. Raw files and GlycoWorkbench files for bPRG4 are available at GPST000060. Structures with associated MIRAGE information are available at UniCarb-DR (390). Glycoproteomics MS raw files and Byonic search files for rhPRG4 are uploaded to GPST000062. Glycoproteomic MS raw files and Byonic search files for patient lubricin are available at GPST000063.

Acknowledgments—We thank Hakon Leffler at Lund University for the gift of recombinant galectin-3, Sofia Grindberg and Paula-Therese Kelly Pettersson at Danderyd Hospital and Lotta Falkendahl at University of Gothenburg for assistance in collecting samples, and the Proteomics Core facility at Gothenburg University for access to Byonics software.

Author contributions—S. A. F., S. C. R., J. H., T. E., R. K., and N. G. K. conceptualization; S. A. F., L. A., S. H., Y. M., S. C. R., and N. G. K. formal analysis; S. A. F., K. A. T., L. A., S. H., Y. M., S. C. R., T. A. S., and N. G. K. investigation; S. A. F., L. A., S. H., Y. M., S. C. R., and N. G. K. visualization; S. A. F., K. A. T., and N. G. K. writing-original draft; S. A. F., S. H., Y. M., S. C. R., J. H., T. A. S.,

O. R., L. I. B., M. S., A. K.-B., G. D. J., T. E., R. K., and N. G. K. writing-review and editing; K. A. T., L. A., S. H., O. R., and R. K. data curation; K. A. T., L. A., S. H., Y. M., S. C. R., J. H., M. S., and A. K.-B. methodology; K. A. T., T. A. S., and N. G. K. project administration; S. H., J. H., T. A. S., M. S., A. K.-B., and N. G. K. supervision; J. H. and N. G. K. funding acquisition; O. R., L. I. B., G. D. J., T. E., R. K., and N. G. K. resources.

Funding and additional information—This study was funded by grants from the Swedish state under the agreement between the Swedish government and the county council, the ALF-agreement Grants ALFGBG-722391 (to N. G. K.) and ALFGBG-726801 (J. H.), the Swedish Research Council Grants 621-2013-5895 (to N. G. K.) and 2018-03077 (A.K.), Kung Gustav V:s 80-year foundation (N. G. K.), Petrus and Augusta Hedlund's foundation Grant M-2016-0353 (to N. G. K.), AFA insurance research fund Grant dnr 150150 (to N. G. K.), and National Institute of Health R01AR067748 (to G. D. J.).

Conflict of interest—G. J., R. K., and T. S. authored patents related to rhPRG4, and G. J. and T. S. hold equity in Lubris BioPharma LLC. T. S. is also a paid consultant for Lubris BioPharma, LLC. S. F. and N. G. K. authored a patent using lubricin for diagnostics. K. T., L. A., S. H., Y. M., S. R., J. H., O. R., R. B., M. S., A. K., and T. E. declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: OA, osteoarthritis; RA, rheumatoid arthritis; rhPRG4, recombinant lubricin; bPRG4, bovine chondrocyte lubricin; SF, synovial fluid; CHO, Chinese hamster ovary; MRM, multiple reaction monitoring; Tn-antigen, GalNAcα1-; T-antigen, Galβ1-3GalNAcα1-; ECM, extracellular matrix; COMP, cartilage oligomeric matrix protein; HCD, higher-energy collisional dissociation; CID, collision-induced fragmentation; SPR, surface plasmon resonance; Hex, hexose; HexNAc, *N*-acetylhexosamine; AAL, Aleuria aurantia lectin; Sulf, sulfate; HAA, Helix aspersa agglutinin; PNA, peanut agglutinin; SNA, Sambucus nigra agglutinin; aa, amino acid; ETD, electron-transfer dissociation; TBS, Tris-buffered saline; CV, coefficient of variation.

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