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Boundary lubrication by lubricin is mediated by O-linked β(1-3)Gal-GalNAc oligosaccharides

Gregory D. Jay[1*,2], Darcy A. Harris[1] and Chung-Ja Cha[1]

[1] *Department of Medicine, Section of Emergency Medicine, Brown University, School of Medicine, RI 02903, USA,*
[2] *Division of Engineering, Brown University, RI 02903, USA*

Lubrication of mammalian joints is mediated by lubricin, a product of megakaryocyte stimulating factor gene (MSF; GenBank accession #U70136) expression. Lubricin ($M_r \sim 240$ kDa) is a mucinous glycoprotein which is 50% (w/w) post-translationally modified with β(1-3)Gal-GalNAc incompletely capped with NeuAc, and lubricates apposed cartilaginous surfaces in the boundary mode through an unknown mechanism. Both bovine and human lubricin were purified from synovial fluid and digested with recombinant glycosidases. Released oligosaccharides were identified and quantified by fluorophore assisted carbohydrate electrophoresis (FACE). Corresponding digests of human lubricin were also assayed in a friction apparatus oscillating latex rubber against polished glass at a pressure of 0.35×10^6 N/m^2 and the coefficient of friction (μ) was measured. Digestion with α2,3-neuraminidase decreased lubricating ability by 19.3%. Partial removal of β(1-3)Gal-GalNAc moieties by endo-α-*N*-acetyl-D-galactosaminidase reduced lubricating ability by 77.2%. Human lubricin digested with combined α2,3-neuraminidase and β1-3,6-galactosidase continued to lubricate at 52.2% of its nominal value. Both bovine and human lubricin released 48.6% and 54.4% of total β(1-3)Gal-GalNAc sidechains following digestion with endo-α-*N*-acetyl-D-galactosaminidase. Biological boundary lubrication by synovial fluid *in vitro* is provided primarily by extensive O-linked β(1-3)Gal-GalNAc.

Keywords: **Lubricin, synovial fluid, megakaryocyte stimulating factor, lubrication**

Introduction

Lubricin, a classical protein first described by Swann, is a mucinous glycoprotein which provides lubrication of cartilaginous surfaces during mammalian locomotion [1]. Lubricin is a product of megakaryocyte stimulating factor (MSF) gene expression by synovial fibroblasts [2], as the lubricant is aspiratable from the synovial cavity and can be purified from synovial fluid. Chondrocytes also express MSF as superficial zone protein (SZP) [3] which covers the surface of articular cartilage. Both synovial fibroblasts and chondrocytes transcribe MSF in a similar manner as both cell types employ alternate splicing of MSF exons 2,4 and 5 giving rise to four different molecular phenotypes [4]. Each contains a central exon 6 which is 904 amino acids long of which 208 are threonine in a repeating degenerate sequence of KEPAPTT and putative sites of extensive O-linked glycosylation. Most recently, another laboratory [5] and later ours independently linked the disease state of camptodactyly-arthropathy-coxa vara-pericarditis syndrome (CACP) [6] to errors in MSF expression. Marcelino and his co-workers have identified several truncating mutations in the CACP protein product, most towards the 3′ end of the MSF gene [5]. Patients with CACP are diagnosed at birth and develop non-inflammatory arthropathy before puberty. Evidently, lubricin/MSF/SZP/CACP protein is important towards diarthrodial joint homeostasis under load, which has been posited previously. Intra-articular contact pressure *in vivo* is typically 5×10^6 N/m^2 which is supported by weeping lubrication and boundary lubrication which provides for asperity separation.

Lubricin is a boundary lubricant and lubrication is provided in the absence of viscosity which is provided by hyaluronate, another synovial fluid constituent [7,8]. By definition boundary lubricants can be dry or wet but work in bearing systems with the following parameters: a slow bearing sliding speed, high contact pressure and reciprocation (which eliminates formation of a lubricant wedge). Biomolecules accomplish this task by the ability to adsorb to the bearing surface and generate a repulsive force of unknown origin.

Bovine lubricin is 50% (w/w) glycosylated with multiple residues of O-linked β(1-3)Gal-GalNAc which are incompletely capped with NeuAc to an unknown degree [9]. Another example of a mucinous glycoprotein with lubricating ability

*To whom correspondence should be addressed: Gregory D. Jay, M.D., Ph.D., Department of Emergency Medicine, Rhode Island Hospital, 593 Eddy Street, Providence, RI 02903, USA. Tel.: 401-444-6656; Fax 401-444-5456; E-mail: gregory_jay_MD@brown.edu

is MG2 [10] which also possesses the same glycosylations. This glycoprotein lubricates dental contacts and originates from submandibular saliva. Preliminary data on the sequential removal of glycans from lubricin have been previously reported using glycosidases which were potentially contaminated with proteases. Removal of NeuAc did not appear to adversely affect lubrication but the enzymatic digestion of the penultimate galactose diminished lubricating ability [7,11].

The present study was undertaken to identify O- and N-linked glycosylations on both bovine and human lubricin which can be removed enzymatically and correlate these findings with lubricating ability. Recombinant glycosidases were used and thus free of proteolytic contamination, supporting reliable digestion experiments. In particular, recombinant endo-α-*N*-acetyl-D-galactosaminidase was selected as it has specificity for O-linked β(1-3)Gal-GalNAc unsubstituted with NeuAc. Fluorophore-assisted carbohydrate electrophoresis (FACE) [12] was used to reveal the glycosylation identity after enzymatic digestion and corresponding digests were assayed for lubricating ability. Lubrication was tested in a bearing of apposed latex rubber and polished glass which isolates the conditions of boundary lubrication without the component of weeping lubrication provided by cartilage. This bearing system has been used previously by a number of investigators studying joint lubrication [13–16].

Methods

Lubricin purification from bovine synovial fluid (BSF)

Bovine synovial fluid was aspirated percutaneously from the lateral aspect of radiocarpal joints of freshly slaughtered cattle with sterile 18-gauge needles after cleansing the skin with alcohol swabs. The cattle were 1 year old and of both sexes (Pel-Freeze Corp., Little Rock, AK). The fluid was pooled within a 500 ml sterile polypropylene container and transported at 4°C. That same day, the fluid was clarified by centrifugation at 20,000× g at 4°C for 30 mins.

Bovine synovial fluid (500 ml) was filtered through 0.22 μm sterile filter units (Nalgene, Rochester, NY) at 4°C over two days. Retentate was scraped off filter membranes and resuspended with 50 mM NaAc buffer, pH 5.5, containing proteolytic inhibitors—1mM phenylmethylsulfonyl fluoride, 1 mM *para*-chloromercuribenzoic acid and 10 mM ethylenediaminetetraacetic acid (EDTA) to the original synovial fluid volume of 500 ml. Digestion of hyaluronic acid was carried out with *Streptomyces* hyaluronidase (Sigma, St. Louis, MO) 1 U/ml of resuspended synovial fluid at 37°C for 18 h. The digest was loaded on a diethyl-aminoethyl (DEAE) column (Whatman International, Maidstone, UK) settled volume of 300 ml, equilibrated with 50 mM NaAc buffer, pH 5.5, and washed with 1.5 L of the same buffer. The desired material was eluted off of the DEAE matrix with 1 M NaCl. A 1 L wash was collected and concentrated via a 500 ml Amicon flow cell with an XM-100 membrane (molecular weight cut-off 100 kDa). The concentrated sample was dialyzed against 25 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) and 0.5 mM $CaCl_2$.

The DEAE-bound concentrate was loaded onto a peanut agglutinin (PNA)-agarose affinity column (EY Labs, San Mateo, CA) with a settled bed volume of 25 ml, equilibrated at room temperature in PBS. Unbound protein was eluted with the same buffer until absorbance at 230 and 280 nm decreased to background. Desired material was maximally eluted in the presence of a step-wise gradient of 0.15 M α-lactose in PBS. Purity was assayed on a pre-cast 5–15% gradient polyacrylamide electrophoresis gel (Biorad, Hercules, CA) stained with Coomassie Blue. Purity was also assayed via high pressure liquid chromatography on a μ Bondapak C18 3.9×150 mm column (Waters, Milford, MA) eluted in reverse phase with 45% (v/v) methanol (Sigma) and 5% (v/v) acetonitrile (Aldrich, Milwaukee, WI) HPLC grade at 1 ml/min at 35°C. Purity was determined by a photo diode array detector PDA 996 (Waters) and purity plot analysis (Millenium 32 software, Waters). Purified bovine lubricin was stored at −20°C

Human synovial fluid (HSF) collection

Aliquots of synovial fluid from 150 patients undergoing diagnostic arthroscopy and total knee joint replacement were collected and assayed in the friction apparatus. The synovial fluid was aspirated prior to either surgical procedure and immediately centrifuged at 10,000g at 4°C for 1 h to remove cellular debris. Samples grossly contaminated with blood were discarded. Aliquots with normal lubricating ability were pooled and stored at −20°C. Harvesting of synovial fluid in this manner was approved by the Institutional Review Board. Additional samples of post-mortem human synovial fluids from male and female subjects without gross degenerative joint disease were obtained *in gratis* from Dr. Martin Lotz at Scripps Research Institute. These samples served as controls to identify normal lubricating ability.

Lubricin purification from human synovial fluid

Human synovial fluid (200 ml) was filtered through 0.22 μm sterile filter units at 4°C over two days, the retentate was digested with hyaluronidase and then fractionated on a separate PNA column as described above. After dialysis against PBS, pre-purified lubricin was fractionated on an Actigel ALD agarose (Sterogene Bioseparations, Arcadia, CA) column 1 × 5 cm which was coupled via amine groups to a murine monoclonal antibody directed against human fibronectin (Zymed Laboratories Inc., San Francisco, CA). Purity was determined as above. Purified human lubricin was stored at −20°C.

Measurement of lubrication

The friction apparatus was an improved version of McCutchen's instrument [16] as modified by Davis et al. [13]. A more detailed methodological description has been published previously [17]. Natural latex was oscillated against a ring of polished glass with

a constant contact area of 1.59 cm^2. The bearing system was axially loaded within a gimbals system free to rotate around two perpendicular horizontal axes. Latex and glass as bearing materials were chosen since they offer a flat surface with small asperity heights on the order of 0.05 mm. Latex, like cartilage, is compliant. Within the gimbals system, these surfaces possess near perfect co-planarity. Accordingly, fluid wedges were not generated and only a thin layer of boundary fluid was present. The entraining velocity (i.e., sliding speed) was 0.37 mm/sec with a constant contact pressure of 0.35×10^6 N/m^2.

The friction apparatus recorded displacements of the gimbals system around the vertical loading axis through a linear displacement voltage transducer, the output voltage of which was directly proportional to the magnitude of the frictional torque. The peak to peak amplitude of this signal was related to μ by a previous calibration with known frictional torque.

Test surfaces were cleaned extensively before use. A 3.8 × 3.8 cm piece of latex strapped onto the stainless steel stud was washed under running distilled deionized water (DDW) for 2 min. It was then placed in a shallow bath of 0.15 M NaCl physiological saline (NS). The glass slide was scrubbed with a 1% (v/v) 7X detergent (Flow Laboratories, McLean, VA) solution in DDW for 10 min and then allowed to soak in the same solution at 100°C. A 5 min scrubbing was also performed with the hot 7X solution followed by rinsing for 2–4 min under running DDW.

The μ was measured at 23°C and was preceded by a baseline measurement of the μ with NS. Lubrication was manifested by a reduction of μ relative to the μ of NS. Negative $\Delta\mu$ values indicate lubrication whereas positive values indicate friction. Addition of 200 μl of NS and later 200 μl of test lubricant was followed by bringing the bearing surfaces close enough so that the solution wet both surfaces. After 5 min for equilibration, the latex-coated bearing was brought to rest on the glass as it was reciprocating under load. Peak to peak voltages were automatically recorded after 1, 3 and 5 min. At this point the surfaces were separated for 2 min and then brought back together for another 5 min session. The 3 and 5 min μ values of the last two 5 min sessions typically stabilized and were recorded. Normal lubricating ability of human synovial fluid was identified by studying the post-mortem synovial fluid aliquots described above. Assays were performed in triplicate and reported as $\Delta\mu \pm$ S.D., and results compared by the Kruskal-Wallis test.

Enzymatic digestions of human lubricin

Human lubricin in 500 μl aliquots, at a concentration of 300 μg/ml in NS, was digested with recombinant α2,3,6,8-neuraminidase, 25mU from *Arthrobacter ureafaciens* (10 mU/μl; Roche Diagnostics, Mannheim, Germany). Simultaneous digestions with α2,3,6,8-neuraminidase and recombinant β1-3,6-galactosidase, 200 mU from *Xanthomas manihotis* (4 mU/μl; Galase II, Glyko Inc., Novato, CA) were also performed. In each case, digestions were conducted at 37°C for 18 h on a gyrating platform (Lab-line, Melrose Park, IL). Dilution of the original lubricin solution was minimized in each case since the enzyme preparations were at relatively high concentrations. Sham digestions with an equivalent volume of NS in lieu of enzyme were performed in parallel which provided a suitable control for the lubrication assays.

Additional digestions were performed with recombinant endo-α-*N*-acetyl-D-galactosaminidase, 100 mU from *Streptococcus pneumoniae* (4 mU/μl; O-glycosidase DS, Glyko), and a combination of recombinant α2,3-neuraminidase, 250 mU from *Streptococcus pneumoniae* (20 mU/μl; NaNase I, Glyko) and endo-α-*N*-acetyl-D-galactosaminidase. In these digestions it was necessary to dialyze the 500 μl aliquots of human lubricin against 25 mM phosphate buffer, pH 7.6, prior to digestion at 37°C for 18 h. Afterwards, these digests were dialyzed against NS in preparation for lubrication assay. The buffer exchange was necessary as chloride has been shown to inhibit endo-α-*N*-acetyl-D-galactosaminidase [18]. Sham digestions with 25 mM phosphate buffer in lieu of enzyme, underwent the same dialysis procedures as above, and were performed as controls for the lubrication assay.

O-linked glycosylation profiling analysis

Both bovine and human lubricin samples were dialyzed against 50 mM sodium phosphate buffer, pH 6.0. Samples were denatured at 100°C for 5 min in 0.05% SDS and 50 mM β-mercaptoethanol and cooled to 23°C and NP-40 was added to a final concentration of 0.4%. O-linked glycosylations were released by adding 4 mU endo-α-*N*-acetyl-D-galactosaminidase (1 mU/μl; Glyko) or following predigestion with 20 mU α2,3-neuraminidase (20 mU/μl; NaNase I, Glyko) to remove ultimate glycosylations. Additional digestions were done with endo-α-*N*-acetyl-D-galactosaminidase and 20 mU β1-3,6-galactosidase (5 mU/μl; Glyko) in combination and β1-3,6-galactosidase alone. Limited profiling was also performed by reduction with hydrazine per previously published procedures [12] to confirm O-linked oligosaccharide length.

N-linked glycosylation profiling analysis

Both bovine and human lubricin samples were dialyzed against 50 mM sodium phosphate buffer, pH 7.7. Samples were denatured at 100°C for 5 min in 0.05% SDS and 50 mM β-mercaptoethanol and cooled to 23°C and NP-40 was added to a final concentration of 0.4%. N-linked glycosylations were released by 5 mU of recombinant peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F from *Flavobacterium meningosetticum* (2.5 mU/μl; PNGase F, Glyko) digestion which was performed at 37°C for 2 h.

Fluorophore-assisted carbohydrate electrophoresis (FACE)

N- and O-linked oligosaccharides released from both bovine and human lubricin by either peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F or endo-α-*N*-acetyl-D-galactosaminidase were precipitated via cold absolute ethanol

(Pharmco, Brookfield, CT) extraction. Following centrifugation at 10,000 g for 15 min at 4°C, pellets were resuspended and labeled by reductive amination with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) using reagents and protocols from Glyko Inc. [12]. Labeling was performed at 37°C for 18 h. After labeling samples were dried in a Speed-Vac for 15 min. The sample was resuspended with 20 μl of loading buffer diluted with distilled deionized water (DDW) and 4 μl/lane was loaded onto either 27 or 30% (w/w) pre-cast polyacrylamide gels for N-link or O-link glycosylation analysis, respectively.

Polyacrylamide gels were subjected to an electric field at 20 mA/gel or 15 mA/gel for N-link or O-link gels respectively for 1.5 h at 4°C in a gelbox provided by Glyko. Images were acquired after electrophoresis was completed using a 16-bit, cooled charge-coupled device fluorescence camera (Glyko).

Preparation of ANTS-labeled oligosaccharide standards

A β(1-3)Gal-GalNAc oligosaccharide standard (Glyko), *N*-acetylgalactosamine (Sigma) and galactose (Sigma) were labeled with ANTS as above. Stock solutions of β(1-3)Gal-GalNAc and the monosaccharide standards (already diluted with gel loading buffer) were at a final concentration of 25 pm/μl. An ANTS-labeled maltotetrose ladder standard was supplied by Glyko. The G4 moiety established the electrophoretic mobility of 4 glucose units or 4 degrees of polymerization (dp) and was at a concentration of 50 pm/band.

N-linked glycosylation sequencing

Bands visualized under UV light in FACE gels were excised and placed in 100% absolute ethanol at 4°C in an autoclaved microtube. Gel slices were removed, after turning white, and then allowed to soak in a minimum of DDW overnight. This procedure was repeated and the DDW pooled each time and lyophilized. Dried oligosaccharides were resuspended in DDW, aliquoted and mixed with a 5X buffer concentrate. The following recombinant exoglycosidases were added alone or in combination to a final volume of 20 μl: recombinant α2,3,6,8-neuraminidase, 0.34 mU from *Arthobacter ureafaciens*; β1-3,6-galactosidase, 0.4 mU; β1-2,3,6-*N*-acetylhexosaminidase, 0.5 mU (HEXase, Glyko); α1-2,3,6-mannosidase, 0.25 mU (MANase II, Glyko). All exoglycosidases were supplied by Glyko and the latter two were recombinant enzymes from *Canavalia ensiformis*. Following overnight incubation at 37°C, digests were lyophilized and resuspended in electrophoresis loading buffer. Digested N-linked oligosaccharides were separated by electrophoresis as noted above. Two standards were run beside each of these samples. The first was the fluorophore-labeled maltotetrose ladder standard noted above. The other contained the following fluorophore-labeled fucosylated and non-fucosylated mannosylchitobiose core: Man(β1-4)GlcNAc(β1-4)GlcNAc. Gels were imaged as described above. Bands were identified based on *a priori* established mobility rules for N-linked oligosaccharides [12].

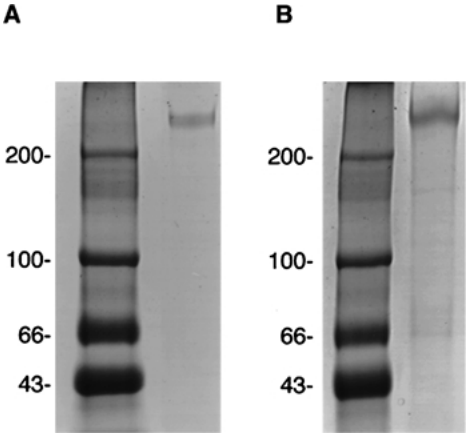

Figure 1. Purified bovine (A) and human lubricin (B) analyzed by electrophoresis on a 4–15% (w/v) polyacrylamide gradient gel stained with Coomassie Blue.

Results

Purification of human and bovine lubricin

Both bovine and human lubricin possessed lubricating ability following the fractionation of synovial fluid. Observed was a product with an apparent molecular weight of ~240 kDa and few lower molecular weight components at ~70 and ~160 kDa which variably appeared on polyacrylamide electrophoresis (Figure 1). Components with a molecular weight above 240 kDa were not observed. The electrophoretic mobility of both proteins were similar. Human lubricin appeared as a single peak in the analytical high pressure liquid chromatography with a retention time of 1.60 min and purity angle of 6° (data not shown). Lubricating ability of both purified bovine and human lubricin were $\Delta\mu = -.082 \pm .010$ and $\Delta\mu = -.085 \pm .008$ respectively. Neither of which differ significantly from the lubricating abilities of whole synovial fluid: $\Delta\mu = -.087 \pm .007$ and $-.090 \pm .011$, respectively for bovine and human synovial fluid.

O-linked glycosylation analyses

Both bovine and human forms of lubricin demonstrated the expected O-linked glycosylations as revealed by FACE (Figures 2 and 3). Digestion with endo-α-*N*-acetyl-D-galactosaminidase alone removed some of the dissacharide β(1-3)Gal-GalNAc (Figures 2 and 3; lane 1). Digestion with α2,3-neuraminidase followed by endo-α-*N*-acetyl-D-galactosaminidase produced a more intense band respectively for bovine and human lubricin (Figure 2; lane 3 and Figure 3; lane 4). Endo-α-*N*-acetyl-D-galactosaminidase will only remove β(1-3)Gal-GalNAc when it is sterically possible and not capped with an ultimate sugar. Densitometry of both β(1-3)Gal-GalNAc bands, comparison with the 100 pM β(1-3)Gal-GalNAc standard and determination of stoichiometric ratios (Table 1) revealed that 48.6 $\pm$ 12.6% and 54.4 $\pm$ 16.6% of the β(1-3)Gal-GalNAc is not capped with NeuAc in bovine and human lubricin respectively

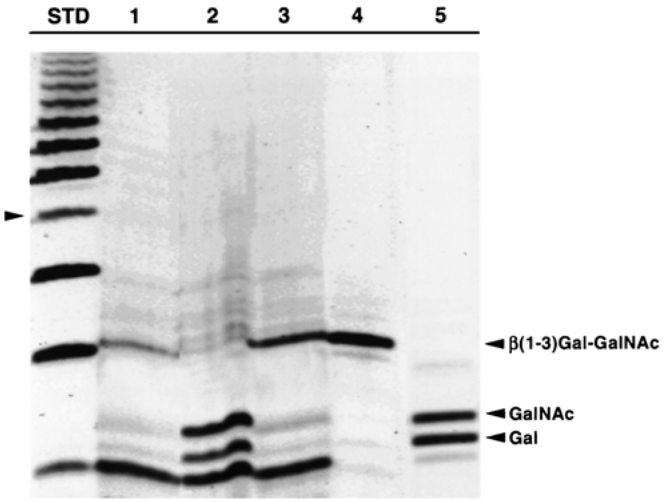

Figure 2. FACE of bovine lubricin O-linked oligosaccharides released enzymatically with endo-α-*N*-acetyl-D-galactosaminidase (lane 1), endo-α-*N*-acetyl-D-galactosaminidase & β1-3, 6-galactosidase (lane 2), α2,3-neuraminidase & endo-α-*N*-acetyl-D-galactosaminidase (lane 3). Lane STD is an oligoglucose ladder standard and the arrowhead denotes the 4.0 dp marker (G4). Lanes 4 and 5 are quantitation standards for β(1-3) Gal-GalNAc (100 pm), and Gal (50 pm) and GalNAc (50 pm) respectively. Imaging was performed under UV and captured using a cooled charge-coupled fluorescence camera.

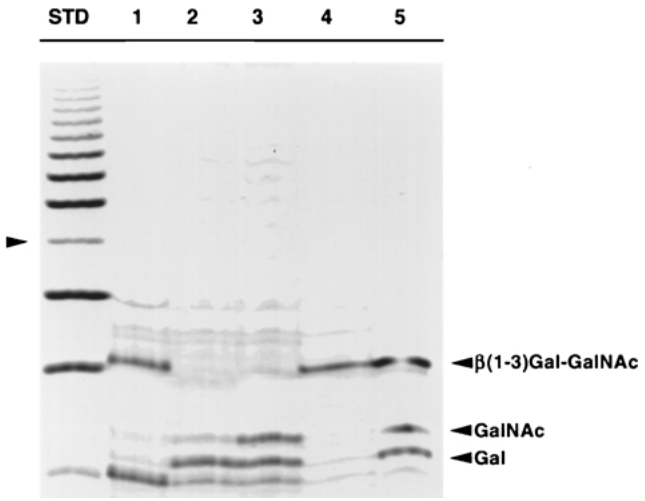

Figure 3. FACE of human lubricin O-linked oligosaccharides released enzymatically with endo-α-*N*-acetyl-D-galactosaminidase (lane 1), β1-3,6-galactosidase (lane 2), endo-α-*N*-acetyl-D-galactosaminidase & β1-3,6-galactosidase (lane 3), α2,3-neuraminidase & endo-α-*N*-acetyl-D-galactosaminidase (lane 4). Lane STD is an oligoglucose ladder standard and the arrowhead denotes the 4.0 dp marker (G4). Lane 5 is a quantitation standard for β(1-3)Gal-GalNAc (100 pm), Gal (50 pm) and GalNAc (50 pm). Imaging was performed under UV and captured using a cooled charge-coupled fluorescence camera.

($F(8,8) = 1.73$; $p = .23$). These results were obtained across three individual digestion and gel electrophoresis experiments. Calculation of the number of oligosaccharides per lubricin monomer was not performed.

Confirmation of the presence of β(1-3)Gal-GalNAc was also evident in the appearance of equimolar quantities of Gal and GalNAc following digestion with endo-$\alpha - N$-acetyl-D-galactosaminidase and β1-3,6-galactosidase (Figure 2, lane 2 and Figure 3, lane 3). Digestion with β-galactosidase alone (Figure 3, lane 2) resulted in the same amount of free galactose, indicating that all of the galactose removable by β1-3,6-galactosidase is likely coming from the same β(1-3)Gal-GalNAc moieties which were accessible to endo-α-*N*-acetyl-D-galactosaminidase. Liberated NeuAc was

Table 1. Densitometry of FACE gels of β(1-3)Gal-GalNAc released oligosaccharides

	BSF Lubricin			*HSF Lubricin*		
β(1-3)Gal-GalNAc digestion → oligosaccharide[a]	*Endo-α-N-acetyl-D-galactosaminidase [β(1-3)Gal-GalNAc]*	*α2,3-neuraminidase & endo-α-N-acetyl-D-galactosaminidase [(α2,3NeuAc)-β(1-3)-Gal-GalNAc]*[b]	*Molar ratios*[c]	*Endo-α-N-acetyl-D-galactosaminidase [β(1-3)Gal-GalNAc]*	*α2,3-neuraminidase & endo-α-N-acetyl-D-galactosaminidase [(α2,3NeuAc)-β-(1-3)Gal-GalNAc]*[b]	*Molar ratios*[c]
FACE Gel no. 1	7.6	21.0	0.36	52.8	117.5	0.45
	7.9	24.8	0.32	58.9	119.2	0.49
	7.4	23.2	0.32	53.6	122.3	0.44
FACE Gel no. 2	6.8	14.1	0.48	48.7	119.7	0.41
	8.5	13.6	0.62	48.8	119.8	0.41
	7.9	15.1	0.52	47.7	115.7	0.41
FACE Gel no. 3	13.0	24.5	0.53	89.90	120.5	0.74
	10.4	17.9	0.58	92.8	118.7	0.78
	12.6	19.2	0.65	90.1	116.4	0.77

[a]Concentration reported in picomoles.
[b]Total [β(1-3)Gal-GalNAc] capped and uncapped with α2,3NeuAc.
[c]Proportion uncapped with α2,3NeuAc {Ratio of [β(1-3)Gal-GalNAc] to [β(1-3)Gal-GalNAc] + [α2,3NeuAc)-β(1-3)Gal-GalNAc]}.

not evident in Figure 2, lane 3 and Figure 3, lane 4 as NeuAc is not labeled by ANTS. Bands with the same electrophoretic mobility as the G1 moiety were observed in both Figures 2 and 3. These are artifactual and associated with the electrophoretic wavefront.

Lubricating ability of human lubricin following digestion with glycosidases

Digestion with endo-α-*N*-acetyl-D-galactosaminidase alone decreased lubricating ability from $\Delta\mu = -.088 \pm .008$ to $\Delta\mu = -.020 \pm .011$ ($\chi^2 = 3.85$; $p = .049$). Neuraminidase alone decreased lubricating ability to $\Delta\mu = -.071 \pm .003$ ($\chi^2 = 3.85$; $p = .049$). Combination of neuraminidase and β-galactosidase decreased $\Delta\mu$ further to $-.046 \pm .009$ (Table 2). Combination of endo-α-*N*-acetyl-D-galactosaminidase and neuraminidase decreased lubricating ability to $\Delta\mu = -.018 \pm .005$ which was not significantly different from digestion with endo-α-*N*-acetyl-D-galactosaminidase alone ($\chi^2 = .04$; $p = .82$). Lubricating ability of control lubricin aliquots remained unchanged following the same digestion conditions without added glycosidases ($\chi^2 = 1.19$, $p = .27$). This control possessed the same lubricating ability as lubricin which did not undergo the dialysis against potassium phosphate buffer and overnight incubation followed by dialysis against 0.15 M NaCl which enabled assay for lubricating ability.

N-linked glycosylation analysis

Limited N-linked oligosaccharide substitution was observed for human lubricin. The N-link was an oligosaccharide 8.5 dp units long in addition to other bands which appeared to either comigrate with N-links from fibronectin and smaller bands in the experimental blank (Figure 4). The largest N-link of 8.5 dp units was digestible by β1-3,6-galactosidase, β1-2,3,6-*N*-acetylhexosaminidase and α1-2,3,6-mannosidase (Figure 5). No band shifting was observed with α2,3-neuraminidase digestion indicating the absence of ultimate NeuAc (Figure 5, lane 2). A 2.0 dp unit shift occurred following digestion with α2,3-neuraminidase and β1-3,6-galactosidase, indicating the loss of two galactose residues (Figure 5, lane 3). Following digestion with α2,3-neuraminidase, β1-3,6-galactosidase and β1-2,3,6-*N*-acetyl hexosaminidase, an additional 1.5 dp unit shift occurred which is consistent with loss of two GlcNAc residues (Figure 5, lane 4). Adding α1-2,3,6-mannosidase, a fourth enzyme, resulted in a band which co-migrated with the N-link core Man(β1-4)GlcNAc[Fuc]GlcNAc standard (Figure 5, lane 5). There was also a larger oligosaccharide which may be the same core structure, incompletely digested with α1-2,3,6-mannosidase and therefore contains one additional mannose residue (Man mobility shift = 0.75 dp units). Overall the above band shifts correspond to an asialo-, bi-antennary Gal-GlcNAc glycan on a standard Man3GlcNAc2 fucosylated core. No N-linked glycoyslations were observed for bovine lubricin, despite repeated attempts (data not shown).

Discussion

Lubricating ability of a single mucinous glycoprotein in a fraction of filtered synovial fluid has been reported previously [1,7]. The ability of recombinant glycosidases to remove lubricating

Table 2. Coefficients of friction (μ) for glycosidase digested human lubricin

Digestion	*Lubricant* μ	*NS* μ	$\Delta\mu$
Lubricin[a]	.0202	.1140	−.0938
	.0256	.1206	−.0950
	.0220	.1250	−.1030
Control[b]	.0296	.1186	−.0890
	.0220	.1180	−.0960
	.0236	.1025	−.0789
Lubricin + (α2,3-neuraminidase)	.0365	.1040	−.0674
	.0380	.1120	−.074
	.0358	.1068	−.071
Lubricin + (endo-α-*N*-acetyl-D-galactosaminidase)	.0790	.1125	−.0334
	.1096	.1252	−.0156
	.0970	.1090	−.0120
Lubricin + (α2,3-neuraminidase & β1-3,6-galactosidase)	.0551	.1080	−.0529
	.0800	.1164	−.0364
	.0542	.1051	−.0509
Lubricin + (α2,3-neuraminidase & endo-α-*N*-acetyl-D-galactosaminidase)	.0834	.1044	−.0209
	.0910	.1115	−.0205
	.0984	.1105	−.0121

[a]Lubricin at a concentration of 300 μg/ml in 0.15 M NaCl.
[b]Lubricin at same concentration which was dialyzed against 25 mM phosphate buffer, incubated at 37°C overnight and then dialyzed against 0.15 M NaCl.

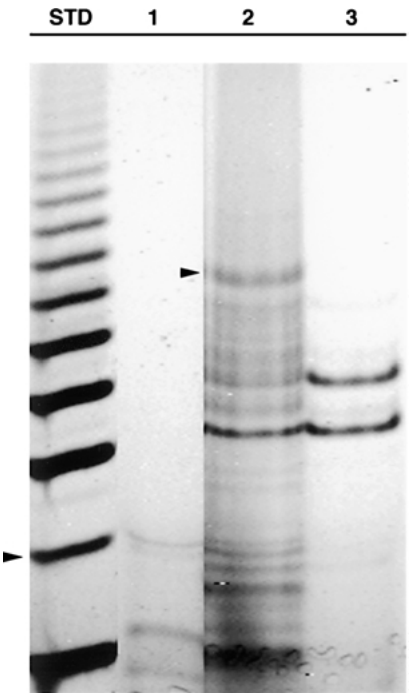

Figure 4. FACE of N-linked oligosaccharides released enzymatically with peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F from purified human lubricin (lane 2). Lane STD is an oligoglucose ladder standard and the arrowhead denotes the 4.0 dp marker (G4). Lane 1 is a blank and underwent the same experimental procedures but did not contain the purified lubricin fraction. Lane 3 is fibronectin. Imaging was performed under UV and captured using a cooled charge-coupled fluorescence camera. The band labeled with an arrowhead in lane 2 at 8.5 dp appears unique to human lubricin.

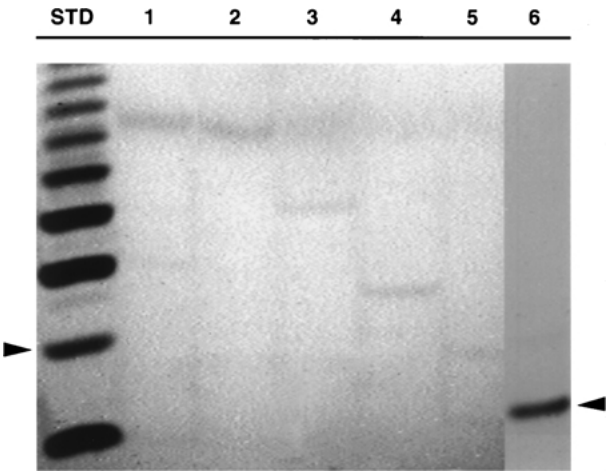

Figure 5. FACE of the 8.5 dp band imaged in Figure 4 after excision from acrylamide (lane 1) and sequential enzymatic digestions (lanes 2–5). Lane 2 is digestion with α2,3,6,8-neuraminidase. Lane 3 is digestion with α2,3,6,8-neuraminidase and β1-3,6-galactosidase. Lane 4 is digestion with α2,3,6,8-neuraminidase, β1-3,6-galactosidase and β1-2,3,6-N-acetylhexosaminidase. Lane 5 is digestion with α2,3,6,8-neuraminidase, β1-3,6-galactosidase, β1-2,3,6-*N*-acetylhexosaminidase and α1-2,3,6-mannosidase. Lane 6 is the core standard ManGlcNAc-[Fuc]GlcNAc at 3.2 dp. Lane STD is an oligoglucose ladder standard and the arrow head denotes the 4.0 dp marker (G4).

ability confirms that lubricin/MSF/SZP/CACP is the lubricating factor in synovial fluid. A critical observation of this investigation is that removal of 54% or less of β(1-3)Gal-GalNAc in human lubricin diminished lubricating ability by almost 80%. These moieties were presumably accessible to endo-α-*N*-acetyl-D-galactosaminidase digestion by virtue of their being uncapped with NeuAc and not sterically hindered by adjacent glycosylations. The degree to which loss of lubrication occurred was similar for lubricin digested with combined endo-α-*N*-acetyl-D-galactosaminidase and α2,3-neuraminidase. This latter digest demonstrated little observable friction reducing properties. Human lubricin which was digested with α2,3-neuraminidase and β1-3,6-galactosidase resulting in a terminal GalNAc, had a 50% decrease in lubricating ability which differed from previous experiments in which enzymes used were not recombinant. It thus appears that boundary lubrication provided by lubricin is dependent upon the β(1-3)Gal-GalNAc moiety and more specifically the penultimate galactose. This conclusion is also supported by a smaller but statistically significant change in lubrication after removal of the ultimate NeuAc by α2,3-neuraminidase. One methodologic limitation of this study is that carbohydrate analyses were carried out on denatured lubricin while the lubrication assays were carried out on proteins that were native during glycosidase digestions.

Corroborating evidence for the importance of β(1-3)Gal-GalNAc is found in a salivary glycoprotein, MG2, also observed to be a boundary lubricant [19] in the same friction apparatus used in the present investigation. However, other glycoproteins which possess large amounts of the β(1-3)Gal-GalNAc moiety, such as antifreeze glycoprotein, do not lubricate. Evidently, tertiary structure is also important as the glycosylations on antifreeze glycoprotein form a straight line on one plane of the molecule [20].

Although our study did not identify the actual number of glycosylations per lubricin monomer, the observed glycosylation on lubricin is qualitatively similar to that previously described by Swann [21] who used different techniques in sugar analysis. Lubricin has been linked to expression of the MSF gene by synovial fibroblasts. Megakaryocyte stimulating factor is a molecule with multifunctional domains, first isolated from the urine of patients with acute thrombocytopenia undergoing bone marrow transplantation [22]. A 25 kDa cysteine rich fragment was purified, corresponding to cDNA obtained from human monocytes, and found to stimulate the development of megakaryocytes. The discoverers of MSF [22] identified 12 exons and a 40% homology to vitronectin but did not identify a function for exon 6 which comprises 85% of the MSF gene and is notably absent from vitronectin. The complete MSF product is theoretically 400 kDa in molecular weight with 6.4% Ser and 20.1% Thr which primarily reside in the exon 6 product and is

therefore the posited site of extensive O-linked glycosylation [2]. Recently, MSF was also identified as the precursor of SZP, a proteoglycan with $M_r \sim 345$ kDa apparent molecular weight expressed by superficial zone chondrocytes.

Expression of MSF by chondrocytes and synovial fibroblasts results in at least 4 phenotypical variants through alternative splicing of exons 2,4 and 5. Exon 6 is common to each and thus all presumably possess the ability to lubricate apposed cartilaginous surfaces. However, lubricating ability may not strictly reside in exon 6 as chymotrypsin digestion removed lubricating activity and no aromatic residues exist in exon 6 [4]. Either end of the lubricant monomer plays an important role in providing lubrication. It is possible that the N- and C-terminal exons play a role in stabilizing the monomer to articular cartilage.

Analysis of the MSF gene by NetOGly 2.0 [23] revealed that 211 of 284 (74.2%) threonine residues and 34 of 87 (39%) of serine residues are likely glycosylated. Further analysis of just exon 6 revealed that 208 of 254 (81.9%) threonine residues and 23 of 50 (46%) of serine residues are likely glycosylated. Assuming that all predicted Ser/Thr residues within exon 6 were glycosylated, then 231 β(1,3)Gal-GalNAc sidechains could exist of which roughly 152 would be substituted with α2,3NeuAc. These hypothesized post-translational modifications could account for 132.8 kDa of the apparent 240 kDa molecular weight of lubricin which is consistent with previous observations. Digestion of human lubricin with endo-α-*N*-acetyl-D-galactosaminidase and α2,3-neuraminidase resulted in halving of the apparent molecular weight to 120 kDa [2].

Exon 6 within the MSF gene has a degenerate repeating structure of KEPAPTT. This sequence possesses both hydrophobic and hydrophilic repeating motifs constrained within a helical structure. Modeling is ongoing to determine if this sequence when glycosylated could have amphipathic character. This question is germane to our understanding of boundary lubrication. A boundary lubricant must adsorb to a substrate and generate a repulsive force through chemical or electrostatic means. Examples of boundary lubricants include graphite, molybdenum sulfide, detergents and lipids. These are known to change the physico-chemical character of surfaces enabling wettability, for example, in the case of detergents or lipid. Surface active phospholipid has been implicated to play a role in lubrication provided by synovial fluid on the basis of loss of lubrication following phospholipase digestion [24]. However, these results have been challenged by the discovery of trypsin-like proteolytic activity in the utilized phospholipase preparations [25]. Lipid, if present in synovial fluid naturally, may be a component of the lamina splendens [26], rendering articular cartilage hydrophobic—an observation supported by others [27]. Lubricin interacts with hydrophobic substrates as evidenced by its measurable activity in the friction apparatus. The present results serve to focus attention on the glycosylations of lubricin as the structural mediator of lubrication. These post-translational modifications appear to be conserved as both bovine and human lubricin share the same accessibility to endo-α-*N*-acetyl-D-galactosaminidase digestion. Structured inter-cartilaginous surface water has been observed by confocal laser scanning microscopy as a fringe pattern (liquid crystal) between apposed normal cartilage surfaces [28]. The implication is that lubricin, by virtue of its extensive glycosylations, somehow organizes water to support contact loading and shear stress.

On a technical note, the extensive glycosylation would appear to have a retarding effect on lubricin electrophoretic mobility. It has been necessary to electrophorese for longer than typical by allowing the wavefront to exit the bottom of slab gels until 5 of 7 high molecular weight markers are left beginning at 29.0 kDa. As a convention, our laboratory allows each electrophoresis experiment 90 min for its completion. Allowing slab gels to electrophorese even longer may artifactually magnify apparent molecular weight determinations. Swann noted a molecular weight of 227.5 kDa by sedimentation equilibrium analysis [1] and 206 kDa by Rayleigh light scattering [9] for bovine lubricin.

The discovery of a small amount of asialo-, bi-antennary Gal-GlcNAc glycan, tri-core fucosylated N-link on human lubricin is important in further establishing that lubrication is mediated by O-linked glycosylations. Complex N-linked glycosylations have been associated with intra-cellular signaling, initiating protein folding and secretion of final glycosylated products [29]. This same asialo- bi-antennary structure on other glycoproteins has been observed in murine macrophage like cells [29]. Despite repeated attempts, we were unable to demonstrate a corresponding N-link on bovine lubricin. The fact that the purified human lubricin contained other N-links, most likely contributed by fibronectin, is not surprising. We have previously reported [2] that the final lubricating fraction of human synovial fluid is contaminated with small amounts of fibronectin.

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