Osteoarthritis and Cartilage 25 (2017) 1496–1504

Osteoarthritis and Cartilage

Cartilage oligomeric matrix protein forms protein complexes with synovial lubricin via non-covalent and covalent interactions

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ARTICLE INFO

Article history:
Received 9 August 2016
Accepted 23 March 2017

Keywords:
Boundary lubrication
Cartilage degradation
Proteomics
O-linked glycoproteins
Lubricin
Cartilage oligomeric matrix protein

SUMMARY

Objective: Understanding the cartilage surface structure, lost in arthritic disease, is essential for developing strategies to effectively restore it. Given that adherence of the lubricating protein, lubricin, to the cartilage surface is critical for boundary lubrication, an interaction with cartilage oligomeric matrix protein (COMP) was investigated. COMP, an abundant cartilage protein, is known to be important for matrix formation.

Design: Synovial fluid (SF) from arthritic patients was used to detect possible COMP–lubricin complexes by immunological methods. Recombinant (RC) COMP and lubricin fragments were expressed to characterize this bonding and mass spectrometry employed to specifically identify the cysteines involved in inter-protein disulfide bonds.

Results: COMP–lubricin complexes were identified in the SF of arthritic patients by Western blot, co-immunoprecipitation and sandwich ELISA. RC fragment solid-phase binding assays showed that the *C*-terminal (amino acids (AA) 518-757) of COMP bound non-covalently to the *N*-terminal of lubricin (AA 105-202). Mass spectrometry determined that although cysteines throughout COMP were involved in binding with lubricin, the cysteines in lubricin were primarily focused to an *N*-terminal region (AA 64-86). The close proximity of the non-covalent and disulfide binding domains on lubricin suggest a two-step mechanism to strongly bind lubricin to COMP.

Conclusion: These data demonstrate that lubricin forms a complex network with COMP involving both non-covalent and covalent bonds. This complex between lubricin and the cartilage protein COMP can be identified in the SF of patients with arthritis conditions including osteoarthritis (OA) and rheumatoid arthritis (RA).

Introduction

Arthritic diseases including osteoarthritis (OA)[1] and rheumatoid arthritis (RA)[2] result in destruction of the joint surface leading to debilitating pain and restricted motion. Although RA treatment is increasing in efficacy[3], OA treatment is limited to symptom management, making understanding joint biomechanics essential for devising treatments to re-establish the lubricating joint surface.

The cartilage extracellular matrix (ECM) in diarthrodial joints has lubricating and shock-absorbing functions. Lubrication is created by two modes: *boundary mode*, created at the cartilage surface, and *mixed mode*, a combination of boundary and hydrodynamic mode lubrication[4,5]. Hydrodynamic mode is generated by major synovial fluid (SF) components including hyaluronic acid and lubricin, a heavily *O*-glycosylated, mucin-like glycoprotein[4,5]. Boundary lubrication at the mechanically vulnerable cartilage surface creates a smooth non-adherent low friction coating vital for cartilage surface structural integrity; studies show that lubricin is essential for these properties[4,6,7].

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http://dx.doi.org/10.1016/j.joca.2017.03.016

Lubricin (PRG4), also known as superficial zone protein and proteoglycan 4, has an expected molecular weight of 150 kDa that is almost doubled by its glycosylation. Although also present in the blood, urine and on the ocular surface[8–10], in the joint, lubricin is synthesized by articular chondrocytes and synoviocytes, and enriched in the superficial zone of articular cartilage, the synovial membrane, tendons and the SF[11,12]. Although it seems that adherence of lubricin to the cartilage surface is necessary for boundary lubrication, the assembly remains unknown.

The SF of arthritic patients provides a unique opportunity to analyse this surface structure as it is lost from the cartilage into the SF during disease. Proteomic analysis of the acidic fraction of SF has identified lubricin and a range of other proteins, including cartilage oligomeric matrix protein (COMP)/thrombospondin 5 (TSP5)[13,14]. COMP is a homopentameric ECM glycoprotein, with five 100–110 kDa subunits[15]. COMP is mainly synthesized by chondrocytes, secreted into cartilage ECM where it is essential for matrix formation through its interactions with a range of molecules including aggrecan, fibronectin, matrilins and collagens[16–20]. COMP and its fragments are released into the SF, where they can interact with and regulate complement factors[21]. COMP in serum is considered a biomarker for early cartilage damage[22].

As COMP has complex forming capabilities and may co-purify with lubricin, we investigated a potential COMP–lubricin complex in arthritic SF. These analyses were able to identify a complex network of non-covalent and covalently bonded COMP and lubricin.

Methods

Patient sample information and study design

Eight independent SF samples from RA ($n = 5$ designated RA1, RA2, RA3, RA4, and RA5), OA ($n = 2$, OA1 and OA2) and Spondyloarthritis (SpA) ($n = 1$, SpA1) patients were collected during aspiration of knee joints at the Rheumatology Clinic, Sahlgrenska University Hospital (Gothenburg, Sweden) and Lund University Hospital (Lund, Sweden). All RA patients fulfilled the American College of Rheumatology 1987 revised criteria for RA[23]. All patients gave informed consent and the procedure approved by the Ethics Committees of Gothenburg and Lund Universities. Acidic fractions, containing lubricin and COMP, were purified from SF as previously described[13].

Discovery Western blot, proteomic, co-immunoprecipitation, size exclusion chromatography and ELISA experiments used human SF samples (*n* described throughout results and dependent on sample volume availability). The binding domain of each protein involved in bond formation was then determined by solid-phase binding assays using truncated recombinant (RC) COMP and lubricin, and MS disulfide bond analyses of SF ($n = 2$).

Immunoassays of synovial lubricin and COMP

Western blotting

Samples were separated using agarose-SDS-PAGE or 3–8% Tris/acetate gels. Transferred PVDF membranes were blocked (3% bovine serum albumin (BSA) in PBS), and probed with mouse anti-human lubricin (mAb 13, Pfizer Research), or rat anti-human COMP (mAb HC484D1, AbD Serotec). HRP conjugated secondary antibodies included rabbit anti-mouse immunoglobulins (DakoCytomation) and rabbit anti-rat IgG and IgM (Jackson ImmunoResearch Europe Ltd.).

Co-immunoprecipitation

SF acidic fractions (8 μg) and anti-COMP antibody or PBS were incubated 4°C overnight. Protein G agarose beads (100 μL, Thermo Scientific) were added for 2 h at RT, washed (3 × 10 min 0.75% Triton X-100 then PBS 4 × 10 min) and resuspended in non-reducing sample buffer for SDS-PAGE and Western blot analysis using anti-lubricin mAb13. ImageJ was used to quantitatively compare bands[26].

Size exclusion chromatograpy

HPLC with a BioSuite 450 column (8 μm HR SEC, 7.8 × 300 mm, Waters) was used to separate a portion of enriched SF glycoproteins, at a flow rate of 0.25 mL/min with a mobile phase of 2 M urea in PBS. The eluents (0.25 mL/fraction) were then analyzed by ELISA using mouse anti-human lubricin-specific mAb (mAb13, Pfizer Research) and rat anti-human COMP-specific mAb (mAb HC484D1, AbD Serotec).

Sandwich ELISA

Anti-COMP mAb (HC484D1, 10 μL/mL in 50 mM sodium carbonate buffer, pH 9.6, 16 h, 4°C) was the capture antibody and coated onto 96 well plate, blocked (1% BSA in PBS-T) and incubated with diluted SF. Anti-lubricin (mAb13) was added and detected with HRP conjugated rabbit anti-mouse Ig at 0.5 μg/mL and 3,3′,5,5′-tetramethylbenzidine before absorbance was measured (450 nm). Assay performed in triplicate.

Solid-phase binding assays

Assays were conducted with conditions as for sandwich ELISAs. RC protein (5 μg/mL) coated plates were incubated with potential interacting proteins (1 μg/mL) and binding detected with mouse anti-His mAb (18184, AbCam) and alkaline phosphatase-conjugated goat anti-mouse and visualized using para-nitrophenylphosphate. Assays were performed in triplicate and repeated twice, statistics described below.

Production of RC lubricin and COMP fragments

RC human lubricin with a FLAG-tag and a truncated mucin-like domain (without amino acids (AA) 403-870) was produced in 293F cells using p3xFLAG-CMV-8 vector (Sigma–Aldrich) and purified on anti-FLAG beads. The same method was used to produce four lubricin fragments named L25-221 (all molecular weights are calculated, MW 21.9 kDa), L220-402 (MW 19.2 kDa), L871-1078 (MW 22.6 kDa), L1079-1404 (MW 37.2 kDa, Fig. 2(A)). GST-tagged fragments of the *N*-terminal domain divided by exon boundaries (exons 2–5), named L25-72 (MW 5.3 kDa), L67-109 (MW 5.0 kDa), L105-160 (MW 6.0 kDa), L155-202 (MW 5.2 kDa), were produced using pGEX-5X-3 vector (GE Healthcare) and Rosetta 2 *E. coli* (Novagen), purified in native conditions using glutathione beads (Pierce). The His-tagged fragments of COMP [Fig. 2(A)] named mCOMP (AA 73-757, MW 79.3 kDa), TII1-TIII8 (AA 73-517, MW 51.7 kDa), TIII1-CG (AA 268-757, MW 59.0 kDa), TIII1-8 (AA 268-517, MW 31.5 kDa) and CG (AA 518-757, MW 31.9 kDa), based on the AA sequence of the human COMP reference sequence (NM_000095.2), were produced in human 293-EBNA cells and purified as previously described[21]. The identity of the proteins was confirmed by mass spectrometry, and purity by SDS-PAGE (Fig. S3).

Mass spectrometry methods

Protein identification from gel bands

Coomassie stained gel bands were excised, destained, dried and digested with 300 ng sequencing-grade lysine-C (50 mM ammonium bicarbonate, 4 h, 37°C, Promega) then 500 ng trypsin (overnight, 37°C, Promega). Samples were dried and resuspended in 50 mM ammonium bicarbonate with 0.2% formic acid for LC-MS/MS analysis. The MassMatrix conversion tool was used for file