Consequences of Disease-causing Mutations on Lubricin Protein Synthesis, Secretion, and Post-translational Processing*

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Lubricin, a protein product of the gene PRG4, is a secreted mucin-like proteoglycan that is a major lubricant in articulating joints. Mutations in PRG4 cause the autosomal recessive, human disorder camptodactyly-arthropathy-cova vara-pericarditis syndrome. We developed rabbit polyclonal antibodies against human lubricin to determine the consequence of disease-causing mutations at the protein level and to study the protein's normal post-translational processing. Antiserum generated against an epitope in the amino-terminal portion of lubricin detected protein in wild-type synovial fluid and in conditioned media from wild-type cultured synoviocytes. However, the antiserum did not detect lubricin in synovial fluid or cultured synoviocytes from several patients with frameshift or nonsense mutations in PRG4. Antiserum generated against an epitope in the protein's carboxyl-terminal, hemopexin-like domain identified a post-translational cleavage event in wild-type lubricin, mediated by a subtilisin-like proprotein convertase (SPC). Interestingly, in contrast to wild-type lubricin, one disease-causing mutation that removes the last 8 amino acids of the protein, including a conserved cysteine residue, was not cleaved within the hemopexin-like domain when expressed in COS-7 cells. This suggests that formation of an intrachain disulfide bond is required for SPC-mediated cleavage and that SPC-mediated cleavage is essential to protein function.

Lubricin, a protein product of the gene PRG4, is a large (>200 kDa) secreted proteoglycan that functions as the major lubricant in articulating joints (1, 2). PRG4 also encodes superficial zone protein (3–5), which is expressed by superficial zone chondrocytes, and two other proteins, megakaryocyte-stimulating factor (6) and hemangiopeitin (7), which have unknown in vivo biologic activities. Produced and secreted by synoviocytes and superficial zone chondrocytes, lubricin is present in synovial fluid and at the cartilage surface (3, 4). Functions attributed to lubricin within articulating joints include protection of cartilage surfaces from protein deposition and cell adhesion, inhibition of synovial cell overgrowth, and boundary lubrication at the articular cartilage surface (8).

Analysis of the amino acid sequence of lubricin reveals that it is a paralog of vitronectin. Both proteins contain somatomedin B (SMB) and hemopexin-like (PEX) domains (6). In the case of vitronectin, the SMB domain was shown to regulate protein binding to the urokinase-type plasminogen activator receptor and to plasminogen activator inhibitor (9, 10). The binding of vitronectin to the urokinase-type plasminogen activator receptor is postulated to regulate cell surface plasminogen activation and cell adhesion (11). In addition to being present in vitronectin, PEX domains are also found in members of the matrix metalloproteinase (MMP) family, where they mediate attachment to extracellular matrix proteins, such as collagen and fibronectin (12). Cleavage by subtilisin-like proprotein convertases (SPCs) has been described for the PEX domains of vitronectin and MMPs, but the biological consequences of this processing event are unknown (13, 14). Lubricin differs from vitronectin by also containing a large, central, extensively O-linked glycosylated, mucin-like domain (2, 15). The abundance of negatively charged sugars within this domain creates strong repulsive hydration forces that enable the protein to act as a boundary lubricant (16).

Mutations in PRG4 cause the rare, autosomal recessive disease camptodactyly-arthropathy-cova vara-pericarditis syndrome (CACP) (17). Individuals with CACP have normal appearing joints at birth but with advancing age develop joint failure associated with noninflammatory synoviocyte hyperplasia and subintimal fibrosis of the synovial capsule (18). Prg4 knock-out mice exhibit phenotypic features similar to patients with CACP and have severe joint pathology, including articular cartilage destruction and marked synovial cell overgrowth (8). Therefore, it appears that lubricin plays an essential role in maintaining healthy joint function and that CACP disease-causing mutations alter the ability of lubricin to perform this role. An allelic series of mutations within PRG4 have been identified in individuals

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The abbreviations used are: SMB, somatomedin B-like; PEX, hemopexin-like; MMP, matrix metalloproteinase; SPC, subtilisin-like proprotein convertase; CACP, camptodactylty-arthropathy-cova vara-pericarditis syndrome; HA, hemagglutinin; RIPA, radioimmune precipitation; TBS, Tris-buffered saline; SZP, superficial zone protein; OA, osteoarthritis.
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with CACP (17,2). These disease-causing mutations can be used to identify regions and/or protein processing events that are important for proper function. We developed polyclonal antibodies against two conserved domains within the amino and carboxyl-terminal regions of lubricin and utilized these antisera on patient-derived synovial fluid and cultured synoviocytes to identify the consequences of disease-causing mutations at the protein level. For one disease-causing mutation, in which affected tissue was unavailable, we expressed recombinant forms of wild-type and mutant protein in COS-7 cells and identified a specific cleavage event within the protein’s PEX domain that appears essential to the protein’s function.

MATERIALS AND METHODS

Collection of Synovial Fluid Aspirates and Cultured Synoviocytes from Patients with Osteoarthritis, Rheumatoid Arthritis, and CACP—CACP samples were obtained as discarded material at the time of diagnostic joint aspiration, synovial biopsy, or joint replacement surgery. All patients gave their informed consent. The Institutional Review Board of University Hospitals of Cleveland approved this project. Osteoarthritis and rheumatoid arthritis samples were obtained as discarded material at the time of diagnostic or therapeutic joint aspiration or joint replacement surgery. Synovial fluid samples that were not sterile and appearance were centrifuged at 10,000 x g for 20 min at 4 °C and then stored frozen at −20 °C. Synoviocytes were recovered from synovial tissue by identifying the intimal surface of the tissue, dissecting it free of adjacent adventitial tissue, and then culturing the cells in the presence of serum-free Dulbecco’s modified Eagle’s medium containing 0.4% collagenase (w/v) (Sigma) for 3 h at 37 °C in a tissue culture incubator. Nonadherent cells in these digested samples were centrifuged and washed three times in growth medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B, all from Life Technologies). Cells were grown in 100-mm dishes to ~80% confluence and then split 1:3. Cells were then maintained in growth medium that contained 20% fetal bovine serum.

Generation of Lubricin Expression Constructs—A pME18S vector, which expresses a common splice variant of mouse lubricin (PrG4) that lacks exons 2, 4, and 5 (19), was generously provided by Dr. Shiro Ikegawa. To perform site-directed mutagenesis that will alter the carboxyl-terminal region of lubricin and utilized these antisera on pEX domain (p.K1230fs; using nomenclature (17)).

To determine whether inhibiting SPC enzymes affected cleavage of lubricin, at 24 h following transfection, cells were switched from growth medium to serum-free medium containing the SPC inhibitor decanoyl-RVRR-chloromethylketone (50 μM) (Bachem). Medium and cell lysate were harvested, and aliquots were analyzed for the presence of protein by Western blot.

Expression and Immunodetection of Recombinant Lubricin and Lubricin Subdomains—COS-7 cells were cultured in growth medium. Cells were then trypsinized and plated in 6-well cell culture plates (Costar 3506) and allowed to grow to confluent. At 80% confluence, the cells had reached confluence and were replaced with 2 ml of fresh growth medium containing 20% fetal bovine serum. Three days later, the medium was collected and cleared of debris by centrifugation at 1,800 x g for 10 min at room temperature. Ten μl of conditioned medium was mixed with 10 μl of 2× loading buffer, boiled, and separated by SDS-PAGE as described above. Cell lysate was also collected by adding 350 μl of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris, pH 8), scraping the cells, and centrifuging the lysate at 12,000 x g for 10 min at room temperature. Ten μl of the cell lysate supernatant and 10 μl of 2× sample loading buffer were mixed, boiled, and separated by SDS-PAGE.

Lubricin was immunodetected using either J108N or J109C anti-lubricin serum. Immunoblots were blocked for 1 h with 10% nonfat dry milk in PBS (pH 7.5), then incubated with either a 1:500 dilution of J108N or a 1:250 dilution of J109C in antibody buffer (5% nonfat dry milk, TBS, 0.05% Tween 20, pH 7.5) for 1 h. After three 5-min washes with RIPA buffer, blots were incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in antibody buffer for 1 h and then washed again three times with RIPA buffer. Immunoreactive proteins were detected with the ECL Plus chemiluminescent detection system (Amersham Biosciences).

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Antisera Generated against Epitopes within the Amino and Carboxyl Domains of Lubricin Recognize Endogenous and Recombinant Lubricin with High Specificity—We generated two polyclonal antisera against epitopes within human lubricin (Fig. 1). The epitopes were chosen to recognize the N-terminal or C-terminal regions of the protein. The N-terminal antisera (J108N) was generated against a peptide motif within the second SMB domain, whereas the C-terminal antisera (J109C) was generated against a peptide motif within the PEX domain.

The J108N antisera could detect a >200-kDa band on SDS-PAGE Western blots, consistent with the size of full-length lubricin, in synovial fluid samples from patients with rheumatoid arthritis and osteoarthritis (Fig. 2A and data not shown). In addition to full-length lubricin, multiple smaller polypeptide species (<39 kDa) were also detected by this antisera in patients with rheumatoid arthritis, which probably represent degradation products (Fig. 2A). Importantly, neither full-length lubricin nor any smaller polypeptides were present in synovial fluid samples from several patients with CACP, although equal amounts of synovial fluid protein were evaluated (Fig. 2A). These data indicate that J108N antisera is specific to lubricin in synovial fluid. Further support for the specificity of the antibody derives from studies using recombinantly expressed protein (Fig. 2B).

The J109C antisera was also tested for its ability to recognize lubricin. Synovial fluid samples from an osteoarthritis patient and from a CACP patient were analyzed by Western blot under reducing conditions (Fig. 3A). Additionally, synovial fluid specimens recovered from healthy cow and pig joints were also evaluated using this antibody (Fig. 3B). In the osteoarthritis sample, the antibody detected full-length protein and also a single 14-kDa species, whereas neither band was detected in the CACP sample (Fig. 3A). The specificity of this antisera for lubricin was further confirmed using cultured synoviocytes and recombinantly expressed protein (data not shown). Intriguingly, antisera J109C only detected the 14-kDa fragment in synovial fluid from a healthy pig and cow (Fig. 3B), suggesting that this fragment results from a normal post-translational processing event. However, an alternative explanation for this finding is that J109C cannot detect full-length pig or cow lubricin because their epitopes are obscured when the protein is not cleaved.

Absence of Lubricin Synthesis or Secretion Is a Common Mechanism of Mutational Effect for CACP Disease-causing Mutations—Synovial fluid aspirates were available from four patients with disease-causing mutations in PRG4 (Fig. 1). In contrast to synovial fluid recovered from patients with osteoarthritis or rheumatoid arthritis, in which lubricin and/or lubricin degradation products were readily detectable by immunoblotting, no immunodetectable lubricin or lubricin fragments were present in the CACP samples (Figs. 2 and 3). This implies that disease-causing frameshift and nonsense mutations commonly affect PRG4 mRNA stability or lead to the production of an unstable, truncated lubricin polypeptide. To distinguish between these two possibilities, lubricin protein was assessed in conditioned medium and cell lysates from cultured wild-type and CACP patient synoviocytes with defined mutations (Figs. 1 and 2C). Each of these mutations occurs downstream of the SMB epitope detected by J108N, so that this antisera should detect truncated forms of lubricin, were they to be expressed. Lubricin was not detected in conditioned medium or cell lysate from either cultured CACP patient synoviocytes, although it was present in conditioned medium or cell lysate from wild-type (i.e. osteoarthritis (OA)) synoviocytes (Fig. 2C). Therefore, nonsense-mediated mRNA decay (21) seems to be the most likely mechanism leading to absent lubricin synthesis in patients with nonsense and frameshift mutations that involve exons upstream of the final coding exon.

Post-translational Processing of the Lubricin PEX Domain Is Functionally Important—Two siblings with CACP were found to be homozygous for a mutation in the final coding exon of PRG4 (17). This mutation creates a premature termination codon 23 nucleotides upstream of the proper termination codon. The effect of this mutation is not predicted to cause nonsense-mediated mRNA decay but is instead predicted to produce a polypeptide that lacks the final 8 amino acid residues of the protein. Because synovial fluid and synoviocytes were not available from these siblings, we expressed this mutant protein (pSI397T) using COS-7 cells. Truncating the final 8 amino acid residues of the protein did not affect the secretion of the protein (Fig. 2B), suggesting that the mutant protein is likely to be present in synovial fluid but be nonfunctional. Because a highly conserved cysteine residue is among the 8 deleted residues...
FIG. 2. J108N antiserum recognizes wild-type lubricin. A, Western blot analysis of synovial fluid samples from two patients with rheumatoid arthritis (RA1 and RA2) and three patients with mutation-confirmed CACP (CA). Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected using J108N antiserum. J108N detects full-length lubricin (arrowhead) and a series of smaller species (bracketed) in the rheumatoid arthritis samples. No immunodetectable full-length lubricin or smaller fragments are detected in the synovial fluid from the three patients with CACP. The Coomassie-stained gel below the Western blot demonstrates that comparable protein was loaded in each lane. B, Western blot analysis of conditioned media (CM) and cell lysates (CL) from untransfected COS-7 cells, cells expressing wild-type mouse lubricin, and cells expressing lubricin mutants corresponding to the human mutations p.S1397X and p.K1230fs. Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected using J108N antiserum. The antibody detects full-length lubricin in cell lysates from all transfectected cells. Wild-type and the p.S1397X mutant proteins are also secreted into the conditioned medium. In contrast, the mutant that lacks half of the PEX domain (p.K1230fs) is poorly secreted, although it is expressed at comparable levels within the cells. Note that the most likely consequence of the p.K1230fs mutation in vivo would be to cause nonsense-mediated mRNA decay rather than synthesis of the truncated polypeptide. However, the p.S1397X mutation lacks only the final 8 amino acid residues and is expected to produce a stable mRNA that is translated in vivo. C and D, Western blot analyses of cell lysates (C) and conditioned medium (D) of the OA synoviocytes recovered from a patient with OA or from patients with mutation-confirmed CACP. Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected using J108N antiserum. Equal loading of cell lysate in each lane is demonstrated by immunodetection of the same blot using a monoclonal anti-tubulin antibody (D-10-HRP; Santa Cruz Biotechnology). Immunodetectable lubricin (molecular mass >200 kDa) is only present in the cell lysate (C) and conditioned medium (D) of the OA synoviocytes. The lubricin schematic diagram denotes the location of the J108N epitope in the second SMB domain.

FIG. 3. J109C antiserum detects a C-terminal fragment of lubricin. A, Western blot analysis of synovial fluid from a patient with OA and a patient with mutation-confirmed CACP. Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected using J109C antiserum. Full-length lubricin (arrowhead) is detected in the synovial fluid from the OA patient but not from the patient with CACP. In addition, a 14-kDa species (double arrowhead) is present in the OA synovial fluid, but not in the CACP fluid, indicating that this is a C-terminal lubricin fragment. B, Western blot analysis of synovial fluid from the patient with OA and from healthy porcine and bovine joints. Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected using J109C antiserum. J109C detects the small C-terminal fragment in all samples (double arrowhead). In contrast to human OA synovial fluid, where full-length lubricin is also detected, only the C-terminal fragment is detected in pig and cow, suggesting that this is a normal lubricin processing step and that incomplete processing may be associated with acquired disease. C, Western blot analysis of conditioned media from COS-7 cells expressing C-terminal Myc-tagged, wild-type mouse lubricin (Lub-Myc). Samples were separated by reducing SDS-PAGE (12% gel) and immunodetected using an anti-Myc antibody. The lubricin C-terminal cleavage product is 14 kDa, a size that corresponds with the lubricin cleavage products seen in A and B. D, J109C immunoprecipitates the 14-kDa cleavage product from the conditioned media of COS-7 cells expressing Lub-Myc. Samples were immunoprecipitated using the J109C antiserum, separated by reducing SDS-PAGE (4–20% gel), and immunodetected using an anti-Myc antibody. The lubricin schematic denotes the location of the J109C epitope and the SPC cleavage site (asterisk).
Lubricin is cleaved within its PEX domain by SPCs. A, Western blot analysis of conditioned media and cell lysates from COS-7 cells expressing C-terminal Myc-tagged, wild-type, mouse lubricin (Lub-Myc) or Lub-Myc and SPC4 (done in duplicate). Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected using an anti-Myc antibody. Full-length lubricin (arrowhead) is present in each cell lysate. The C-terminal cleavage product (double arrowhead) is present in the cell lysates and increases in abundance when SPC4 is co-expressed. Occasionally, a <52-kDa band is detected in some of the transfected cell lysates; the identity of this species is not known. Only the C-terminal cleavage product is detected in the conditioned media. It too increases in abundance when SPC4 is co-expressed. B, Western blot analysis of conditioned media and cell lysates from COS-7 cells expressing Lub-Myc and cultured in the presence or absence of decanoyl-RVKR-chloromethyl ketone (dRVKR-CMK), a peptide inhibitor of SPC enzymes. Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected using an anti-Myc antibody. Full-length lubricin (black arrowhead) is detected in the cell lysates and faintly in the conditioned media of cells treated with the peptide inhibitor (gray arrowhead). Treatment with the peptide inhibitor of SPCs reduced the amount of the lubricin C-terminal cleavage fragment in the conditioned media (double arrowhead).

When we expressed this construct in COS-7 cells, we detected a 14-kDa polypeptide following SDS-PAGE under reducing conditions (Fig. 3C). This size is consistent with the polypeptide being the C-terminal fragment previously observed in human, cow, and pig synovial fluid (Fig. 3B). Under nonreducing conditions, this fragment migrated as a >200-kDa polypeptide, suggesting that it is disulfide-linked to a larger polypeptide (Fig. 6B). To confirm the identity of this fragment, we used the J109C antiseraum and were able to immunoprecipitate the fragment from conditioned medium (Fig. 3D).

Post-translational processing by subtilisin-like proprotein convertases has been observed within the PEX domains of other proteins (14, 22). We tested whether the PEX domain of lubricin was also cleaved by SPCs. Whereas endogenous cleavage of recombinant lubricin occurred in COS-7 cells, the amount of cleavage was increased when SPC4 was co-expressed (Fig. 4A). Furthermore, decanoyl-RVKR-chloromethyl ketone, a peptide inhibitor of SPCs, reduced cleavage in COS-7 cells (Fig. 4B).

Several potential SPC consensus cleavage sites are present within the PEX domain of lubricin. The likely cleavage site that would yield a 14-kDa C-terminal fragment is in an arginine-rich region between amino acid residues 947 and 954 of the mouse protein; the amino acid sequence in this region is RRRRFEA in humans, mice, rats, chimpanzees, and cows (Fig. 5A). To identify the preferred cleavage site, we expressed recombinant forms of Myc-tagged lubricin or the PEX domain of lubricin present in humans, chimpanzees, mice, rats, and cows. The first arginine (R) represents Arg947 in the mouse sequence. The LubR949/951AMyc construct mutates both Arg949 and Arg951 residues to alanines, thereby removing any potential RR cleavage sites and the first possible RXXR cleavage site. The LubR949/951/955AMyc construct mutates the Arg949, Arg951, and Arg955 residues to alanines, thereby removing all RR or RXXR cleavage sites. B, Western blot analysis of conditioned media and cell lysates from COS-7 cells expressing Lub-Myc, LubR949/951AMyc, or LubR949/951/955AMyc alone or co-expressed with SPC4. Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected with an anti-Myc antibody. The anti-Myc antibody detects full-length lubricin (black arrowhead) in the cell lysates of all transfected cells. A faint band, corresponding to full-length lubricin, is present in the conditioned media of cells co-transfected with Lub-Myc and LubR949/951/955AMyc, even when co-expressed with SPC4. This indicates that SPC-mediated cleavage of lubricin probably occurs after the Arg955 residue of the mouse protein. C, Western blot analysis of conditioned media from COS-7 cells expressing either the wild-type PEX domain of lubricin (HA-PEX) or the PEX domain of lubricin with Arg955 mutated to an alanine (HA-PEX R955A). Both constructs were tagged with an N-terminal HA epitope. Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected using an anti-HA antibody. The wild-type PEX domain is fully cleaved, yielding a 25-kDa, N-terminal fragment (double arrowhead). The HA-PEX R955A construct is only partially cleaved so that the majority of the expressed protein is the 38-kDa uncleaved polypeptide (arrowhead). A small amount of this polypeptide is cleaved by SPC4, implying that less preferred cleavage sites do exist.

Cleftage site follows Arg955, since mutation of this residue resulted in the greatest impairment of cleavage (Fig. 5, B and C).

An Intrachain Disulfide Bond Involving the First and the Final Cysteine Residues within the PEX Domain Is Required for C-terminal Cleavage—In other proteins with PEX domains, the domain folds into a four-bladed propeller structure, in which a disulfide bond forms between a cysteine residue in the first blade and the final cysteine residue in the fourth blade (Fig. 6A) (23). In vitronectin and MMP25, following the cleavage event within these PEX domains, the two resulting polypeptides remain tethered together by this disulfide bond (14, 22).

Our data suggest that a similar event occurs in lubricin, since the C-terminal fragment detected with J109C or with the anti-Myc antibody (when Lub-Myc protein was expressed) ran as a high molecular weight species under nonreducing conditions (Fig. 6B). This same high molecular weight species was detected with J108N (data not shown), implying that following
cleavage, the amino and carboxyl polypeptides remain disulfide-linked and that this linkage is biologically important.

Because two siblings with CACP have a mutation that removes the final cysteine in the PEX domain, we tested whether the disulfide bond involving this cysteine was required for SPC-mediated cleavage. We mutated the final cysteine residue to alanine in Lub-Myc and LubC1053A-Myc (in which the last cysteine in Lub-Myc has been mutated to alanine). Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected with an anti-Myc antibody. Under reducing conditions, only the 14-kDa C-terminal fragment is detected. Under nonreducing conditions, the amount of the 14-kDa fragment is reduced, and a species migrating at the size of full-length lubricin appears. This indicates that much of the C-terminal fragment maintains the disulfide bond following cleavage. Western blot analysis of conditioned media and cell lysates from COS-7 cells expressing Lub-Myc or LubC1053A-Myc (in which the last cysteine in Lub-Myc has been mutated to alanine) were shown to be reduced when Lub-Myc was expressed (Fig. 6B), similar to what is observed when Lub-Myc was expressed (Fig. 6C). Single residue mutations involving the third or fourth cysteine residues in the PEX domain do not impair C-terminal cleavage. Western blot analysis is shown of conditioned media from COS-7 cells expressing Lub-Myc or Lub-Myc with the third (Lub-C853A-Myc) or fourth (Lub-C930A-Myc) cysteine in the PEX domain mutated to alanines. Samples were separated by reducing SDS-PAGE (4–20% gel) and immunodetected using an anti-HA antibody. Mutating either the third or fourth cysteine residue did not impair C-terminal cleavage.

**DISCUSSION**

Lubricin is a protein that is essential to joint and tendon function. Mutations affecting this protein cause CACP in humans and a similar disorder in mice (8, 17). We produced antisera against polypeptide epitopes within lubricin that are present in all known isoforms (15, 19) (Fig. 1). These antibodies demonstrate that most patients with CACP disease-causing mutations do not have lubricin or lubricin fragments in their synovial fluid (Figs. 2 and 3). Furthermore, their synovocytes do not produce truncated forms of lubricin in cell culture (Fig. 2). This is in contrast to synovial fluid obtained from patients with osteoarthritis and rheumatoid arthritis, in which lubricin is produced by cultured synoviocytes, and lubricin and its amino-terminal degradation fragments are easily detected in synovial fluid. Consequently, immunoblot analysis of synovial fluid may be a useful diagnostic adjunct in children suspected of having CACP before many signs and symptoms of disease are present. Immunodetection of lubricin and its degradation products may also have clinical application in monitoring therapeutic responses in patients with inflammatory joint diseases, such as rheumatoid arthritis. Enzymes released into the joint space during inflammation, such as neutrophil elastase and matrix metalloproteinases, have been demonstrated to degrade lubricin in vitro (24). Therefore, the efficacy of therapies aimed at decreasing inflammation or inhibiting enzymatic degradation of synovial and cartilage constituents might be monitored by determining the abundance of full-length lubricin in synovial fluid and the ratio between full-length protein and its degradation fragments.

Other investigators have generated monoclonal antibodies against bovine and human superficial zone protein (SZP) (5, 25). Two monoclonal antibodies generated against bovine SZP were able to detect a >250-kDa band by Western blot in the conditioned medium of cultured bovine chondrocytes. One antibody also demonstrated that SZP was present at the surface of cartilage and produced by superficial zone chondrocytes (5). This same antibody demonstrated SZP expression in tendons (26). Interestingly, one of the antibodies generated...
against the final 400 amino acid residues of SZP detected full-length protein along with smaller SZP species migrating at ~60–70 and ~140–150 kDa by Western blot (5). These data suggest that lubricin/SZP may undergo additional C-terminal processing events beside the SPC-mediated cleavage event that we describe. However, we did not detect intermediate sized fragments in human, bovine, or porcine synovial fluid or in conditioned medium from cultured wild-type human synoviocytes using J108N (Fig. 2) or J109C (data not shown), suggesting that the processing detected in bovine chondrocytes may be a consequence of the agarose cell culture system the investigators employed (5). Purified human SZP, recovered from human synovial fluid or from the conditioned medium of cultured human superficial zone chondrocytes, was also used to generate monoclonal antibodies (25). Several clones were recovered that had different characteristics with respect to binding affinity of antibody to native, complexed, and denatured protein. Several of the antibodies could identify lubricin/SZP in other species including, cow, dog, rabbit, and guinea pig. Some of these antibodies were also able to detect a slightly faster migrating band in human plasma and serum. Whether these particular bands represent products of PRG4 or cross-reacting proteins could be tested using plasma/serum from patients with CACP, since we have shown that cells and tissues from many of these patients do not express the protein (Fig. 2).

Using an antiserum against an epitope within the C-terminal PEX domain of lubricin, we found that this domain is normally post-translationally modified by a cleavage event. We demonstrated that this cleavage is likely to be mediated by SPC enzymes at a highly conserved amino acid sequence (RFERA). Other proteins, such as vitronectin and MMP-25, undergo SPC-mediated cleavage within their respective PEX domains (13, 14). Cleavage in MMP-25 may alter the ability of this protein to bind to claudin (14). Cleavage in vitronectin abolishes phosphorylation of serine 378 by protein kinase A (27). Although the biologic consequences of these processing events have not been determined in vivo, their occurrence implies physiologic relevance and suggests that similar processing within lubricin will be biologically important. Similar to vitronectin and MMP-25, we have found that the first and last cysteine residues within the PEX domain of lubricin remain covalently linked following SPC-mediated cleavage (Fig. 6B).

Lubricin has been postulated to form higher order structures (28). Other mucinous glycoproteins form higher order structures by establishing intermolecular disulfide linkages, although they do not contain PEX domains (29). It is possible that the PEX domain of lubricin has been co-opted for this function, since it contains three additional cysteine residues, which are not involved in intrachain disulfide binding and do not affect C-terminal cleavage when mutated. Cleavage within the PEX domain may enable them to participate in intermolecular interactions.

Support for the in vivo functional importance of cleavage within the PEX domain of lubricin derives from two observations. First, only the cleaved fragment was present in synovial fluid from a healthy pig and cow joint, suggesting that complete cleavage of the protein is a normal event. Second, two siblings with CACP have a mutation that is predicted to eliminate the final 8 amino acid residues of the protein. Whereas it is possible that this mutation causes mRNA instability and nonsense-mediated mRNA decay, mutations occurring within the final coding exon that are less than 50 nucleotides upstream of the proper termination codon generally escape this surveillance process (21). Therefore, it is probable that lubricin is produced by the synovia of these children and is nonfunctional. We expressed this mutant protein in COS-7 cells and found that it was secreted at rates comparable with wild-type protein (Fig. 2). However, in contrast to wild-type protein, mutant protein lacking the final cysteine residue did not undergo SPC-mediated cleavage within the PEX domain (Fig. 6C). We showed that a disulfide bond between the first and last cysteine residues within the PEX domain is required for SPC-mediated cleavage (Fig. 6D).

Our studies have only addressed lubricin expression and PEX domain cleavage in synovial fluid, cultured synoviocytes, and COS-7 cells expressing recombinant protein. Alternatively spliced or modified forms of PRG4 (e.g. SZP) are likely to be expressed by cartilage surface chondrocytes (4). It is possible that lubricin/SZP molecules with intact PEX domains are better able to bind to other proteins at the cartilage surface, enhancing their role as boundary lubricants. Transforming growth factor β has been shown to increase expression of lubricin (5) and the SPC family member furin (30). Transforming growth factor β is produced during active inflammation (31). By increasing SPC expression and PEX domain cleavage, transforming growth factor β could be involved in the release of lubricin/SZP from the cartilage surface, causing diminished boundary lubrication and surface protection.

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REFERENCES

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