Matrix-degrading protease ADAMTS-5 cleaves inter-α-inhibitor and releases active heavy chain 2 in synovial fluids from arthritic patients

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Destruction of the cartilage matrix in joints is an important feature of arthritis. Proteolytic degradation of cartilage glycoproteins can contribute to the loss of matrix integrity. Human inter-α-inhibitor (IαI), which stabilizes the extracellular matrix, is composed of the light-chain serine proteinase inhibitor bikunin and two homologous heavy chains (HC1 and HC2) covalently linked through chondroitin 4-sulfate. Inflammation promotes the transfer of HCs from chondroitin 4-sulfate to hyaluronan by tumor necrosis factor–stimulated gene-6 protein (TSG-6). This reaction generates a covalent complex between the heavy chains and hyaluronan that can promote leukocyte invasion. This study demonstrates that both IαI and the HC–hyaluronan complex are substrates for the extracellular matrix proteases ADAMTS-5 and matrix metalloprotease (MMP) -3, -7, and -13. The major cleavage sites for all four proteases are found in the C terminus of HC2. ADAMTS-5 and MMP-7 displayed the highest activity toward HC2. ADAMTS-5 degradation products were identified in mass spectrometric analysis of 29 of 33 arthropathic patients, indicating that ADAMTS-5 cleavage occurs in synovial fluid in arthritis. After cleavage, free HC2, together with TSG-6, is able to catalyze the transfer of heavy chains to hyaluronan. The release of extracellular matrix bound HC2 is likely to increase the mobility of the HC2/TSG-6 catalytic unit and consequently increase the rate of the HC transfer reaction. Ultimately, ADAMTS-5 cleavage of HC2 could alter the physiological and mechanical properties of the extracellular matrix and contribute to the progression of arthritis.

Human inter-α-inhibitor (IαI) is a heterotrimer consisting of the serine protease inhibitor bikunin (UniProt P02760) and two homologous heavy chains referred to as heavy chain 1 (HC1) (UniProt P19827) and heavy chain 2 (HC2) (UniProt P19823) (1). Bikunin is a proteoglycan with an undersulfated chondroitin 4-sulfate (CS) chain attached to Ser-10 by a typical tetrasaccharide linkage (Fig. 1) (2, 3). The HCs contain both N- and O-linked glycans (4) and are covalently linked to the CS chain by a unique ester bond between the α-carboxyl group of the C-terminal Asp residues and the C6 atoms of internal N-acetylgalactosamines in the CS chain. This interaction is referred to as a protein–glycosaminoglycan–protein (PGP) cross-link (2, 3, 5). HC2 is positioned closer to bikunin than HC1 on the CS chain, and the two HCs are attached one to two disaccharides apart (6, 7). The unique covalent linkages between the HCs and the CS are assemled intracellularly (6) and are referred to as PGP cross-links (2). Bikunin CS can also link to a third heavy chain (HC3) (UniProt Q06033) to form the heterodimeric pre-α-inhibitor (PαI) (1, 2). In addition, indirect evidence based on sequence identity suggests that the related HC5 (UniProt C9J2H1) (8) and possibly HC6 (UniProt Q6UX5S), but not HC4 (UniProt Q14624), can also become attached to the bikunin CS chain.

IαI is produced primarily in the liver and is found in plasma at concentrations up to ~0.5 mg/ml (9). This protein is involved in diverse biological processes such as ovulation, cell migration, and inflammation (10) in addition to playing roles in the stabilization and remodeling of extracellular matrix (ECM) (11). In the ECM, IαI stabilizes hyaluronan (HA)–rich matrices in a process requiring HC2 (12) and the tumor necrosis factor–stimulated gene-6 protein (TSG-6) (UniProt P98066) (13–15). HC2/TSG-6 catalyzes two sequential transesterifications, thereby transferring HCs from the CS of IαI or Pol to HA, generating a covalent HA–HC complex (Fig. 1) (16). The presence of HC2/TSG-6 and divalent cations is essential for this catalytic activity (12). The HC transfer reaction takes place during inflammation and inflammation-like processes in which TSG-6 expression is induced and colocalizes with IαI (17). The functional characteristics of HA are modified within HC–HA and metalloprotease with thrombospondin type 1 motifs; MMP, matrix metalloproteinase; HA, hyaluronan; RA, rheumatoid arthritis; OA, osteoarthritis; SpA, spondyloarthropathy; PGP, protein–glycosaminoglycan–protein; Pol, pre-α-inhibitor.
_lαl is a substrate for ADAMTS-5, MMP-3, -7, and -13_

 complexes, which can consequently change cell adhesive properties as well as matrix organization (18–20). A documented role of the HC–HA complex is in the formation and stabilization of the ECM in the expanding cumulus oocyte complex. The cumulus matrix contains HA, which becomes modified by enzymes involved in cartilage degradation cleave lαl as part of the progression of arthritis. This hypothesis is supported by the observation of truncated HCs in both arthritic cartilage and synovial fluid (29). In addition, ADAMTS-5 and lαl colocalize in equine degenerative suspensory ligament desmitis, and truncated versions of the HCs are observed (30). In the present study, we show that lαl is a substrate for ADAMTS-5 and MMP-3, -7 and -13. The main cleavage site is found in the C terminus of HC2, and the corresponding degradation product is present in arthritic synovial fluid. Furthermore, we present data showing that the ADAMTS-5–released HC2 retains the capacity to form an active HC2/TSG-6 complex and is therefore able to influence arthritis by increasing the catalytic transfer of heavy chains to hyaluronan.

**Results**

**lαl is a substrate for metalloproteinases**

We initially tested whether lαl was proteolytically processed by MMP-1, MMP-3, MMP-7, MMP-13, ADAMTS-4, or ADAMTS-5. The ability of the proteases to carry out cleavage was determined by titrating a fixed amount of lαl with the different proteases at 0–20 nM (Fig. 2). lαl was cleaved by MMP-7 or ADAMTS-5 at 0.5 nM (Fig. 2, C and F, lane 2). MMP-3 and MMP-13 cleaved lαl to a lesser extent, whereas MMP-1 and ADAMTS-4 showed no or a very low level of activity toward lαl.

To evaluate the relative rates of proteolysis, the amount of noncleaved lαl was measured by densitometry at different protease concentrations and normalized to nontreated lαl (Fig. 2G). From these data, it was inferred that ADAMTS-5 and MMP-7 are the most efficient proteases, whereas MMP-3 and MMP-13 display intermediate rates, and MMP-1 and ADAMTS-4 exhibit little or no activity toward lαl.

The proteases that cleaved lαl produced two initial reaction products of ~80 and 116 kDa, which were further degraded at higher protease concentrations. The 80- and 116-kDa reaction products displayed migration similar to that of free HCs (80 kDa) and also migrated as a single HC linked to bikunin (120 kDa), indicating that the initial cut was near the C terminus of one of the two lαl HCs.
ADAMTS-5 are able to cleave IαI by the tested proteases was evaluated by densitometry. Major bands at 80 and 116 kDa were observed after autohydrolysis of the PGP cross-link and represent free HC1 (80 kDa) and HC1–bikunin (Fig. 3B, lane 1). However, the intensity of the HC1–bikunin band was significantly increased after MMP-3, MMP-7, MMP-13, or ADAMTS-5 treatment (Fig. 3B, lanes 3, 4, 5, and 7), indicating that the HC–bikunin product contains HC1–bikunin (Fig. 3B, lane 6). The digest analyzed with the HC1+2 antibody exhibited the HC–bikunin species, the free HCs, and some minor degradation products after MMP-3, MMP-7, or MMP-13 treatment (Fig. 3C). These additional degradation products all present lower molecular weights than the full-length HCs and are not observed when an anti-HC1 antibody is used, indicating that these products are HC2-related. Moreover, the free HC2 band observed after autohydrolysis (Fig. 3C, lane 1) was reduced or disappeared after treatment with MMP-3, MMP-7, MMP-13, or ADAMTS-5 (Fig. 3C, lanes 3, 4, 5, and 7). These data show that the initial and major cleavage site in IαI is within the C terminus of HC2. After cleavage, the released HC2 most likely comigrates with HC1.

The C terminus of HC2 is susceptible to proteolysis

An N-terminomics approach was applied to identify the neo-N termini after protease treatment. Purified IαI was treated with MMP-1, MMP-3, MMP-7, MMP-13, ADAMTS-4, or ADAMTS-5 was analyzed by immunoblotting using a bikunin antiserum (Fig. 3A), an HC1 antiserum (Fig. 3B), or an antiserum recognizing both HC1 and HC2 (HC1+2) to visualize HC2 by subtraction (Fig. 3C). As nonenzymatic autohydrolysis of the ester in the PGP cross-link releases free full-length HCs, the protease digest was compared with IαI incubated for a similar time at a similar temperature to the protease-treated samples (lane 1). When the samples were probed with the bikunin antibody, one major product and full-length IαI were observed (Fig. 3A). The migration of the major degradation product corresponded to bikunin linked to a single HC. Additional bikunin reactive products were observed after MMP-7 treatment (Fig. 3A, lane 4), indicating additional cleavage sites in the HC–bikunin product. Bikunin remained associated with an HC (the 120-kDa band), suggesting that only one of the HCs contained a C-terminal cleavage site through which the 80-kDa band was released. The HC1-specific antibody revealed two major bands at 80 and 116 kDa (Fig. 3B). These bands were also observed after autohydrolysis of the PGP cross-link and represent free HC1 (80 kDa) and HC1–bikunin (Fig. 3B, lane 1). However, the intensity of the HC1–bikunin band was significantly increased after MMP-3, MMP-7, MMP-13, or ADAMTS-5 treatment (Fig. 3B, lanes 3, 4, 5, and 7), indicating that the HC–bikunin product contains HC1–bikunin (Fig. 3B, lane 6). The digest analyzed with the HC1+2 antibody exhibited the HC–bikunin species, the free HCs, and some minor degradation products after MMP-3, MMP-7, or MMP-13 treatment (Fig. 3C). These additional degradation products all present lower molecular weights than the full-length HCs and are not observed when an anti-HC1 antibody is used, indicating that these products are HC2-related. Moreover, the free HC2 band observed after autohydrolysis (Fig. 3C, lane 1) was reduced or disappeared after treatment with MMP-3, MMP-7, MMP-13, or ADAMTS-5 (Fig. 3C, lanes 3, 4, 5, and 7). These data show that the initial and major cleavage site in IαI is within the C terminus of HC2. After cleavage, the released HC2 most likely comigrates with HC1.

The C terminus of HC2 is susceptible to proteolysis

An N-terminomics approach was applied to identify the neo-N termini after protease treatment. Purified IαI was
I\textalpha I is a substrate for ADAMTS-5, MMP-3, -7, and -13

| A) Anti-Bikunin | B) Anti-HC1 | C) Anti-HC1+2 |
|-----------------|-------------|---------------|
| I\textalpha I  | MMP-1       | MMP-1         |
| MMP-3           | MMP-3       | MMP-3         |
| MMP-7           | MMP-7       | MMP-7         |
| MMP-13          | MMP-13      | MMP-13        |
| ADAMTS-4        | ADAMTS-4    | ADAMTS-4      |
| ADAMTS-5        | ADAMTS-5    | ADAMTS-5      |

Figure 3. HC2 contains the initial cleavage site. I\textalpha I was treated with the indicated proteases. The products were analyzed by SDS-PAGE and visualized by immunoblotting using an antibody recognizing bikunin (A), HC1 (B), or HC1 + 2 (C). Due to the hydrolysis of the PGP cross-link, I\textalpha I alone (lane 1) contained some HC–bikunin and free HCs when analyzed by SDS-PAGE. The schematics on the right side of the gels show the migration of intact I\textalpha I, HC–bikunin, HC2, and HC1 alone. In the anti-bikunin blot (A), MMP-3, MMP-7, MMP-13, and ADAMTS-5 treatment (lanes 3–5 and 7) reduced the level of I\textalpha I and increased the amount of a product migrating similarly to HC–bikunin. Using anti-HC1 (B), we similarly observed an increase in HC–bikunin, whereas the intensity of free HC1 appeared to be stable (lanes 3–5 and 7). The anti-HC1 + 2 blot (C) revealed a reduction in the intensity or a complete absence of the HC2 band (lanes 3–5 and 7). The variation in the intensity of the HC1-containing bands between B and C is a result of antibody efficiency and exposure time and does not represent variations in the amount of HC1. The proteolytic digestion of I\textalpha I was repeated more than 10 times, whereas immunoblotting was repeated two times for A and B and more than six times for C. Taken together, the observations indicate that the major MMP-3, MMP-7, MMP-13, and ADAMTS-5 cleavage sites are located in the C terminus of HC2.

titrated with MMP-3, MMP-7, MMP-13, or ADAMTS-5, and all N termini were acetylated to label the neo-N termini. The labeled samples were digested with either trypsin or a combination of trypsin and the endoproteinase Glu-C and subsequently analyzed by LC-MS/MS. N termini originating from MMP or ADAMTS cleavage events were identified as acetylated N termini, which were not observed in a control sample of non-treated I\textalpha I (Table 1). To verify the N-terminomics results, we determined the N-terminal sequence by Edman degradation of the major HC–bikunin product after transfer to polyvinylidene difluoride membranes (Fig. 2 and Table 1). In line with the previous results, all proteases preferred a substrate site in the C terminus of HC2. Significantly, residues Ser-625 to Ala-628 were particularly susceptible to proteolysis. At this site, MMP-13 uniquely cleaved Ser-625 → Met-626, MMP-3 and MMP-7 cleaved Met-626 → Leu-627, and ADAMTS-5 cleaved the Leu-627 → Ala-628 peptide bond (Fig. 4). In addition to the main cleavage sites, there are two other regions in HC2 (308–331 and 436–438) that are susceptible to cleavage to a minor degree. The 420–438 region has previously been shown to be stable ([81] of HC2. The 420–438 region has previously been shown to be stable ([81] of HC2. The 420–438 region has previously been shown to be stable ([81] of HC2. The 420–438 region has previously been shown to be stable ([81] of HC2.

HC2 is a substrate for MMP-3, MMP-7, MMP-13, and ADAMTS-5 when covalently linked to HA

It has previously been shown that MMP-3, MMP-13, ADAMTS-5, and I\textalpha I are present in the cartilage and synovial fluid of arthropathic patients (27, 29, 32, 33). Although these proteases are able to act directly on I\textalpha I, the major I\textalpha I-related substrate is most likely the HC–HA complex, which shows increased abundance in arthritic patients. Consequently, we analyzed whether the proteases were able to cleave the HCs when linked to HA (Fig. 5). Preincubation with I\textalpha I (Fig. 5, lane 1), TSG-6, and a short HA oligomer was performed for 4 h (Fig. 5, lane 3) before proteases were added to the sample (Fig. 5, lanes 4–7). Subsequently, all samples were incubated for 16 h (lanes 3–7) and analyzed by SDS-PAGE and immunoblotting using antibodies recognizing both HC1 and HC2. I\textalpha I, TSG-6, and HA oligomers generated two bands corresponding to a single HA oligomer linked to either HC1 or HC2 (Fig. 5, lane 3). The four proteases that cleaved I\textalpha I (MMP-3, MMP-7, MMP-13, and ADAMTS-5) also specifically cleaved HC2 when transferred to HA as demonstrated by a significant reduction in the HC2–HA band (Fig. 5, lanes 4–7).

Cleavage of HC2 by ADAMTS-5 is identified in synovial fluid from arthropathic patients as the major degradation product

Our in vitro analysis revealed that I\textalpha I is a substrate for MMP-3, MMP-7, MMP-13, and ADAMTS-5 as they all cleave HC2 within three residues, with P1 located at Ser-625 (MMP-13), Met-626 (MMP-3 and -7), or Leu-627 (ADAMTS-5). These proteases are known to be involved in tissue degradation during pathological conditions such as RA and OA. In addition, inflammation increases the number of HCs linked to HA. To determine whether HC2 is cleaved by MMPs or ADAMTS during inflammatory conditions in vivo, an MS-based assay was employed. The cleaved C-terminal HC2 peptide was isolated after hydrolysis of the PGP cross-link in synovial fluid from 10 OA patients, nine RA patients, and 14 spondyloarthropathy (SpA) patients and identified by MS (Table 2). The ADAMTS-5 cleavage site was detected in 29 of the 33 patient samples (Table 2) (OA, 9 of 10; RA, 8 of 9; and SpA, 12 of 14). The ADAMTS-5 degradation product was found in all types of arthritic synovial...
Iσ1 is a substrate for ADAMTS-5, MMP-3, -7, and -13

Table 1
Identification of metalloproteinase cleavage sites in Iσ1

| Metalloproteinase | 2 nm | 10 nm | 20 nm | Edman sequencing |
|------------------|------|-------|-------|-----------------|
| MMP-3            | HC2: 308, 313, 397, 627 | HC2: 308, 313, 397, 432, 436, 511, 571, 574, 627 | HC2: 313, 627, 65, 308, 313, 362, 397, 432, 437, 438, 511, 574, 627 | L_QGSQVLE__PP (HC2) |
| MMP-7            | HC1: 461 | HC1: 399, 461, 572 | HC1: 22, 367, 399, 572 | LAQG_QVLES_PP (HC2) |
| MMP-13           | ADAMTS-5 | HC2: 313, 437, 628 | HC2: 313, 330, 436, 437, 628 | ML_QGSQVLE_P (HC2) |
|                  | ADAMTS-5 | HC3–TSG-6 complex | ADAMTS-5–released HC2 retains the ability to induce the HC3–TSG-6 complex. | AQGSQVLE_PPP (HC2) |

a Thr-637 is glycosylated and is not identified by Edman degradation. Unidentified residues are indicated with "__".
b Bold residues are major cleavage sites based on the intensity of the MS signal.

Figure 4. Overview of the neo-N termini in heavy chain 2 after proteolytic treatment. A summary of the N-terminal sequencing results and LC-MS/MS data are given in Table 1. The schematics represent HC2 with the location of the neo-N termini indicated with vertical lines, and the relevant protease is indicated. The major and initial cleavage sites are located in the C terminus close to the PGP cross-link. Cleavage in the C terminus of HC2 releases HC2 from the Iσ1 complex and is likely to facilitate HC2/TSG-6 activity and increase the rate of the HC transfer reaction.

We have previously shown that complex formation between TSG-6 and HC3 depends on HC2 (12). Although the PGP cross-link is necessary for transesterification, HC2 in its free form can induce complex formation between glycosaminoglycans (HA or CS)-bound HCs and TSG-6 (12). To determine whether HC2 released by ADAMTS-5 proteolysis was able to induce complex formation between HCs and TSG-6, we incubated TSG-6 with Iσ1 cleaved by ADAMTS-5 (Fig. 6). Following incubation with ADAMTS-5, all the HC2 was cleaved (Fig. 6A, lane 2). After incubation with TSG-6, the samples were separated by SDS-PAGE and visualized by immunoblotting using a TSG-6 antibody (Fig. 6B). TSG-6 incubated with Iσ1 usually forms complexes with both HCs (Fig. 6B, lane 4). When Iσ1 was preincubated with ADAMTS-5, only a single complex corresponding to HC1–TSG-6 was apparent. We cannot exclude the possibility that HC1–bikunin is able to form a complex with TSG-6 independent of HC2. Consequently, ADAMTS-5–cleaved HC2 was isolated and incubated with Pol and TSG-6 (Fig. 6C). A previous study showed that TSG-6 and Pol alone are unable to form the HC3–TSG-6 complex (Fig. 6C, lane 4). However, when purified and cleaved HC2 was included, the HC3–TSG-6 complex was observed (Fig. 6C, lane 5). Because HC2 is required for complex formation, we conclude that ADAMTS-5–released HC2 retains the ability to induce the HC3–TSG-6 complex.

Discussion

In this study, we demonstrated that Iσ1 is a substrate of ADAMTS-5 in vivo. HC2 was cleaved in vitro at an enzyme concentration (2 nm) equivalent to the physiological ADAMTS-5 concentration (34). In addition, MMP-3, -7, and -13 cleaved Iσ1 in vitro. The major cleavage sites of all of these proteases are located within three residues (VISQ625 ↓ MMP-13 M ↓ MMP-3/7 L ↓ ADAMTS-5 AQGSQ). After the initial cleavage, additional N- and C-terminal cleavage may occur. To identify degradation in vivo, we used a novel isolation technique exploiting the ability to selectively release HCs from HA by hydrolyzing the PGP cross-link using 100 mM NaOH (2). In synovial fluid from SpA, RA, and OA patients, we were able to identify the ADAMTS-5–cleaved C terminus of HC2 in 33 of 37 patient samples.

In general, MMPs prefer to cleave peptide bonds preceding residues containing hydrophobic side chains (P1': Leu, Ile, Met, Phe, or Tyr) and those with a proline as the third residue prior to the cleavage site (P3) (36). For both MMP-3 and MMP-7, Leu is favored in the P1' position. Based on the substrate cleavage sites reported in MEROPS (37), the frequencies of Leu at P1' for MMP-3 and MMP-7 are 0.28 and 0.55, respectively, which is in agreement with the cleavage site in HC2. MMP-13 also shows specificity toward Leu (frequency 0.34), whereas the frequency of Met at P1 is 0.08. The best-described ADAMTS-5 substrate is aggrecan, containing five sites, all of which harbor a Glu residue at P1 and a Gly, Ala, or Leu...
TSG-6 expression facilitates the generation of HC–HA complexes. It might be responsible for the observed accumulation of Iα (11) in the joints of OA, RA, and SpA patients. In a previous study, truncated versions of the HCs were detected in synovial fluid and osteoarthritic cartilage. It was suggested that they might have been synthesized by the chondrocytes (29). Alternatively, these truncated HC species, which migrated with apparent molecular masses between 55 and 75 kDa, might result from ADAMTS-5 or MMP proteolysis as described in the present study.

The ADAMTS-5–mediated release of HC2 from either HA or Iα is likely to have a number of functional implications (Fig. 7). This HC–HA processing may change the stability as well as the cell-binding properties of HA with multiple downstream consequences (20, 23). In addition, the bioavailability of HC2 and TSG-6 will be affected as HC2 is released from the HA-coated ECM surface (Fig. 7). This is likely to affect the kinetics of the HC transfer reactions and, thus, the physical properties of HA. In synovial fluids from RA patients, up to five HCs are found attached to a single HA chain of 2 × 10^6 Da. This covalent HC modification influences the propensity of HA to aggregate compared with unmodified HA and may, thus, alter HC–HA matrix stabilization (25). In addition, the transfer of HCs to HA in arthritis (11) increases the binding of HA to its major cell surface receptor, CD44, on leukocytes (20), although it is not clear how this might influence the pathology of arthritis (15, 39).

One of the most significant physiological functions of the HCs is stabilization of the ECM in the expanding cumulus oocyte complex. During the maturation of the oocyte, the granulosa cells in the follicle expand the ECM and form an HC-dependent HA-rich matrix that is essential for fertilization (21–23). In a study analyzing the expression of ADAMTS-5 in the cumulus oocyte complex, ADAMTS-5 was expressed by granulosa cells in most follicles (40). However, after ovulation, ADAMTS-5 expression was absent in the released oocytes, whereas it was still expressed during follicular atresia. The ovulation-dependent regulation of ADAMTS-5 may therefore govern the formation of the HC–HA-rich matrix by cleaving and, thus, releasing HC2 from the ECM. Matured follicles that are not ovulated undergo atresia (apoptosis), and ADAMTS-5 may participate in this process by dissolving the hyaluronan-rich matrix, offering an explanation and role for the continued expression of ADAMTS-5.

It has been shown previously that the HC–HA complex mediates cellular adhesion to the HA-rich matrix (18–20). This interaction is regulated by thrombin-induced cleavage of HC1 and reduces leukocyte binding to an HA-rich matrix during inflammation (41). The ADAMTS-5–mediated release of HC2 could have a similar effect and, hence, regulate leukocyte adhesion and migration by dissolving the HC–HA matrix. An additional study has shown that the HCs of Iα inhibit the classical pathway of the complement system, with HC2 acting as the most potent inhibitor (42). We speculate that the release of HC2 acts as a mechanism for local control of the activation of the complement system. In conclusion, the release of HC2 by ADAMTS-5 could influence inflammation by regulating both leukocyte infiltration and complement activation.
In conclusion, the data presented in this study show that HC2 from Iαl is a substrate for matrix-degrading proteases, with ADAMTS-5 being the most efficient. Our in vitro data are supported by the identification of the ADAMTS-5 degradation product in synovial fluids from arthritic patients. The release of HC2 can influence both the mechanical and physiological properties of the ECM.

**Experimental procedures**

**Materials**

Iαl and Pol were purified from human plasma obtained from Aarhus University Hospital, Skejby, Denmark (1). Human TSG-6 was expressed in insect cells and purified as described previously (43). MMP-1, -3, -7, -13 (44-46), ADAMTS-4 lacking the C-terminal spacer domain, and ADAMTS-5 lacking the C-terminal thrombospondin domain (38) were expressed, activated, and purified as described previously. HA oligomers were produced and purified as described previously (12). Briefly, a 13-disaccharide-long HA oligosaccharide was generated and purified following limited chondroitinase ABC digestion of HA. Subsequently, HA was fractionated by anion exchange and desalted by size-exclusion chromatography. All reagents and chemicals were from Sigma-Aldrich/Merck unless otherwise stated.

**SDS-PAGE and immunoblotting**

Samples were boiled in SDS sample buffer in the presence of 50 mM dithiothreitol (DTT). SDS-PAGE was performed in 5–15% gradient gels (10 × 10 × 0.15 cm) using the glycine/2-amino-2-methyl-1,3-propanediol/HCl system (47). The gels were either stained for protein using Coomassie Blue or electroblotted to an Immobilon-P membrane (48). Immunoblotting was carried out using standard protocols and a rabbit anti-bikunin (3), rabbit anti-TSG-6 (49), or rabbit anti-HC antibody (3) as the primary antibody. The secondary antibody was goat anti-rabbit IgG-peroxidase. The membranes were developed using ECL reagents (GE Healthcare). Densitometry measurements of the Coomassie Blue–stained gels were performed using NIH ImageJ 1.52 (50).

**Proteolytic digestion of Iαl**

Fixed concentrations of Iαl (1.1 μM) were incubated with either fixed (20 nM) or increasing amounts of activated MMP-1, MMP-3, MMP-7, MMP-13, ADAMTS-4, or ADAMTS-5 (0, 0.5, 2, 10, and 20 nM). All samples were dissolved in 50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl₂, pH 7.6, supplemented with 0.05% Brij-35. The samples were incubated for 16 h at 37 °C and analyzed by either LC-MS/MS or SDS-PAGE.
Iαl is a substrate for ADAMTS-5, MMP-3, -7, and -13

**Figure 7. Potential consequences of ADAMTS-5–mediated HC2 release from HA.** ADAMTS-5–mediated release of HC2 may change the stability and cell-binding properties of HA. In addition, it has previously been shown that the transfer of HCs to HA increases the binding of HA to its primary cell surface receptor, CD44, on leukocytes (20). Consequently, the release of HC2 may inhibit leukocyte adhesion to the HA-rich matrix. HC2 also affects complement activation (42), and the ADAMTS-5–mediated release of HC2 may therefore provide a mechanism for local regulation of the immune response. Furthermore, the bioavailability and mobility of the HC2–TSG-6 complex will be affected when HC2 is released from HA in the ECM.

**Degradation of HA–associated HCs**

Iαl (2.5 μg), TSG-6 (0.8 μg), and 25 μg of HA oligomers (HA14) were incubated for 4 h at 37 °C in 50 mM Tris-HCl, 100 mM NaCl, 1 mM MgCl₂, pH 7.4. Subsequently, MMP-3, MMP-7, MMP-13, or ADAMTS-5 was added to a final concentration of 0.2, 10, and 20 nM, respectively, and the reactions were quenched by adding EDTA to a final concentration of 10 mM. The released HC2 was separated from HC1–bikunin by anion-exchange chromatography using a Mono Q 4.6/100 PE column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7.4. Fractions containing cleaved HC2 were pooled and concentrated by ultrafiltration (30 kDa; Amicon). LC-MS/MS analysis of these fractions confirmed the presence of HC2.

**Isolation of ADAMTS-5–cleaved HC2**

Limited proteolysis of Iαl using 160 nM ADAMTS-5 completely released HC2 from Iαl as confirmed by Western blotting. The released HC2 was separated from HC1–bikunin by anion-exchange chromatography using a Mono Q 4.6/100 PE column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7.4. Proteins were eluted using a linear gradient from 0 to 0.6 M NaCl over 60 min with a flow rate of 1 ml/min. Fractions containing cleaved HC2 were pooled and concentrated by ultrafiltration (30 kDa; Amicon).

**HC–TSG-6 complex formation**

Iαl (0.6 μg), Iαl (0.6 μg) pretreated with ADAMTS-5 (160 nM), or Pol (0.6 μg) was incubated with 0.2 μg of TSG-6 for 2 h at 37 °C. The samples were dissolved in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4. To verify the activity of cleaved HC2, purified HC2 was incubated with TSG-6 and Pol under the same conditions. The samples were analyzed by SDS-PAGE and immunoblotting using an anti-TSG-6 antibody.

**N-terminal sequencing**

Automated Edman degradation was performed in a PPSQ-31B protein sequencer (Shimadzu Biotech) with in-line phenylthiohydantoin analysis in an LC-20AT HPLC system equipped with a CTO-20A column heater and an SPD20A UV detector (Shimadzu Biotech). SDS-PAGE–separated samples were transferred to polyvinylidene difluoride membranes and applied to TFA-treated glass fiber membranes that had been precelled with Polybrene (Shimadzu Biotech). Data were recorded using Shimadzu PPSQ-31B software, and the sequence was determined by visual inspection of the UV 269 nm chromatograms.

**Identification of cleavage sites by N-terminomics**

Iαl was titrated with increasing amounts of MMP-3, MMP-7, MMP-13, or ADAMTS-5 (0, 2, 10, and 20 nM) for 4 h at 37 °C in 50 mM phosphate, 100 mM NaCl, 1 mM MgCl₂, 0.05% Brij-35, 10 mM CaCl₂, pH 7.4. The reactions were quenched by adding EDTA to a final concentration of 10 mM. Subsequently, all N termini and lysine residues were acetylated using 10 mM sulfono-NHS-acetate. The samples were incubated for 60 min at 22 °C before the reaction was quenched with 150 mM Tris-HCl, pH 7.4. The sample was denatured and reduced in 6 M urea containing 5 mM DTT for 1 h and subsequently alkylated with 15 mM iodoacetamide for 1 h. The urea concentration was reduced by diluting the sample 8 × with 50 mM NH₄HCO₃. The samples were treated either with trypsin alone or with endoproteinase Glu-C followed by trypsin. The proteases were added at a ratio of 1:20 (w/w), and the samples were incubated for 16 h at 37 °C. The digested samples were analyzed by LC-MS/MS.

**Qualitative identification of degradation products in arthropathic patient samples**

Synovial fluid from 10 OA patients, nine RA patients, and 14 SpA patients was collected at Aarhus University Hospital and the Musculoskeletal Tissue Bank of the University of Liverpool, UK. The collection of patient samples was approved by the Danish Data Protection Agency (2011-41-6863), the Regional Ethics Committee, Denmark (1-10-72-291-12), and the National Research Ethics Service, UK (15/NW/0661). The subjects' written consent was obtained according to the Declaration of Helsinki.

Proteins and hyaluronic acid were precipitated by adding 9 volumes of ice-cold 90% ethanol followed by incubation at −18 °C for 16 h. The pellet was isolated by centrifugation at 17,000 × g for 30 min. The C-terminal product resulting from the in vivo cleavage of HC2 was released from HA and/or CS by hydrolyzing the ester bond with 100 mM NaOH for 30 min on ice. Subsequently, undigested protein and HA were removed by precipitation with TCA and centrifugation at 17,000 × g for 30 min. The supernatant containing the potential C terminus of HC2 was analyzed by LC-MS/MS.

**LC-MS/MS analysis**

Mass spectrometry—All samples were desalted by micropurification using Empore™ solid-phase extraction Disks of C₁₈ octadecyl packed in 10-μl pipette tips (51). LC-MS/MS was performed using either an EASY-nLC 1000 system (Thermo Fisher Scientific, San Jose, CA) connected to a Q Exactive™ hybrid quadrupole–orbitrap mass spectrometer (Thermo Fisher Scientific). The digested samples were analyzed by LC-MS/MS.
Data Converter beta 1.1 or RawConverter (verted to generic Mascot format (MGF) using the Sciex MS phase B (0.1% formic acid, 90% acetonitrile or 0.1% formic acid, flow rate of 250 nl/min and a 50-min gradient from 5 to 35% separated in a 15-cm analytical column (75-). The peptides were eluted from the trap column and separated in a 15-cm analytical column (75-µm inner diameter) packed in-house in a pulled emitter with ReproSil-Pur C18-AQ packed in-house in a pulled emitter with ReproSil-Pur C18-AQ

3-N and 3-acetylhexoseamine, and N-acetylneuraminic acid) of Ser and Thr were selected as variable modifications. Additional settings for the identification of N termini using sulfo-NHS-acetate were fixed acetylation of Lys and variable acetylation of the peptide N terminus.

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