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SLRP interaction can protect collagen fibrils from cleavage by collagenases

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Abstract

Decorin, fibromodulin and lumican are small leucine-rich repeat proteoglycans (SLRPs) which interact with the surface of collagen fibrils. Together with other molecules they form a coat on the fibril surface which could impede the access to collagenolytic proteinases. To address this hypothesis, fibrils of type I or type II collagen were formed in vitro and treated with either collagenase-1 (MMP1) or collagenase-3 (MMP13). The fibrils were either treated directly or following incubation in the presence of the recombinant SLRPs. The susceptibility of the uncoated and SLRP-coated fibrils to collagenase cleavage was assessed by SDS/PAGE. Interaction with either recombinant decorin, fibromodulin or lumican results in decreased collagenase cleavage of both fibril types. Thus SLRP interaction can help protect collagen fibrils from cleavage by collagenases.
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Keywords: Decorin; Fibromodulin; Lumican; Type I collagen; Type II collagen; Collagenase-1; Collagenase-3

1. Introduction

Decorin, fibromodulin and lumican belong to the family of small leucine-rich repeat proteoglycans (SLRPs). This family is characterized by a core protein of 6 to 10 adjacent leucine-rich repeats flanked by disulfide-bonded domains, and the presence of attachment sites for substitution of chondroitin sulfate or keratan sulfate (Hocking et al., 1998; Iozzo, 1999). In the case of decorin a single chondroitin sulfate attachment site is present near the amino terminus of the core protein (Fisher et al., 1989; Krusius and Ruoslahti, 1986; Roughley and White, 1989), whereas fibromodulin and lumican possesses several potential keratan sulfate attachment sites in the central leucine-rich repeat region (Dunlevy et al., 1998; Grover et al., 1995; Oldberg et al., 1989; Plaas et al., 1990). The SLRPs are present within the extracellular matrix of connective tissues, where they interact with a variety of other proteins. Foremost amongst these interactions are those with growth factors (Hildebrand et al., 1994; Iozzo et al., 1999; Schönherr et al., 2005) and collagen fibrils (Brown and Vogel, 1989; Hedbom and Heinegård, 1993; Neame et al., 2000; Svensson et al., 2000). In this manner, the SLRPs can play a role in controlling the synthesis, structure and interactions of the collagen fibrils.

Collagen fibrils are formed by types I, II, III, V and XI collagens (Burgeson and Nimni, 1992; Van der Rest and Bruckner, 1993), with the fibrils formed by types I and II collagen being most abundant. The collagen fibrils form the structural framework of all connective tissues and are responsible for tissue strength and integrity. The fibrillar collagens are characterized by the presence of a long central triple helical region composed from three polypeptide chains. This triple helical region facilitates the lateral association of the collagen molecules to form the collagen fibrils (Kadler et al., 1996; Wess, 2005). The collagen fibrils do not exist in isolation, but interact with a number of other proteins, including the fibril-associated (FACIT) collagens (Shaw and Olsen, 1991; Van der Rest et al., 1991) and SLRPs (Scott, 1996). These molecules essentially form a protein coat on the surface of the fibrils.

The fibrillar collagen triple helix is resistant to degradation by most proteinases, with the exception of collagenases (Lauer-

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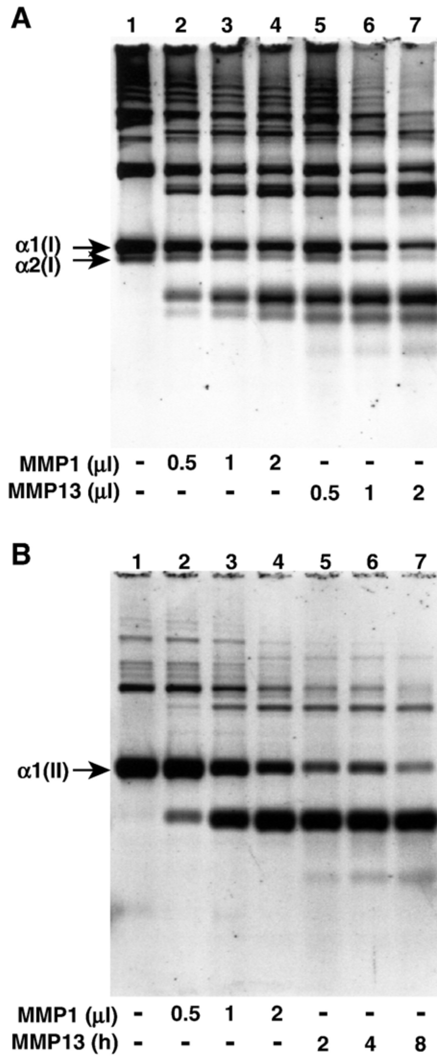

Fig. 1. Cleavage of types I and II collagen by collagenases. Type I collagen fibrils (A) or type II collagen fibrils (B) were incubated in the presence of either MMP1 and MMP13, and the digestion products analyzed by SDS/PAGE. In the case of MMP1, digestion was carried out overnight with increasing volumes of enzyme. MMP13 digestion of type I collagen was carried out in a similar manner, but with type II collagen a fixed amount of enzyme was used with increasing incubation times. The migration positions of the collagen α-chains are indicated.

Fields et al., 2002) and cathepsin K (Kafienah et al., 1998). The collagenases are responsible for collagen fibril degradation in all soft connective tissues by cleavage of each collagen molecule at a single site. Three mammalian collagenases have been described, which belong to the family of matrix metalloproteinases (MMPs). These are collagenase-1 (MMP1), collagenase-2 (MMP8) and collagenase-3 (MMP13). MMP8 is mainly associated with polymorphonuclear leukocytes and collagen degradation in inflammation, whereas MMP1 and MMP13 are produced by many connective tissue cells and mediate collagen fibril turnover under both physiological and pathological conditions. While MMP1 and MMP13 will degrade both types I and II collagen fibrils, they do so with different avidities, as MMP13 favors type II collagen as a substrate (Martel-Pelletier and Pelletier, 1996).

It is feasible that the SLRP coat on the surface of the collagen fibrils could act as a steric barrier limiting the access of the collagenases to their cleavage site. The purpose of this work was to address this hypothesis by determining whether the ability of MMP1 and MMP13 to degrade type I or type II collagen fibrils could be retarded by the presence of decorin, fibromodulin or lumican on the fibril surface.

2. Results

In initial experiments types I and II collagen were digested with differing amounts of MMP1 or MMP13 to establish a collagenase concentration able to give about 80% collagen degradation. This would enable subsequent experiments to establish whether SLRP interaction could either enhance or retard collagenase action. It was determined that when collagen fibrils were formed from 10 μg type I collagen, about 80% of the collagen could be degraded in an overnight digestion using either 100 ng MMP1 or 75 ng MMP13 (Fig. 1A). MMP1 showed a similar response towards type II collagen, with 100 ng MMP1 being able to cleave about 80% of the collagen in fibrils formed from 10 μg type II collagen (Fig. 1B). However, MMP13 reactivity towards type II collagen was greater than that for MMP1 and shorter incubation times were required to attain the desired results. Thus 50 ng MMP13 was able to cleave about 80% of the collagen in fibrils formed from 10 μg type II collagen after a 2-h incubation (Fig. 1B). These digestion conditions were used in all subsequent experiments.

The ability of the recombinant SLRPs to interact with the collagen fibrils was determined by incubating the SLRPs in the presence of pre-formed fibrils. This would enable subsequent collagenase digests to be performed under conditions where SLRP coating of the fibril surface was maximal. In

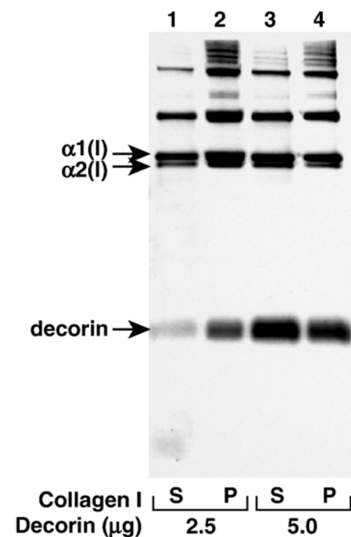

Fig. 2. Binding of decorin to type I collagen. Type I collagen fibrils were incubated in the presence of differing amounts of decorin. After centrifugation, the fibril pellet (P) and the supernatant (S) were recovered and the presence of decorin was analyzed by SDS/PAGE. The migration positions of the collagen α-chains and decorin are indicated.

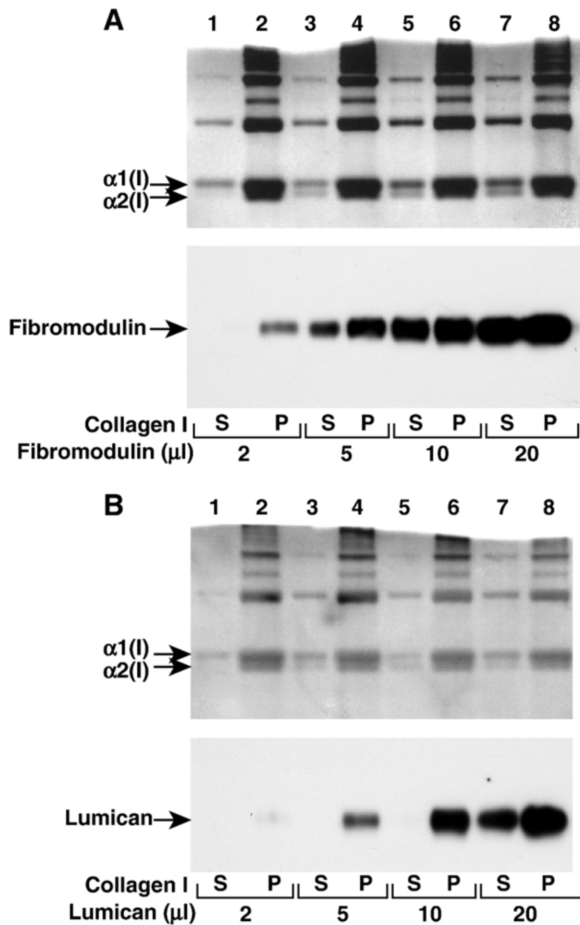

Fig. 3. Binding of fibromodulin and lumican to type I collagen. Type I collagen fibrils were incubated in the presence of different amounts of recombinant fibromodulin (A) or lumican (B). After centrifugation, the fibril pellet (P) and the supernatant (S) were recovered and the samples analyzed by SDS/PAGE. Collagen was identified by staining of the gels with Coomassie orange, and fibromodulin and lumican were identified by immunoblotting. The migration positions of the collagen α-chains, fibromodulin and lumican are indicated.

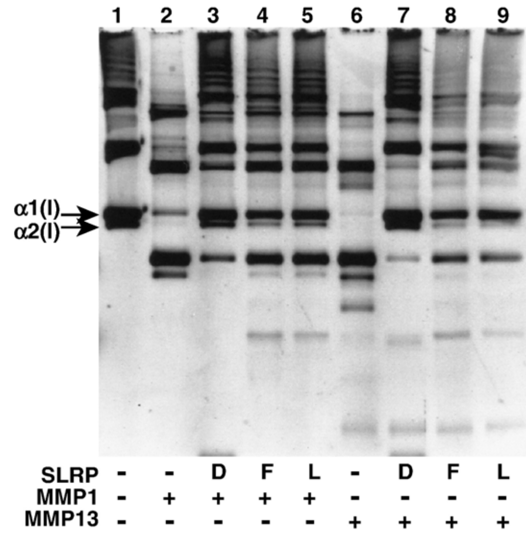

Fig. 5. Action of collagenases on type I collagen fibrils in the presence of SLRPs. Type I collagen fibrils were incubated with MMP1 or MMP13 in the presence or absence of decorin (D), fibromodulin (F) or lumican (L), and the digestion products analyzed by SDS/PAGE. Lane 1, no MMP; lanes 2–5, incubation with MMP1; lanes 6–9, incubation with MMP13. The migration positions of the collagen α-chains are indicated.

the case of type I collagen, 5 μg decorin added to the fibrils formed from 10 μg collagen was necessary to ensure maximal interaction (Fig. 2). With fibromodulin and lumican the precise concentration of recombinant SLRP was not known due to the presence of bovine serum albumin in the recombinant preparations, and binding studies were performed with increasing volumes of the concentrated preparations. In both cases 20 μl of the SLRP solution was sufficient to give maximal binding (Fig. 3). In the case of type II collagen, 5 μg decorin and 10 μl of either recombinant fibromodulin or lumican were found to give maximal binding with collagen fibrils formed from 10 μg collagen (Fig. 4). These SLRP/collagen ratios were used in all subsequent experiments. Under these conditions all SLRP binding sites on the collagen fibrils should be occupied.

To study the ability of the SLRPs to influence collagenase digestion of the collagen fibrils, fibrils formed in the presence or

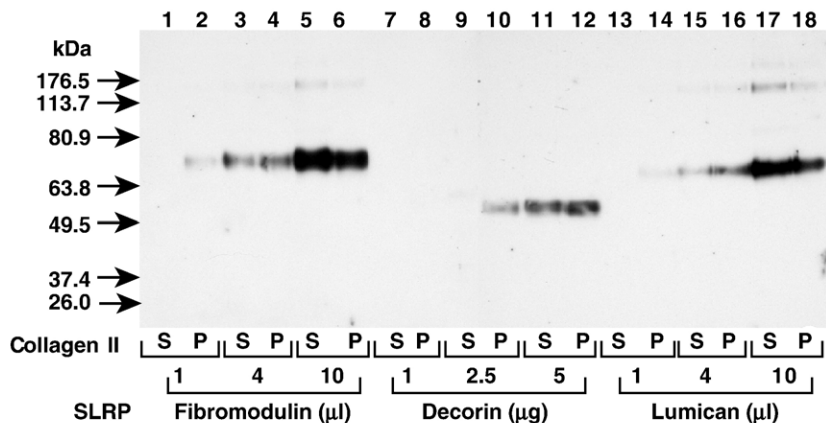

Fig. 4. Binding of SLRPs to type II collagen. Type II collagen fibrils were incubated in the presence of differing amounts of fibromodulin, decorin or lumican. After centrifugation, the fibril pellet (P) and the supernatant (S) were recovered and the samples analyzed by SDS/PAGE. The presence of the SLRPs was identified by immunoblotting. The migration positions of molecular weight markers are indicated.

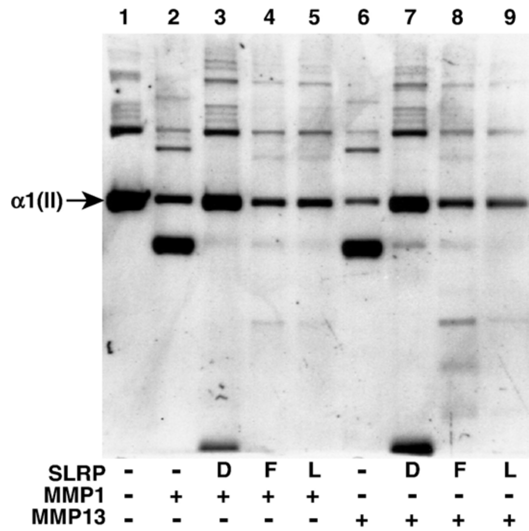

Fig. 6. Action of collagenases on type II collagen fibrils in the presence of SLRPs. Type II collagen fibrils were incubated with MMP1 or MMP13 in the presence or absence of decorin (D), fibromodulin (F) or lumican (L), and the digestion products analyzed by SDS/PAGE. Lane 1, no MMP; lanes 2–5, incubation with MMP1; lanes 6–9, incubation with MMP13. The migration positions of the collagen α-chains are indicated.

absence of SLRPs were incubated under identical conditions. The presence of all recombinant SLRPs was found to diminish the degree of type I collagen fibril degradation by either MMP1 or MMP13 (Fig. 5), with similar degrees of inhibition of cleavage being observed for both enzymes. It was apparent that under the conditions used, decorin gave a greater degree of inhibition than fibromodulin or lumican. A similar effect was seen with SLRP interaction with type II collagen fibrils (Fig. 6). Degradation was diminished to a similar extent for all recombinant SLRPs for both MMP1 and MMP13 treatment. It was, however, apparent that the amount of type II collagen fibrils in the reaction mixture was lower for the digestions with recombinant fibromodulin and lumican, even though the fibrils had been formed from 10 μg collagen as with other samples being analyzed. This was due to partial solubilization of the type II collagen fibrils in the presence of fibromodulin or lumican. This would effectively increase the collagenase to collagen fibril ratio over the other samples, yet inhibition of collagenase activity was still observed. Increasing the quantity of SLRP-coated fibrils in the digestion mixture did not influence the result (data not shown).

It was also important to establish whether the recombinant SLRPs could be acting as competitive substrates for the collagenases and thereby diminishing their reactivity towards the collagen fibrils. The recombinant SLRPs were therefore incubated with either MMP1 or MMP13 in the presence or absence of the collagen fibrils. Neither decorin, fibromodulin nor lumican alone were degraded by MMP1 or MMP13 under the conditions used in this work (Fig. 7). When the SLRPs were interacting with type I collagen fibrils, there was also no degradation evident by MMP1 (Fig. 7A). A similar result was obtained with MMP13 for decorin or lumican coating type I collagen fibrils (Fig. 7B). However, fibromodulin did show evidence of proteolysis by MMP13 when bound to the type I collagen fibrils. Identical results were obtained for recombinant SLRP interaction with type II collagen fibrils, with only fibromodulin being susceptible to cleavage by MMP13 (Fig. 8). In this work fibromodulin degradation is evident as a loss of immunoreactivity on immunoblotting. However, immunoreactive degradation products were not observed. Presumably, the immunoreactive epitope on the fibromodulin either is cleaved

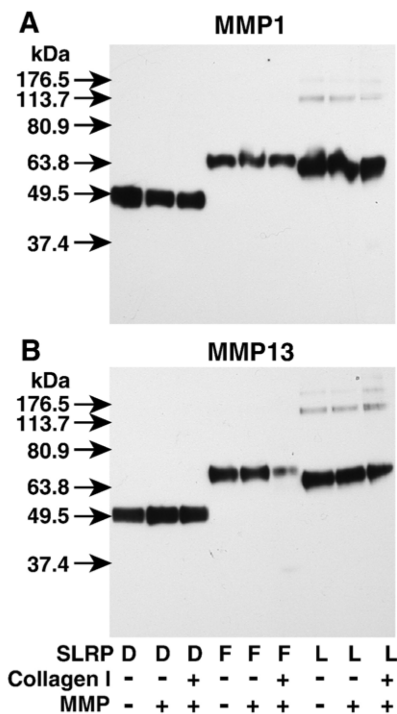

Fig. 7. Action of collagenases on SLRPs in the presence of type I collagen fibrils. Decorin (D), fibromodulin (F) or lumican (L) were incubated with MMP1 (A) or MMP13 (B) in the presence or absence of type I collagen fibrils, and the digestion products analyzed by SDS/PAGE. The presence of the SLRPs was identified by immunoblotting. The migration positions of the molecular weight markers are indicated.

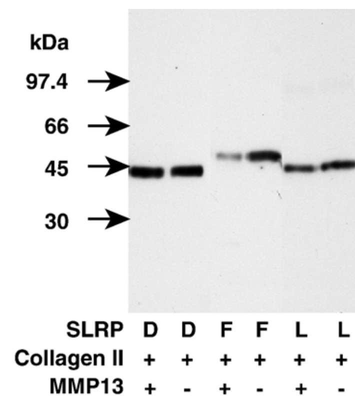

Fig. 8. Action of MMP13 on SLRPs in the presence of type II collagen fibrils. Decorin (D), fibromodulin (F) or lumican (L) were incubated with MMP13 in the presence of type II collagen fibrils, and the digestion products analyzed by SDS/PAGE. The presence of the SLRPs was identified by immunoblotting. The migration positions of the molecular weight markers are indicated.

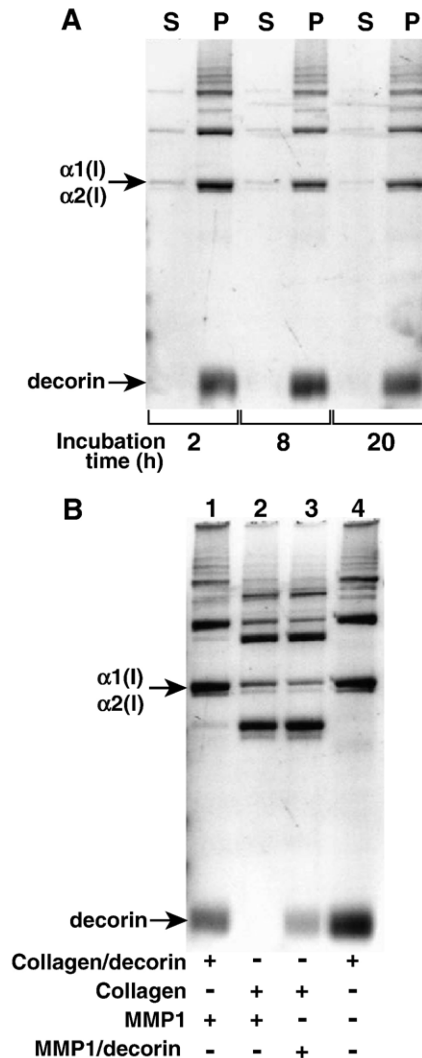

Fig. 9. Stability of SLRP binding to collagen fibrils. (A) Decorin-coated collagen fibrils were incubated in collagenase buffer for 2, 8 or 20 h and the fibril pellet (P) and supernatant (S) were then analyzed by SDS/PAGE. (B) Type I collagen fibrils were incubated with MMP1, either alone or following preincubation with decorin, and the results compared to the incubation of decorin-coated fibrils with MMP1 directly. The extent of collagen cleavage was assessed by SDS/PAGE. The migration positions of the collagen α-chains and decorin are indicated.

by the MMP13 or resides on a peptide fragment which is too small to be resolved by SDS/PAGE.

Finally, the possibility that SLRP binding was not stable under the collagenase digestion conditions and that soluble SLRP released from the coated fibrils could inhibit collagenase activity were assessed. For this study decorin-coated collagen fibrils were incubated in the collagenase buffer alone for up to 20 h, and the supernatant and fibril pellet were then examined for the presence of decorin (Fig. 9A). It was apparent that the SLRP remained stably bound during the incubation. In addition, type I collagen fibrils that were uncoated were incubated in the presence of a mixture of decorin and MMP1 that had been preincubated together to allow any potential interaction to occur (Fig. 9B). It was apparent that prior coating of the collagen fibrils was essential for the prevention of their cleavage by collagenase, and that the presence of soluble SLRP had no inhibitory effect on collagenase activity.

3. Discussion

The present work demonstrates that decorin, fibromodulin or lumican interacting at the surface of either type I or type II collagen fibrils can diminish the susceptibility of the collagen fibrils to both MMP1 and MMP13 cleavage. The current data suggest that decorin may have a greater propensity for inhibition than fibromodulin or lumican, although it is not possible to say whether this is true on a molar basis as the precise quantity of each SLRP bound to the collagen fibrils is not known. However, SLRP binding was carried out under conditions where there was excess SLRP and this should result in saturation of the binding sites in all three cases. Thus when all decorin binding sites are occupied there appears to be greater inhibition of collagen fibril cleavage than when all fibromodulin/lumican sites are occupied.

In the case of fibromodulin it does itself undergo proteolytic cleavage by MMP13 when bound to either type I or type II collagen fibrils. This latter observation is in agreement with a previous study (Heathfield et al., 2004), where proteolytic cleavage of fibromodulin bound to collagen fibrils in cartilage could be induced by treatment with MMP13 or IL-1 (which is known to stimulate MMP13 synthesis). Cleavage was reported to occur between Tyr-63 and Ala-64 in the bovine fibromodulin core protein. This site is within the amino terminal region of fibromodulin, prior to the first disulfide-bonded domain and the leucine-rich repeat region. While the amino-terminal fragment is lost from the cartilage matrix, the larger fragment is retained within the cartilage presumably via continued interaction with the collagen fibrils. Similar cleavage was observed in earlier work on IL-1-stimulated cartilage using the carboxy terminal antibody used in the present work (Sztrolovics et al., 1999). Yet while the large fibromodulin fragment was observed in the cartilage extracts, it was not observed in the present study where only a decrease in intact fibromodulin was evident on immunoblotting. Thus it is likely that in the in vitro system additional cleavage may be occurring within or near the carboxy terminal epitope. Such cleavage may be sterically blocked within the tissue extracellular matrix, where numerous other proteins reside with fibromodulin on the collagen fibril surface.

It is perhaps surprising that free soluble fibromodulin is not cleaved by MMP13, whereas the collagen fibril bound form is cleaved. One explanation for this phenomenon could be the dimeric conformation that the SLRPs may adopt in solution. The SLRPs have been proposed to form horse-shoe-like structures with concave and convex surfaces due to the folding of their leucine-rich repeats (Scott, 1996; Weber et al., 1996). In the case of decorin, X-ray crystal data reveals a more banana-shape conformation and the occurrence of dimerization by interlocking of two molecules via their concave surfaces (Scott et al., 2004). Similar dimerization has also been shown to occur in solution (Scott et al., 2003), and has been proposed to be

feature of all SLRPs. Although decorin dimers can be dissociated to monomers by elevation of temperature or the addition of guanidinium chloride, such dissociation is reversible and under physiological conditions dimerization is a stable conformation (Scott et al., 2006). If so, then dimerization might sterically block access of MMP13 to its cleavage site. One could then speculate that monomeric SLRPs may be involved in interaction with the collagen fibrils, as the concave surface is thought to mediate the interaction. In such a monomeric state the cleavage site may be exposed.

As decorin or lumican do not act as competitive substrates for or inhibitors of the collagenases, it is plausible that they act by sterically blocking access of the collagenases to their cleavage site in the monomeric collagen molecules that form the fibrils. All three SLRPs interact with both types I and II collagen fibrils at sites located within the gap zones of the fibrils. In the case of fibromodulin and lumican, interaction occurs at the same sites at the a and c bands, whereas decorin interaction occurs at the d and e bands (Scott, 1988). The a and c bands are located at opposite ends of the gap zone near the boundary with the overlap zones, whereas the d and e bands are located within the central region of the gap zone. Interestingly, MMP1 and MMP13 cleave at identical sites on both type I and type II collagen, with cleavage occurring between Gly-775 and Leu/Ile-776 (Lauer-Fields et al., 2002). This site is close to the border between the gap and overlap zones. It is therefore not difficult to envisage that the presence of the SLRPs may impede access of the collagenases, as long as they remain stably bound to the collagen fibrils. At present it is unclear what proteinases may be capable of degrading decorin, fibromodulin and lumican to the extent that their interaction with the collagen fibrils is abolished. Certainly aggrecanases, which are produced in conjunction with the collagenase in response to IL-1 are incapable of this action (Sztrolovics et al., 1999).

The ability of the SLRPs to protect collagen fibrils from catabolism by collagenases provides an additional function for the SLRP/collagen interaction in addition to previously characterized roles in collagen fibril formation and fibril–fibril interaction. It is unclear at present whether this protective role is by design or fortuitous, or whether in the tissue it matters whether all or only one of the SLRPs is present. It is feasible that SLRP interaction helps protect the connective tissue matrix from excessive damage by collagenases during normal physiological remodeling. This could be envisaged as a benefit during tissue development and growth as it may stabilize the newly formed collagen fibrils prior to their maturation by intermolecular cross-linking. Certainly all connective tissues studied to date contain one or more of the SLRPs used in this work. Moreover, as protection occurs for both types I and II collagens, it is possible that this is a common feature in both fibrous and cartilaginous connective tissues. Under pathological conditions where increased collagenase levels and additional proteinase types will be present, the protective barrier will likely be bridged and tissue damage result. It is however apparent that there is redundancy in SLRP function, and therefore SLRP deficiency, whether by enhanced degradation or impaired synthesis, would need to affect several family members.

4. Experimental procedures

4.1. Source of reagents

Pepsin-solubilized bovine skin type I collagen (Vitrogen) was obtained from Angiotech. Pepsin-solubilized bovine nasal cartilage type II collagen was obtained from Southern Biotech. MMP1 was obtained from Sigma, and MMP13 and recombinant human decorin were obtained from R&D Systems. The pCEP4-BGN plasmid was provided by Dr. David McQuillan. Recombinant human fibromodulin and lumican were generated as part of this study.

4.2. Generation of pCEB4-FBN and pCEB4-LUM

Plasmid pCEB4-BGN contains an insulin signal peptide sequence, a 6His tag, and a factor Xa cleavage site upstream of the human biglycan coding sequence. The fusion protein expression cassette had been excised from the a vaccinia expression vector used previously for the generation of recombinant biglycan (Hocking et al., 1996) then inserted into pCEB4 (Invitrogen). In the present work the biglycan sequence was replaced by that for human fibromodulin or lumican (Fig. 10). The fibromodulin and lumican coding sequences were prepared by RT–PCR using a total RNA preparation isolated from human articular chondrocytes (Recklies et al., 2001). Reverse transcription (RT) was carried out in a 20 µl reaction, containing 1 µl RNA (180 ng), 2 µl RT buffer, 1 µl RNasin (10 U), 2 µl dNTPs (10 mM each of dATP, dTTP, dCTP and dGTP), 0.5 µl downstream PCR primer, 1 µl Omniscript reverse transcriptase (Qiagen) and 12.5 µl DEPC-treated water. The reaction was carried out at 42 °C for 1 h, then the mixture was heated to 65 °C for 5 min to denature the enzyme. Polymerase chain reaction (PCR) amplification of the resulting cDNA was carried out in a 50 µl reaction, containing 5 µl cDNA, 1 µl 5′-primer (25 mM), 1 µl 3′-primer (25 mM), 1 µl dNTP mixture (10 mM each), 1 µl Pfu DNA polymerase (Stratagene, 2.5 U/µl), 5 µl PCR buffer and 36 µl water. Following denaturation at 95 °C for 2 min, PCR amplification was carried out for 35 cycles – denaturation at 95 °C for 30 s, annealing at

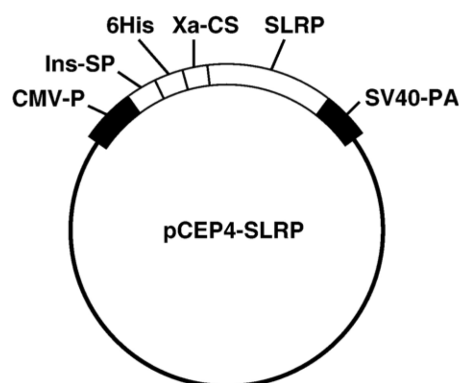

Fig. 10. The structure of pCEB4-SLRP. The pCEB4 plasmid was modified to insert the insulin signal peptide (Ins-SP), a six histidine sequence (6His), a factor Xa cleavage sequence (Xa-CS), and the coding sequence of the mature fibromodulin or lumican (SLRP) between the CMV promoter (CMV-P) and the SV40 polyadenylation sequence (SV40-PA).

58 °C for 1 min, and extension at 72 °C for 1 min. The 5′- and 3′-primers for fibromodulin were 5′-ccgctcgagcagtatgaagatgaccct and 3′-tccggatccggctgctcagatctcgat and for lumican were 5′-ccgctcgagcagtactatgattatgatttt and 3′-tccgaatccaggatacagatattaat-taag, respectively.

The RT–PCR products were TA-cloned by ligation into the pcDNA3.1 plasmid (Stratagene), as described by the manufacturer. The recombinant plasmid was then used to transform TOP10 competent cells, and positive clones were used to isolate the SLRP coding sequence inserts by digestion with *Xho*II and *Bam*H1. The gel purified inserts were then ligated into the gel-purified *Xho*II/*Bam*H1-treated pCEB4-BGN plasmid. Ligation was carried out in a 21 μl reaction, containing 1 μl plasmid, 9 μl insert, 10 μl ligase buffer, 1 μl Quic1 ligase (New England BioLabs) at 20 °C for 15 min. The recombinant plasmids were then used to transform TOP10 competent cells and positive clones used to prepare pCEB4-FBN and pCEB4-LUM midi preps (Qiagen).

4.3. Generation of recombinant fibromodulin and lumican

Recombinant SLRPs were prepared by transient transfection in HEK293 cells. For transfection, plasmid DNA (8 μg) in 0.5 ml DMEM was mixed with lipofectamine 2000 (Invitrogen, 20 μl) in 0.5 ml DMEM and left at 20 °C for 30 min. The mixture was then diluted to 5 ml with DMEM, and used to treat monolayers of HEK293 cells (2×10^6) in 60 mm culture plates. After incubation at 37 °C for 5 h, the DNA/lipofectamine solution was replaced by 5 ml DMEM containing 10% FCS. Cells were then cultured at 37 °C, collecting and replacing the medium every 48 h. The collected media were supplemented with proteinase inhibitors to a final concentration of 0.4 mM PMSF, 1 mM *N*-ethylmaleimide, 1 mM *p*-aminobenzamidine, 5 mM EDTA and 10 μg/ml leupeptin, and then stored at −20 °C in 2 ml aliquots.

For purification, the media were dialyzed against 0.9% NaCl to reduce the EDTA concentration, and the recombinant SLRPs were recovered by binding to Ni–NTA agarose beads (Qiagen, 0.1 ml beads per 2 ml medium). After washing the beads in 50 mM Na_2HPO_4, 300 mM NaCl, 0.05% Tween 20, pH 8.0, containing 20 mM imidazole, the SLRPs were eluted with 4 × 1 ml of the same buffer containing 250 mM imidazole. The eluent was then concentrated to 100 μl using a Centricon-30 (Amicon) and stored at −20 °C. As an assessment of their functional state, all recombinant SLRPs were shown to retard type I collagen fibrillogenesis in an in vitro assay (Vogel et al., 1984).

4.4. Formation of collagen fibrils

Type I collagen fibrils were formed by mixing 3.5 μl collagen (2.9 μg/μl in 12 mM HCl) with 21.5 μl 10 mM HCl and 25 μl TES buffer (60 mM TES, 20 mM Na_2HPO_4, 0.56 M NaCl, pH 7.6) (Rada et al., 1993). After incubating at 37 °C for 1 h, the fibrils were recovered as a pellet by centrifugation. For type II collagen, fibrils were formed by mixing 20 μl collagen (0.5 μg/μl in 4.5 mM Na_2HPO_4, 2 mM HCl) with 80 μl buffer (50 μl buffer 1, 16.2 μl buffer 2, 13.8 μl buffer 3) (Gray et al., 2004). Fibrils were again recovered by centrifugation after incubation at 37 °C for 1 h. The type II collagen solution was obtained in 0.5 M acetic acid and was dialyzed into the phosphate buffer for use.

To coat the collagen fibrils with a SLRP, the fibril pellets were resuspended in 25 μl TES buffer and 10 μl recombinant SLRP (0–5 μg) was added together with 2 μl 10 mM $ZnSO_4$ to facilitate binding (Dugan et al., 2003). The volume was adjusted to 50 μl with water. After leaving the mixture at 37 °C overnight, the coated fibrils were recovered by centrifugation and used immediately for collagenase digestion. In one experiment the SLRP-coated collagen fibrils were incubated in the collagenase buffer alone to verify stable binding.

4.5. Collagenase digestion

Collagen fibril/SLRP pellets were resuspended in 25 μl 100 mM Tris, 300 mM NaCl, 20 mM $CaCl_2$, pH 7.5 (Krane et al., 1996), to which was added 2 μl collagenase (50 ng/μl), 2 μl 25 mM APMA and water to 50 μl. The collagenase concentration was optimized to give about 80% degradation of uncoated collagen fibrils. For MMP1, optimization studies were carried out using 25–100 ng enzyme for both types I and II collagen with incubation being carried out overnight at 32 °C. For MMP13, optimization with type I collagen was carried out in a similar manner, whereas with type II collagen incubations were carried out from 2 to 8 h. In one experiment MMP1 was preincubated with recombinant decorin for 10 min prior to the digestion of uncoated type I collagen fibrils to check that soluble SLRP has no direct inhibitory affect on the collagenase.

4.6. SDS/PAGE and immunoblotting

For analysis of collagen cleavage, samples following collagenase digestion were loaded onto Novex 8% Tris/Glycine gels (Invitrogen) and subjected to electrophoresis under reducing conditions. Following electrophoresis, proteins were visualized in the gel by staining with Coomassie orange (Invitrogen) and exposing the stained gel to UV light.

For analysis of SLRP binding and response to collagenase, samples were loaded onto NuPage 4–20% Bis/Tris gels (Invitrogen) and subjected to electrophoresis under reducing conditions. Following electrophoresis, proteins were transferred to nitrocellulose (Bio-Rad) by electroblotting (Towbin et al., 1979). The SLRPs were identified by immunoblotting using rabbit anti-peptide antibodies recognizing the carboxy terminus of the SLRP core proteins (Grover et al., 1995; Roughley et al., 1993, 1996). Subsequently, blots were incubated with a biotinylated anti-rabbit IgG (Amersham Biosciences), followed by a streptavidin-biotinylated horseradish peroxidase complex. Finally, immunoreactive proteins were visualized using an enhanced chemiluminescent (ECL) detection reagent (Amersham Biosciences) and exposure to hyperfilm.

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