CMLS, Cell. Mol. Life Sci. 57 (2000) 859–863
1420-682X/00/010859-5 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Independent modulation of collagen fibrillogenesis by decorin and lumican

P. J. Neame[a,b,*], C. J. Kay[a,b], D. J. McQuillan[c,‡], M. P. Beales[b] and J. R. Hassell[a,b]

[a]Department of Biochemistry and Molecular Biology, College of Medicine and Institute of Biomolecular Sciences, University of South Florida, 4202 E. Fowler Avenue, Tampa (Florida 33620, USA)
[b]Shriners Hospital for Children, 12502 N. Pine Dr., Tampa, (Florida 33612, USA), Fax + 1 813 975 7127, e-mail: pneame@com1.med.usf.edu
[c]Texas A + M University, Institute of Biomolecular Science, 2121 West Holcombe Boulevard, Houston (Texas 77030, USA)

Received 25 January 2000; received after revision 16 March 2000; accepted 3 April 2000

Abstract. The leucine-rich proteoglycans (also known as "small, leucine-rich proteoglycans," or SLRPs) lumican and decorin are thought to be involved in the regulation of collagen fibril assembly. Preparation of these proteoglycans in chemical amounts without exposure to denaturants has recently been achieved by infecting HT-1080 cells with vaccinia virus that contains an expression cassette for these molecules. Addition of lumican and decorin to a collagen fibrillogenesis assay based on turbidity demonstrated that lumican accelerated initial fibril formation while decorin retarded initial fibril formation. At the end of fibrillogenesis, both proteoglycans resulted in an overall reduced turbidity, suggesting that fibril diameter was lower. The presence of both proteoglycans had a synergistic effect, retarding fibril formation to a greater degree than either proteoglycan individually. Competitive binding studies showed that lumican did not compete for decorin-binding sites on collagen fibrils. Both proteoglycans increased the stability of fibrils to thermal denaturation to approximately the same degree. These studies show that lumican does not compete for decorin-binding sites on collagen, that decorin and lumican modulate collagen fibrillogenesis, and that, in the process, they also enhance collagen fibril stability.

Key words. Decorin; lumican; collagen; extracellular matrix.

Introduction

The majority of extracellular matrix depends on collagen fibrils for many of its physical properties. Collagen provides tensile strength in tissues such as bone and skin and provides shear strength in cartilage. Fibrillogenesis of collagen types I and II is thought to be regulated by a number of factors, including the so-called minor collagens and the leucine-rich proteoglycans. For example, in cartilage, decorin is associated with relatively thicker collagen type II fibrils, while collagen type IX is associated with thinner fibrils [1]. Collagen type V has been shown to inhibit collagen type I fibrillogenesis [2]. A small proteoglycan isolated from tendon (now known to be decorin) inhibited collagen type I and type II fibrillogenesis in a core protein-dependent fashion [3], reducing the diameter of the collagen fibrils [4]. Transgenic mice lacking decorin exhibit fragile skin of low tensile strength and irregular collagen ultrastructure [5].

* Corresponding author.
‡ Present address: Lifecell, One Millenium Way, Branchburg (New Jersey 08876, USA)

Lumican, the major leucine-rich proteoglycan of the corneal stroma, has also been shown to inhibit collagen type I fibrillogenesis [6], although these preparations were subsequently shown to be contaminated by keratocan. Transgenic mice lacking lumican exhibit fragile skin of low tensile strength and irregular collagen ultrastructure with the development of corneal opacity after 4–5 weeks [7]. These results demonstrated the importance of lumican in collagen fibril formation and in maintenance of corneal transparency.

Recently, leucine-rich proteoglycans have been made recombinantly in eukaryotic cells (HT-1080) using a vaccinia virus-based system [8, 9]. The vaccinia-based system has made it possible to radiolabel these proteoglycans biosynthetically and to prepare and purify the proteoglycans in milligram amounts without exposing them to denaturants. These recombinantly produced proteoglycans are thought to have a structure essentially identical to that found in tissues. By using circular dichroism, denaturants have been shown to unfold recombinant decorin and biglycan produced in a eukaryotic system, essentially irreversibly [10].

We report here an analysis of the effects of recombinant lumican and decorin, separately and together, on collagen fibrillogenesis using assays that mimic the process of collagen fibril formation in vivo, and avoid some of the disadvantages of solid-phase assays. These assays are essentially as described by Williams et al. [11], using purified rat tail tendon collagen which is free of contaminants that might be expected to modify fibrillogenesis ('minor' collagens and leucine-rich proteoglycans). We show for the first time that these proteoglycans act independently on fibril formation, that lumican does not compete for decorin sites on collagen fibrils and that their action on modification of fibril assembly also increases the stability of collagen fibrils to thermal denaturation.

Materials and methods

Preparation of recombinant decorin and lumican. The vaccinia virus/T7 bacteriophage expression system was used to express the proteoglycans as recombinant products in HT-1080 cells (eukaryotic cells of connective tissue origin) by coinfection with vTF7-3 encoding T7 RNA polymerase, as previously described [8, 10]. Plasmid pDCN1 was constructed from a cDNA encoding human decorin core protein fused at the N terminus to a polyhistidine-insulin signal sequence fusion protein cassette [8]. Plasmid pLUM1 was similarly constructed from a cDNA coding for chick lumican core protein linked at its N terminus to the same fusion protein cassette. Homologous recombination was used to generate a recombinant *vaccinia* virus from plasmid pDCN1 and pLUM1 [8, 10]. [^{35}S]-labeled decorin and lumican were prepared by culturing cells in the presence of [^{35}S]methionine. Secreted recombinant proteins were purified from extracellular medium using a Ni^{2+}-chelate column and eluted with a gradient of imidazole [8]. The protein content of the decorin and lumican samples was determined colorimetrically using the DC Protein Assay kit (BioRad, Hercules, Calif.) with bovine serum albumin as the standard.

Fibrillogenesis assays. Fibril formation was measured using a Pharmacia Ultrospec 4000 spectrophotometer, in a 1-cm-path-length, thermostated cuvette, in a total volume of 1 ml at 34 °C. Turbidity was measured as absorbance at 400 nm, in 30 mM Na-phosphate buffer, pH 7.4, 0.15 M NaCl. Each assay contained 800 μg/ml of acid-extracted, pepsin-digested type I bovine dermal collagen (Collagen Corporation, Palo Alto, Calif.) and different amounts of recombinant proteoglycans. All solutions were at 4 °C and thoroughly degassed immediately before use. Collagen was added to proteoglycan in phosphate buffer, mixed by pipetting and transferred to the spectrophotometer which was maintained at 34 °C. Turbidity was monitored every 4 min for 8 h. Experimental turbidity curves were analyzed in terms of the maximum rate of turbidity change observed, the lag prior to fibrillogenesis, and the maximum turbidity attained. In a typical experiment, a control cuvette (collagen without proteoglycan) and up to four sample cuvettes with either varying proteoglycans or varying amounts of proteoglycan were monitored simultaneously. The maximum slope (at the midpoint) was determined and normalized to maximum turbidity [(1/maximum turbidity) × (change in turbidity/time)].

Fibril-melting assays. Fibrils formed in the presence and absence of proteoglycans were analyzed for thermal stability. Sample temperature was manually increased at 0.5° C/min from an initial value of 34° C. Sample temperature was measured using a thermocouple (Cole Palmer) inserted into a duplicate cuvette. Melting curves were analyzed in terms of the maximum rate of turbidity change (at the midpoint) and normalized to maximum turbidity [(1/maximum turbidity) × (change in turbidity/time)].

Binding assays. Competitive assays to determine whether lumican competes for decorin-binding sites on collagen were performed in duplicate at 32 °C. Combinations of [^{35}S]-labeled decorin (10 μg), unlabeled decorin (0, 10, or 20 μg) and unlabeled lumican (0, 10, or 20 μg) were added to buffer containing 800 μg/ml bovine dermal collagen, 30 mM Na-phosphate buffer, and 0.15 M NaCl, pH 7.4. Samples (1 ml total volume) were incubated overnight to permit completion of collagen fibrillogenesis. Collagen and associated proteoglycans were pelleted by centrifugation at 12,000 g for 5 min, and the pellet resuspended in 0.5 ml of glacial acetic acid. Radiolabeled decorin in the supernatant

and resuspended pellet fractions was determined by scintillation counting. Collagen content was measured by the Sircol method according to the manufacturer's instructions (Biocolor, Belfast, UK).

Results and discussion

The ability to prepare leucine-rich proteoglycans in chemical amounts using the vaccinia virus system has made it possible to investigate the effects of leucine-rich proteoglycans on extracellular matrix assembly. One of the primary roles of leucine-rich proteoglycans is thought to be regulation of collagen fibril diameter [4]: mice with null mutations for either lumican [7] or decorin [5] have collagen fibrils of irregular diameter. While it is probable that collagen fibril growth is regulated by both leucine-rich proteoglycans and 'minor' collagens, the three-component free-solution system of collagen type I, decorin, and lumican we used here makes it possible to test the hypothesis that each of the leucine-rich proteoglycans has a different effect on fibril formation.

Initially, we investigated the effects of decorin and lumican on collagen fibril formation. The increase in turbidity of a denatured collagen solution placed into phosphate buffer was measured in a thermostatically controlled spectrophotometer. The absolute time for initial fibril formation varied slightly from experiment to experiment, but both lumican and decorin had consistent effects on the process of fibrillogenesis relative to each other and to a control sample lacking proteoglycan, as shown in figure 1A. Lumican consistently reduced the time taken before initial fibril formation occurred, relative to the control. In contrast, decorin consistently delayed fibrillogenesis, as measured by the onset of turbidity with respect to a control sample containing no proteoglycan. Both proteoglycans reduced fibril diameter as assessed by maximum turbidity, as has been shown previously for proteoglycans extracted from tendon [4] or corneal tissue [6] with chaotropic solvents. The effects on fibrillogenesis were dependent on the proteoglycan concentration (tested at 20 and 40 μg/ml, not shown) as previously demonstrated [4, 6]. The rate of fibrillogenesis, relative to the final fibril diameter, was slower than controls in the presence of either proteoglycan, with decorin slowing fibrillogenesis more than lumican (table 1). Decorin and lumican together, at a concentration of 20 μg/ml each, had a synergistic effect, retarding fibril growth more than either individual proteoglycan at 40 μg/ml (fig. 1A). This suggests that lumican and decorin bind to different sites on the fibril. The effects observed with lumican or decorin are distinct from those observed with polyethylene glycol (not shown), for which the time taken to initiate fibrillogenesis was reduced.

To determine whether lumican could compete for decorin-binding sites on collagen during fibrillogenesis, we used an assay in which the collagen fibrils formed in the presence of radiolabeled proteoglycan were pelleted in a microfuge tube and counted. Equal amounts of unlabeled and labeled decorin resulted in an approximately 35% reduction in label in the pellet compared to the amount of label found in the pellet when only labeled decorin was used (fig. 2). Addition of unlabeled lumican had no statistically significant effect on decorin binding. These findings indicate that lumican does not compete for decorin-binding sites on the collagen type I fibril.

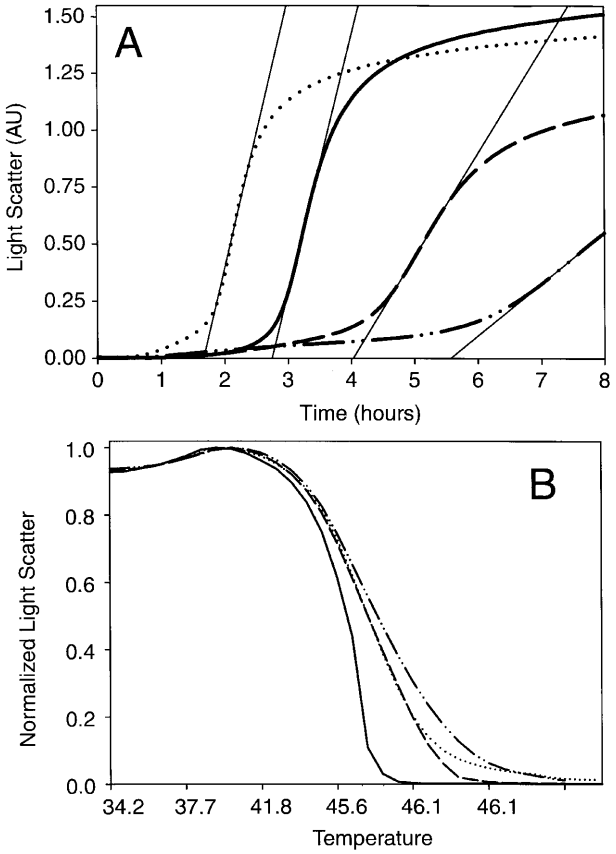

Figure 1. Effects of recombinant decorin and lumican, separately and in combination, on collagen fibrillogenesis (*A*) and melting (*B*). Recombinant human lumican or decorin was added to degassed buffer containing 800 μg/ml bovine dermal collagen, 30 mM Na-phosphate, and 0.15 M NaCl, pH 7.4. Turbidity was measured at 34 °C by absorbance at 400 nm (*A*). After overnight incubation, the sample chamber was heated and the reduction of turbidity caused by melting of collagen fibrils was measured to a maximal temperature of 46.1 °C (*B*). The melting curves have been normalized to the same starting turbidity. Solid line, collagen alone; dotted line, collagen + 40 μg/ml lumican; dashed line, collagen + 40 μg/ml decorin; dotted and dashed line, collagen + 20 μg/ml lumican and 20 μg/ml decorin.

Table 1. The effect of lumican and decorin on collagen fibril formation and melting.

	Collagen alone	+Lumican 40 μg/ml	+ Decorin 40 μg/ml	+Lumican+Decorin 20 μg/ml each
Fibril formation rate (slope of fibrillogenesis curve at midpoint)	0.74 ± 0.02	0.68 ± 0.03	0.43 ± 0.03	0.41 ± 0.03
Fibril formation initiation	–	promoted	delayed	delayed
Fibril thermal stability (slope of melting curve at midpoint)	6.2 ± 0.06	2.2 ± 0.04	2.4 ± 0.04	2.2 ± 0.06

Fibril formation and stability rates were measured by determining the slope of the curves shown in figure 1 followed by normalization to maximal turbidity and normalizing this to the slope of the control. Results are expressed as the average of two experiments.

The presence of leucine-rich proteoglycans on collagen fibrils might be expected to increase the amount of energy required to dissociate the fibril. This effect would be similar to that observed in collagens with differing percentages of hydroxyproline [12] and would result from an overall decrease of Gibbs free energy in the complex as a result of the interaction between collagen and proteoglycan. To test this, we used thermal dissociation of collagen to measure the degree to which decorin and/or lumican stabilized collagen fibrils. When cuvettes in which maximum collagen fibril formation had occurred at 34° C were heated, the solution cleared, consistent with melting of fibrils with elevated temperature (fig. 1B). Both lumican and decorin delayed this process and thus can be assumed to increase the stability of collagen fibrils. By analyzing the slope of the melting curve at its midpoint, the rate of melting could be estimated. Both proteoglycans slowed the melting of collagen fibrils to approximately half the control rate (table 1). There was also a small enhancement of stability when both proteoglycans were present, suggesting that they have approximately the same number of contacts with adjacent collagen triple helices.

These observations suggest that both lumican and decorin stabilize the fibril at the same time as they limit fibril growth. In vivo, this would reduce rearrangement of collagen molecules within collagen fibrils, preventing fibril anastomosis and reducing heterogeneity of fibril diameter. The data from lumican-null mice [7] supports both the increased thermal stability and enhanced formation of smaller fibrils shown here.

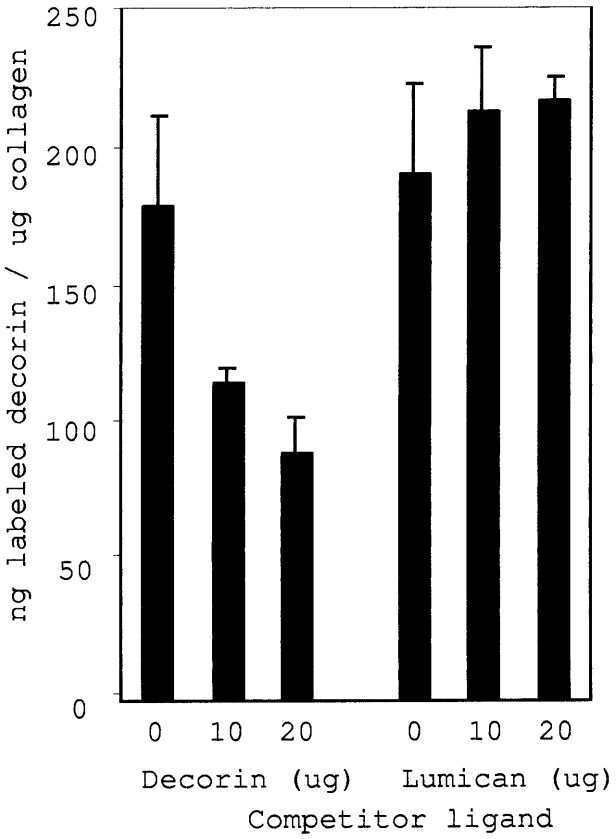

Figure 2. Competitive analysis of decorin binding to collagen fibrils. Biosynthetically labeled decorin (10 μg) was utilized in fibrillogenesis assays as described in the text in the presence and absence of unlabeled decorin (0, 10, 20 μg) or lumican (0, 10, or 20 μg). The precipitating collagen fibrils were collected by centrifugation and counted. Results are the average of two values and are presented as nanograms of labeled decorin bound/μg of collagen.

Acknowledgements. This work was supported by grants from the National Eye Institute, and National Institutes of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health (R37 EY08104-11 to J.R.H. and RO1 AR42826 to D.J.M.), and from Shriners of North America (to J.R.H., P.J.N., and C.J.K.).

1 Hagg R., Bruckner P. and Hedbom E. (1998) Cartilage fibrils of mammals are biochemically heterogeneous: differential distribution of decorin and collagen IX. J. Cell Biol. **142:** 285–294
2 Birk D. E., Fitch J. M., Babiarz J. P., Doane K. J. and Linsenmayer T. F. (1990) Collagen fibrillogenesis in vitro: interaction of types I and V collagen regulates fibril diameter. J. Cell Sci. **95:** 649–657
3 Vogel K. G., Paulsson M. and Heinegård D. (1984) Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. Biochem. J. **223:** 587–597

4 Vogel K. G. and Trotter J. A. (1987) The effect of proteoglycans on the morphology of collagen fibrils formed in vitro. Collagen Rel. Res. **7:** 105–114
5 Danielson K. G., Baribault H., Holmes D. F., Graham H., Kadler K. E. and Iozzo R. V. (1997) Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. J. Cell Biol. **136:** 729–743
6 Rada J. A., Cornuet P. K. and Hassell J. R. (1993) Regulation of corneal collagen fibrillogenesis in vitro by corneal proteoglycan (lumican and decorin) core proteins. Exp. Eye Res. **56:** 635–648
7 Chakravati S., Magnuson T., Lass J. S., Jepsen K. J., LaMantia C. and Carroll H. (1998) Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican. J. Cell Biol. **141:** 1277–1286
8 Hocking A. M., Strugnell R. A., Ramamurthy P. and McQuillan D. J. (1996) Eukaryotic expression of recombinant biglycan: post-translational processing and the importance of secondary structure for biological activity. J. Biol. Chem. **271:** 19571–19577
9 Ramamurthy P., Hocking A. M. and McQuillan D. J. (1996) Recombinant decorin glycoforms: purification and structure. J. Biol. Chem. **271:** 19578–19584
10 Krishnan P., Hocking A. M., Scholtz J. M., Pace C. N., Holik K. K. and McQuillan D. J. (1999) Distinct secondary structures of the leucine-rich repeat proteoglycans decorin and biglycan: glycosylation-dependant conformational stability. J. Biol. Chem. **274:** 10945–10950
11 Williams B.R., Gelman R.A., Poppke D.C. and Piez K.A. (1978) Collagen fibril formation: optimal in vitro conditions and preliminary kinetic results. J. Biol. Chem. **253:** 6578–6585
12 Sakakibara S., Inouye K., Shudo K., Kishida Y., Kobayashi Y. and Prockop D. J. (1973) Synthesis of $(Pro\text{-}Hyp\text{-}Gly)_n$ of defined molecular weights: evidence for the stabilization of collagen triple helix by hydroxypyroline. Biochim. Biophys. Acta **303:** 198–202