View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 470 (2000) 178–182 FEBS 23454

Fibromodulin and lumican bind to the same region on collagen type I fibrils

Liz Svensson, Ingmar Närlid, Åke Oldberg*

Department of Cell and Molecular Biology, University of Lund, P.O. Box 94, S-220 00 Lund, Sweden

Received 31 January 2000

Edited by Pierre Jolles

Abstract Fibromodulin and lumican are closely related members of the extracellular matrix leucine-rich repeat glycoprotein/proteoglycan family. Similar to decorin, another member of this protein family, they bind to fibrillar collagens and function in the assembly of the collagen network in connective tissues. We have studied the binding of recombinant fibromodulin, lumican and decorin, expressed in mammalian cells, to collagen type I. Using a collagen fibril formation/sedimentation assay we show that fibromodulin inhibits the binding of lumican, and vice versa. Fibromodulin and lumican do not affect the binding of decorin to collagen, nor does decorin inhibit the binding of fibromodulin or lumican. Binding competition experiments and Scatchard plot analysis indicate that fibromodulin binds to collagen type I with higher affinity than lumican.

© 2000 Federation of European Biochemical Societies.

Key words: Extracellular matrix; Collagen; Decorin; Fibromodulin; Lumican; Collagen fibrillogenesis

1. Introduction

Fibromodulin [1], lumican [2], and decorin [3] belong to the family of leucine-rich repeat (LRR) glycoproteins/proteoglycans, which includes about 10 members found in extracellular matrices [4]. All the members have an overall similar structure, which can be divided into three main structural domains. The N-terminal domains are least conserved, but all members contain four conserved cysteine residues involved in intrachain disulfide bonds [5]. Frequently this domain is negatively charged due to substitutions with glycosaminoglycan chains, as in decorin [3] and biglycan [6], or with tyrosine-sulfate residues in fibromodulin [7] and most likely also in lumican. The most pronounced amino acid sequence homology is in the central domain, which constitute 60–80% of the total amino acid residues containing 10 repeats of a leucine and asparagine rich motif. This domain contains asparagine-linked carbohydrates. Fibromodulin and lumican have four or five potential sites for carbohydrate substitution, depending on species [2,7–9]. In a single fibromodulin molecule only two of these sites carry keratan sulfate chains [10]. This substitution is more variable in lumican, which is a keratan sulfate proteoglycan primarily in the cornea [11]. The C-terminal domains in the LRR-glycoproteins/proteoglycans are less conserved containing two cysteine residues forming an intrachain disulfide bond.

The extracellular matrix LRR-glycoproteins/proteoglycans can be further divided into subfamilies based on sequence similarities and gene organization. Decorin [3] and biglycan [6], which both are chondroitin/dermatan-sulfate proteoglycans, are encoded by genes consisting of eight exons with conserved exon/intron junctions [12]. They show 57% amino acid sequence identity and constitute one subfamily. Fibromodulin [1] and lumican [2] belong to another subfamily showing 47% amino acid sequence identity. Their genes consist of three exons with conserved exon/intron junctions [13,14].

Fibromodulin and lumican synthesized by different species are highly conserved. In the present study we have used bovine fibromodulin and mouse lumican. Bovine and mouse fibromodulin show 93% amino acid sequence identity. The protein is least conserved in the signal peptide sequence and in the N-terminal domain substituted with tyrosine-sulfate residues. This region is unlikely to be involved in the binding to collagen, since the collagen binding site has been localized to the C-terminal region [15]. Sequences located C-terminal of the first cysteine residue (Cys-75) show 96% sequence identity and the amino acid substitutions are mostly conserved (e.g. Lys to Arg and Ser to Thr), except for a Ser to Gly mutation (Ser171Gly).

Decorin [16], fibromodulin [17] and lumican [18] bind to fibrillar collagens in vitro, thereby retarding the rate of fibril formation and leading to the synthesis of thinner fibrils [19]. This binding is mediated by the core proteins in decorin [20] and in fibromodulin [15].

Fibromodulin and decorin have been shown to bind to different sites on the collagen fibril. The binding of fibromodulin can not be inhibited by decorin and vice versa [21].

Ultrastructural analysis of collagen fibrils in tissues show that both fibromodulin and decorin bind to the gap region of the D-period in the collagen fibril [22].

Also in vivo the LRR-glycoproteins/proteoglycans affect collagen fibril formation. Decorin- and lumican-null mice have fragile skin with reduced tensile strength [23,24].

Ultrastructural analysis of skin and tendon from these mice show irregular and thicker than normal collagen fibrils. In addition the lumican-null mice show corneal opacity and the collagen fibrils in the cornea are also thicker than normal. Mice lacking fibromodulin show irregular collagen fibrils in tendons, with an average thinner diameter than in wild type mice [25]. The fibromodulin-null mice show a four-fold increase of lumican in tendon, whereas decorin levels are unchanged [25]. This suggested to us that lumican binds to the same site as fibromodulin on collagen fibrils and that the absence of fibromodulin in fibromodulin-null mice allows more lumican to bind to collagen.

In this study we show that fibromodulin and lumican com-

*Corresponding author. Fax: (46)-46-2223128.
E-mail: ake.oldberg@medkem.lu.se

0014-5793/00/$20.00 © 2000 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(00)01314-4

pete for the same binding site on collagen type I fibrils in vitro.

2. Materials and methods

Mouse lumican cDNA was prepared from RNA isolated from mouse tail tissue using RT-PCR, as previously described [25]. The mRNA was converted to cDNA using oligo-dT primers and reverse transcriptase (Boehringer Mannheim). Primers for the amplification of mouse lumican cDNA were; 5′-CGGGATCCATGAATGTATGT-GCGTTCTCTC and 5′-CGGATATCTTAGTTAACGGTGATTT-CATTG (GenBank accession number AF013262). The PCR product, representing full length mouse lumican cDNA, was subjected to nucleotide sequence analysis to confirm the correct sequence. Bovine fibromodulin cDNA [1], bovine decorin cDNA [26] and the mouse lumican cDNA were ligated in the Epstein–Barr based pCEP4 expression vector (Invitrogen, San Diego, CA, USA) and used for stable transfection of HeLa cells as previously described [20]. This vector replicates extrachromosomally and gives a high yield of recombinant protein in transformed cells [27]. Cells producing fibromodulin, lumican and decorin were cultured in serum free media (DMEM, GIBCO) containing 0.1 mCi/ml [^{35}S]sulfate or 20 μCi/ml [^{35}S]Met. After incubation for 24 h, culture media were collected and phenylmethylsulfonyl fluoride added as protease inhibitor.

Antisera against bovine fibromodulin [1], the N-terminal region of mouse lumican [25] and bovine decorin [28] were used for immunoprecipitations, as described [29]. The antisera were also used for determining the concentration of recombinant glycoproteins/proteoglycans by ELISA, as described [25].

Immunoprecipitates were treated with *N*-glycosidase F to remove Asn-linked oligosaccharides as recommended by the manufacturer (Boehringer Mannheim). Collagen binding assays were performed as previously described [20]. Conditioned culture media were concentrated about 20-fold by Centricon-30 filtration (Amicon, Beverly, MA, USA). Briefly, 0.1 ml of concentrated medium was incubated with 5 μl of acid solubilized rat tail tendon collagen (3 mg/ml, Sigma). After 5 h at 37°C, the sample was centrifuged at 10 000×*g* for 5 min, the supernatant removed and the pellet washed once with phosphate-buffered saline. The pellet and supernatant were electrophoresed on a SDS polyacrylamide gel [30] and the amounts of radiolabeled glycoprotein/proteoglycan in the pellet and supernatant were determined using a Bio Imaging Analyzer (Fuji Photo Film, Japan).

Scatchard plot analysis [31] was used to determine dissociation constants as previously described [20].

3. Results

3.1. Characterization of recombinant fibromodulin and lumican

Recombinant fibromodulin and lumican were produced in mammalian cells in order to study their collagen-binding properties. An Epstein–Barr based mammalian expression vector containing the cDNA encoding fibromodulin or lumican was used to transform HeLa cells. Cell clones expressing fibromodulin or lumican were grown in the presence of [^{35}S]Met and the radiolabeled cell culture media were subjected to immunoprecipitation. The fibromodulin antiserum precipitated a 53–65 kDa glycoprotein, which after treatment with *N*-glycosidase F migrated as a 42 kDa protein (Fig. 1, lanes 1 and 2). The lumican antiserum precipitated a 52–58 kDa glycoprotein, which was reduced to 39 kDa after *N*-glycosidase F treatment (Fig. 1, lanes 3 and 4). These molecular weights are in agreement with those estimated from fibromodulin cDNA (42 kDa) [1] and from lumican cDNA (39 kDa) [2,8]. The HeLa cells expressing fibromodulin or lumican were also radiolabeled with [^{35}S]sulfate. After an approximately 20-fold concentration of the [^{35}S]sulfate labeled media, samples were analyzed by SDS–PAGE (Fig. 1, lanes 5 and 6). [^{35}S]sulfate is incorporated as tyrosine-sulfates and/or keratan sulfate in fibromodulin and lumican, which represent major

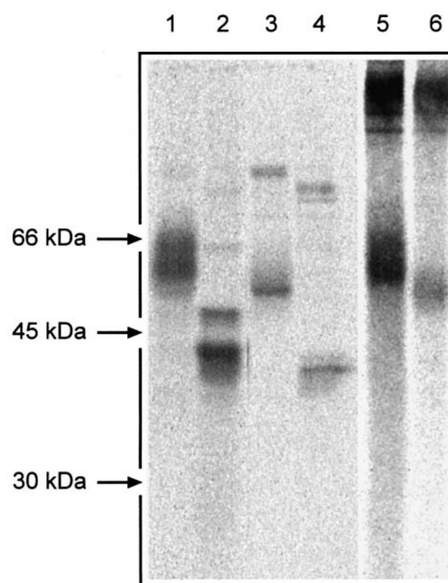

Fig. 1. Characterization of recombinant fibromodulin and lumican. Immunoprecipitation of [^{35}S]Met labeled recombinant fibromodulin (lane 1) and lumican (lane 3) synthesized by transformed HeLa cells. *N*-glycosidase F treatment of fibromodulin (lane 2) and lumican (lane 4) show the core proteins. [^{35}S]sulfate labeled culture media from HeLa cells synthesizing fibromodulin (lane 5) and lumican (lane 6). The media were concentrated 20-fold prior to SDS–PAGE.

[^{35}S]sulfate labeled components in the media. Digestion of [^{35}S]sulfate labeled cell media with *N*-glycosidase F show similar results as digestion of the immunoprecipitated [^{35}S]Met labeled media (results not shown). The [^{35}S]sulfate labeled >200 kDa product represents a chondroitin sulfate proteoglycan, which does not interfere in the collagen binding assay [20].

3.2. Competition experiments

The [^{35}S]sulfate labeled HeLa-cell culture media were used in the collagen binding analysis. Acid solubilized rat tail tendon collagen was allowed to form fibrils in the presence of [^{35}S]sulfate labeled fibromodulin or lumican. We performed inhibition assays to study the binding of fibromodulin, lumican and decorin to collagen (Fig. 2). [^{35}S]sulfate labeled glycoprotein/proteoglycan were incubated with varying amounts of non-labeled glycoproteins/proteoglycan as competitors. The binding of fibromodulin to collagen was inhibited by fibromodulin and lumican. Also, the binding of lumican to collagen was inhibited by fibromodulin and lumican. Decorin binding to collagen type I was unaffected by the addition of fibromodulin or lumican and only decorin inhibited the binding. Likewise, decorin did not affect the binding of fibromodulin or lumican to collagen. Fibromodulin appears to be a more potent inhibitor of collagen-binding than lumican. A three-fold higher concentration of lumican, as compared to fibromodulin, was required for equal inhibition of the binding of [^{35}S]sulfate labeled fibromodulin or lumican to collagen.

3.3. Scatchard plot analysis

To compare the binding of fibromodulin and lumican to collagen, we performed binding assays with increasing concentrations of fibromodulin or lumican (Fig. 3). Saturation was achieved at fibromodulin or lumican concentrations of

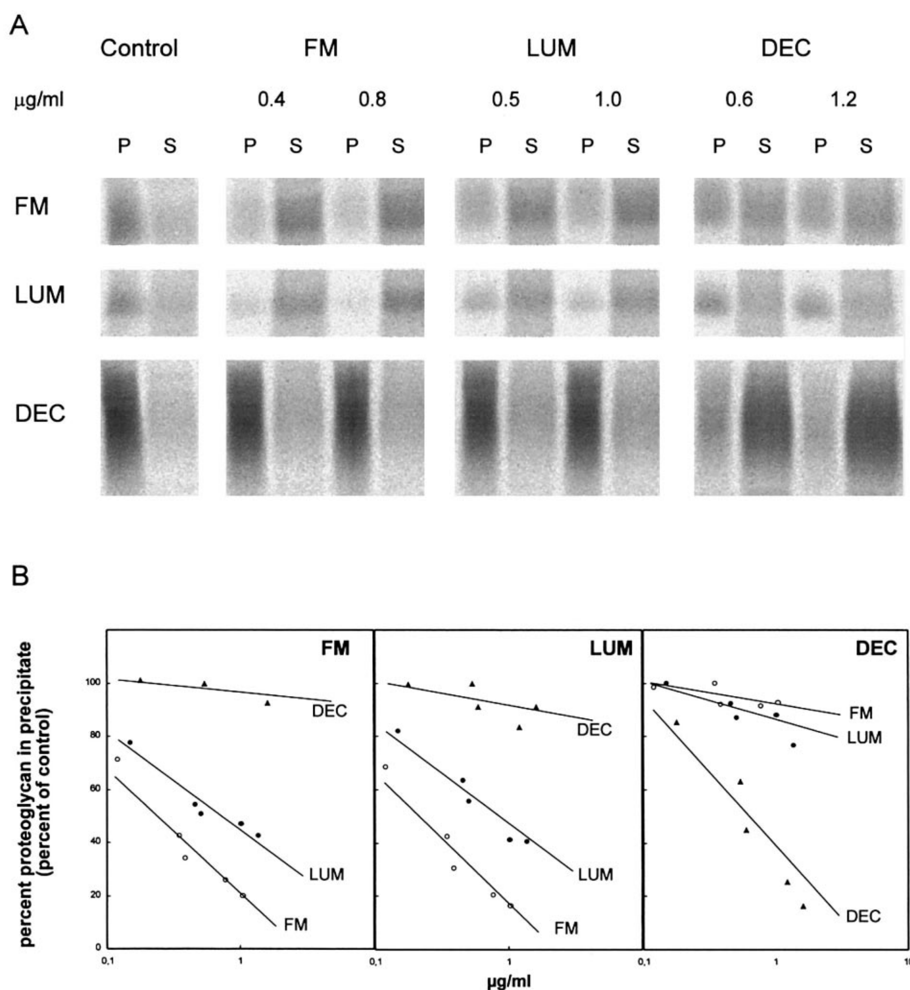

Fig. 2. Inhibition of fibromodulin, lumican and decorin binding to collagen. A: Radiolabeled recombinant fibromodulin (FM), lumican (LUM) and decorin (DEC) were incubated with acid solubilized collagen and with the indicated concentrations of non-labeled recombinant proteins as inhibitors. After centrifugation, radiolabeled proteins bound to collagen fibrils in the pellet (P) and non-bound in the supernatant (S) were analyzed by SDS–PAGE. Control incubations were done without inhibitor. B: The amounts of collagen fibril bound radiolabeled glycoproteins/proteoglycans in the SDS-polyacrylamide gel (A) were determined using a Bio Imager analyzer after inhibition by varying concentrations of non-labeled fibromodulin (○), lumican (●) and decorin (▲). The inhibition curves include additional results not shown in (A).

10–20 nM. The maximum binding of fibromodulin and lumican were 0.40 and 0.34 pmol, respectively. Scatchard plot analysis indicated the presence of low and high affinity collagen binding sites (Fig. 4). The fibromodulin binding to collagen type I showed dissociation constants of 0.2 and 1.6 nM, whereas lumican bound with dissociation constants of 0.5 and 5.4 nM.

4. Discussion

The LRR-glycoproteins/proteoglycans decorin, fibromodulin and lumican bind to fibrillar collagens and affect the collagen matrix assembly in connective tissues. Null-mutations in the genes for decorin [23], fibromodulin [25] and lumican [24] show that these LRR-glycoproteins/proteoglycans function in the collagen fibrillogenesis. Collagen fibrils from these null-mice are irregular in shape and size compared to collagen fibrils from wild type mice. Tendons from fibromodulin-null mice contain four-fold more lumican than wild type tendons. This indicates that fibromodulin and lumican complete for the same binding site on collagen type I fibrils. Decorin and fibromodulin bind to separate sites on the collagen fibril [21]. In this report we show that fibromodulin and lumican share the same binding sites on collagen type I fibrils, which is different from the decorin binding site. Our results also indicate that fibromodulin binds with higher affinity to collagen than does lumican. In the competition experiments fibromodulin was a three-fold more potent inhibitor than lumican. The collagen binding assays show that similar amounts of fibromodulin and lumican bind to collagen. At saturation 0.34–0.40 pmol of fibromodulin or lumican bound to approximately 50 pmol collagen. Fibromodulin and lumican bind to the surface of collagen fibrils and the formation of large fibrils during the assay presumably explains the estimated fibromodulin/lumican:collagen molar ratio 1:140. The Scatchard plot analysis indicate low and high affinity fibromodulin and lumican collagen binding sites. The binding sites show a higher affinity for fibromodulin than for lumican. Previous studies of decorin binding have also suggested at least two collagen binding sites [20].

It has been proposed that the rapid growth of collagen fibrils in the chick metatarsal tendon between day 16 and

18 in embryonic development, is due to a differential synthesis of LRR-glycoproteins/proteoglycans [32]. Hypothetically, decorin, fibromodulin and lumican bind to small extracellular collagen fibril segments leading to inactivation or activation of the assembly into larger fibrils. Our results support a scenario where collagen bound lumican is replaced by fibromodulin leading to tendon collagen fibril growth. Since fibromodulin binds with higher affinity than lumican to the same site on collagen fibrils, a change in collagen-bound LRR-glycoprotein/proteoglycan could be accomplished by an increase in the ratio of fibromodulin/lumican synthesis. This fibril segment assembly hypothesis predicts that the LRR glycoproteins/proteoglycans are pivotal in determining the structure of the final collagen fibril. Indeed, lumican is abundant in cornea, which has very thin collagen fibrils. Fibromodulin is absent from the cornea but prominent in tendon, which contains thicker collagen fibrils. Possibly, the orchestrated action of LRR-glycoproteins/proteoglycans may be important for the generation of diverse collagen type I matrices.

Interestingly, the increased deposition of lumican in fibromodulin-null mice tendons leads to a considerable decrease in lumican mRNA [25]. This indicates that fibroblasts adjust the synthesis of LRR-glycoproteins/proteoglycans in response to the composition and/or structure of the extracellular matrix. This regulation may involve unknown LRR-glycoprotein/proteoglycan cell surface receptors or collagen receptors, such as integrins [33,34] or DDR1/DDR2 tyrosine kinase receptors [35].

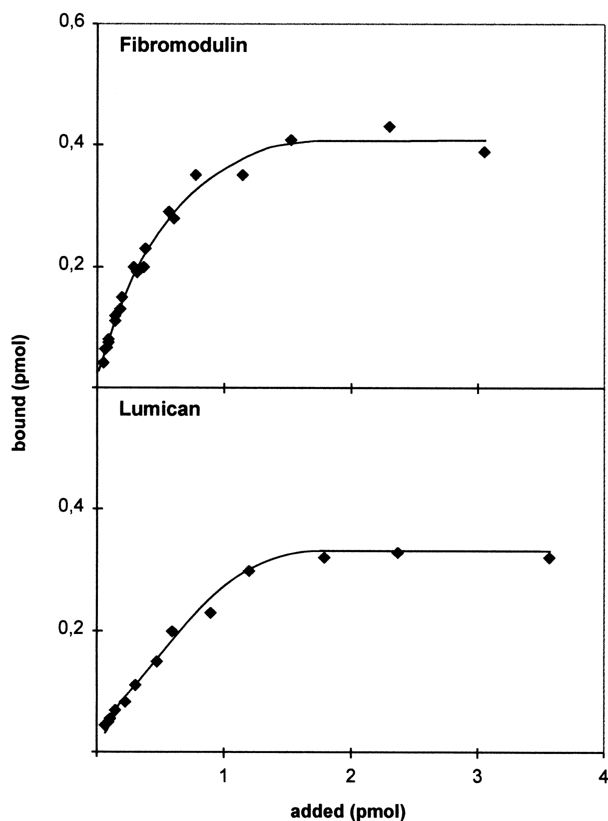

Fig. 3. Binding of fibromodulin and lumican to collagen. Increasing amounts of radiolabeled fibromodulin (top) and lumican (bottom) were incubated with 15 μg collagen (50 pmol) in a final volume of 0.1 ml. After incubation and centrifugation the amounts of bound fibromodulin or lumican in the pellets were determined and plotted against the amounts of added proteins.

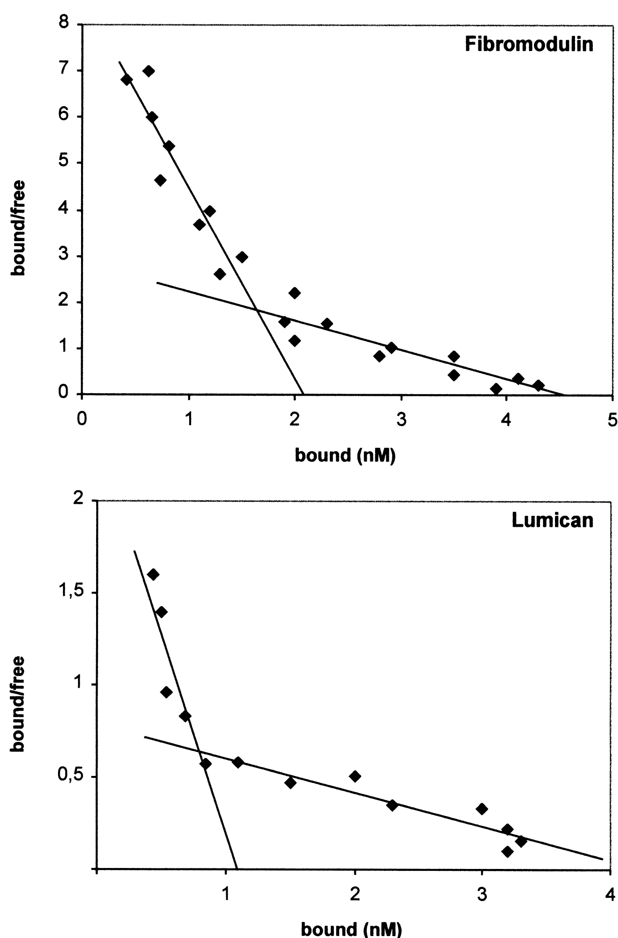

Fig. 4. Scatchard plot analysis. The results from the binding experiments in Fig. 3 were subjected to Scatchard plot analysis. Fibromodulin (top) show higher affinity for type I collagen fibrils than lumican (bottom). Both show biphasic patterns indicating multiple collagen binding sites.

Acknowledgements: This work was supported by the Swedish Medical Research Council, Anna-Greta Crafoord's Stiftelse, Greta och Johan Kock's Stiftelse, Gustav V 80-års Fond and Alfred Österlund's Stiftelse.

References

[1] Oldberg, Å., Antonsson, P., Lindblom, K. and Heinegård, D. (1989) EMBO J. 8, 2601–2604.
[2] Funderburgh, J.L., Funderburgh, M.L., Brown, S.J., Vergnes, J.-P., Hassell, J.R., Mann, M.M. and Conrad, G.W. (1993) J. Biol. Chem. 268, 11874–11880.
[3] Krusius, T. and Ruoslahti, E. (1986) Proc. Natl. Acad. Sci. USA 83, 7683–7687.
[4] Iozzo, R.V. (1997) Crit. Rev. Biochem. Mol. Biol. 32, 141–174.
[5] Neame, P.J., Choi, H.V. and Rosenberg, L.C. (1989) J. Biol. Chem. 264, 8653–8661.
[6] Fisher, L.W., Termine, J.D. and Young, M.F. (1989) J. Biol. Chem. 264, 4571–4576.
[7] Antonsson, P., Heinegård, D. and Oldberg, Å. (1991) J. Biol. Chem. 266, 16859–16861.
[8] Blochberger, T.C., Vergnes, J.-P., Hempel, J. and Hassell, J.R. (1992) J. Biol. Chem. 267, 347–352.

[9] Dunlevy, J.R., Neame, P.J., Vergnes, J.-P. and Hassell, J.R. (1998) J. Biol. Chem. 273, 9615–9621.
[10] Plaas, A.H.K., Neame, P.J., Nivens, C.M. and Reiss, L. (1990) J. Biol. Chem. 265, 20634–20640.
[11] Grover, J., Chen, X.-N., Korenberg, J.R. and Roughley, P.J. (1995) J. Biol. Chem. 270, 21942–21949.
[12] Fisher, L.W., Heegaard, A.M., Vetter, U., Vogel, W., Just, W., Termine, J.D. and Young, M.F. (1991) J. Biol. Chem. 266, 14371–14377.
[13] Antonsson, P., Heinegård, D. and Oldberg, Å. (1993) Biochim. Biophys. Acta 1174, 204–206.
[14] Grover, J., Chen, X.-N., Korenberg, J.R. and Roughley, P. (1995) J. Biol. Chem. 270, 21942–21949.
[15] Font, B., Eichenberger, D., Goldschmidt, D., Boutillon, M.-M. and Hulmes, D.J.S. (1998) Eur. J. Biochem. 254, 580–587.
[16] Vogel, K.G., Paulsson, M. and Heinegård, D. (1984) Biochem. J. 223, 587–597.
[17] Hedbom, E. and Heinegård, D. (1989) J. Biol. Chem. 264, 6898–6905.
[18] Rada, J.A., Cornuet, P.K. and Hassell, J.R. (1993) Exp. Eye Res. 56, 635–648.
[19] Vogel, K.G. and Trotter, J.A. (1987) Collagen Relat. Res. 7, 105–114.
[20] Svensson, L., Heinegård, D. and Oldberg, Å. (1995) J. Biol. Chem. 270, 20712–20716.
[21] Hedbom, E. and Heinegård, D. (1993) J. Biol. Chem. 268, 27307–27312.
[22] Scott, J.E. (1988) Biochem. J. 252, 313–323.
[23] Danielson, K.G., Baribault, H., Holmes, D.F., Graham, H., Kadler, K.E. and Iozzo, R.V. (1997) J. Cell Biol. 136, 729–743.
[24] Chakravarti, S., Magnuson, T., Lass, J.H., Jepsen, K.J., LaMantia, C. and Carroll, H. (1998) J. Cell Biol. 141, 1277–1286.
[25] Svensson, L., Aszódi, A., Reinholt, F.P., Fässler, R., Heinegård, D. and Oldberg, Å. (1999) J. Biol. Chem. 274, 9636–9647.
[26] Day, A.A., McQuillan, C.I., Termine, J.D. and Young, M.R. (1987) Biochem. J. 248, 801–805.
[27] Young, J.M., Cheadle, C., Fulke Jr., J.S., Drohan, W.N. and Sarver, N. (1988) Gene 62, 171–185.
[28] Larsson, T., Sommarin, Y., Paulsson, M., Antonsson, P., Hedbom, E. and Heinegård, D. (1991) J. Biol. Chem. 266, 20428–20433.
[29] Oldberg, Å., Antonsson, P., Moses, J. and Fransson, L.-Å. (1996) FEBS Lett. 386, 29–32.
[30] Laemmli, U.K. (1970) Nature 227, 680–685.
[31] Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672.
[32] Nurminskaya, M.V. and Birk, D.E. (1998) Arch. Biochem. Biophys. 350, 1–9.
[33] Kuhn, K. and Eble, J. (1994) Trends Cell Biol. 4, 256–261.
[34] Clark, E.A. and Brugge, J.S. (1995) Science 268, 233–239.
[35] Schlessinger, J. (1997) Cell 91, 869–872.