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The Family of the Small Leucine-Rich Proteoglycans: Key Regulators of Matrix Assembly and Cellular Growth

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ABSTRACT: The focus of this review is on conceptual and functional advances in our understanding of the small leucine-rich proteoglycans. These molecules belong to an expanding gene class whose distinctive feature is a structural motif, called the leucine-rich repeat, found in an increasing number of intracellular and extracellular proteins with diverse biological attributes. Three-dimensional modeling of their prototype protein core proposes a flexible, arch-shaped binding surface suitable for strong and distinctive interactions with ligand proteins. Changes in the properties of individual proteoglycans derive from amino acid substitutions in the less conserved surface residues, changes in the number and length of the leucine-rich repeats, and/or variation in glycosylation. These proteoglycans are tissue organizers, orienting and ordering collagen fibrils during ontogeny and in pathological processes such as wound healing, tissue repair, and tumor stroma formation. These properties are rooted in their bifunctional character: the protein moiety binding collagen fibrils at strategic loci, the microscopic gaps between staggered fibrils, and the highly charged glycosaminoglycans extending out to regulate interfibrillar distances and thereby establishing the exact topology of fibrillar collagens in tissues. These proteoglycans also interact with soluble growth factors, modulate their functional activity, and bind to cell surface receptors. The latter interaction affects cell cycle progression in a variety of cellular systems and could explain the purported changes in the expression of these gene products around the invasive neoplastic cells and in regenerating tissues.

KEY WORDS: decorin, biglycan, fibromodulin, lumican, keratocan, osteoadherin, epiphycan, osteoglycin.

Abbreviations: SLRP, small leucine-rich proteoglycan; **LRR,** leucine-rich repeat; **GAG,** glycosaminoglycan; **TGF-β,** transforming growth factor-β; **TNF-α,** tumor necrosis factor-α; **IL-1,** interleukin-1; **D,** the 67-nm periodicity in the quarter-staggered fibrillar collagen assemblies.

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I. INTRODUCTION

Invariably, all connective tissues contain one or more members of a family of proteoglycans originally termed nonaggregating or small dermatan-sulfate proteoglycans due to their inability to interact with hyaluronan or to the type of glycosaminoglycan (GAG), respectively. Both terms, however, are confusing, given the fact that these proteoglycans can interact with a variety of proteins and that they can be substituted with carbohydrate moieties other than dermatan sulfate. With the unraveling of their protein core sequences, it is now possible to group these molecules into distinct families and subfamilies predicated on the structural properties of their protein cores and their genomic organization. The nomenclature used in this review is based on the intrinsic protein core homology shared by the eight members of the small leucine-rich proteoglycan (SLRP) gene family. Special emphasis is put on the most recent conceptual advances pertaining to the structure of their protein backbones, their interactions with fibrillar collagens and growth factors, and their involvement in the control of cellular proliferation. Potential mechanisms that link the ectopic expression of decorin with arrest in the G_1 phase of the cell cycle are discussed in light of current knowledge of cell cycle regulation.

II. THE FAMILY OF THE SMALL LEUCINE-RICH PROTEOGLYCANS

Our understanding of SLRP biology has increased exponentially over the past decade, due largely to the meticulous characterization of their primary protein and genomic structures and to the unfolding roles these proteoglycans play in fundamental cellular processes essential for morphogenesis and tissue homeostasis. The SLRPs encompass a class of secreted proteoglycans that include at least nine members (Table 1): decorin (Krusius and Ruoslahti, 1986), biglycan (Fisher et al., 1989; Neame et al., 1989), fibromodulin (Oldberg et al., 1989), lumican (Blochberger et al., 1992b), keratocan (Funderburgh et al., 1996a), PRELP, an acronym for proline arginine-rich end leucine-rich repeat protein (Bengtsson et al., 1995), osteoadherin (Heinegård, D., personal communication), epiphycan (Deere et al., 1996; Johnson et al., 1977), also known as PG-Lb (Shinomura and Kimata, 1992; Kurita et al., 1996), and osteoglycin, formerly called osteoinductive factor (Madisen et al., 1990). It should be pointed out, however, that there is only weak evidence for PRELP being a proteoglycan, even though its closest relatives are SLRPs. Some SLRPs (e.g., fibromodulin) are "part-time" proteoglycans, and it is possible that PRELP is more "part-time" than most. Their structural hallmark is the presence of tandem arrays of leucine-rich repeats (LRRs) flanked by two cysteine-rich regions with highly conserved spacings. Posttranslational modifications account for different functionalities in these molecules. A further dimension of complexity derives from the existence of different SLRPs with one or several GAG side chains, variations in the protein core sequence (i.e., hypervariable regions, mutable number of LRRs, or differences in the arrangement of the disulfide bonds), and diverse patterns of N-glycosylation. The prototype structure of the best characterized SLRP, decorin, comprises four distinct domains (Figure 1). Each domain is discussed below *vis á vis* those found in other SLRP family members.

A. Domain I: Signal Peptide and Propeptide

The primary translation product of human decorin consists of 359 amino acids

TABLE 1
General Structural Characteristics of the Small Leucine-Rich Proteoglycans

Proteoglycan (gene)	Protein core (kDa)	LRR[a] (number)	GAG[b] (type)	Sulfo-Tyr consensus	N-Glycosylation[c] (number)	Chromosomal mapping	
						Human	Mouse
Class I							
Decorin (DCN)	40	10	CS/DS[d]	–	3	12q23	10
Biglycan (BGN)	40	10	CS/DS	–	2	Xq28	X
Class II							
Fibromodulin (FMOD)	42	10	KS	+	3–5	1q32	
Lumican (LUM)	38	10	KS	+	3–5	12q21.3–22	10
Keratocan[e]	38	10	KS	+	3–5		
PRELP[f]	44	10	KS	–	3–4	1q32	
Osteoadherin	42	10	KS	+	3–5		
Class III							
Epiphyca (DSPG3)	35	6	CS/DS	+	3–4	12q21	
Osteoglycin (OG)[g]	35	6	KS	+	2–3		

[a] The number of LRRs can be increased if less stringent criteria are used.
[b] DS, dermatan sulfate; CS, chondroitin sulfate; KS, keratan sulfate.
[c] The number of N-linked oligosaccharides will vary in the KS-containing proteoglycans.
[d] Decorin can also contain KS in the adult avian cornea (Blochberger et al., 1992a).
[e] Keratocan appears to be mapped to a murine chromosome other than chromosome 10 (see footnote 2 in Corpuz et al., 1996).
[f] The presence of KS in bovine PRELP (Bengtsson et al., 1995) has been suggested by the shift in electrophoretic mobility following keratanase digestion; however, it may also be due to the keratanase sensitivity of a nonsulfated polylactosamine.
[g] Osteoglycin has been recently shown to be a KS proteoglycan in bovine cornea (Funderburgh et al., 1996a).

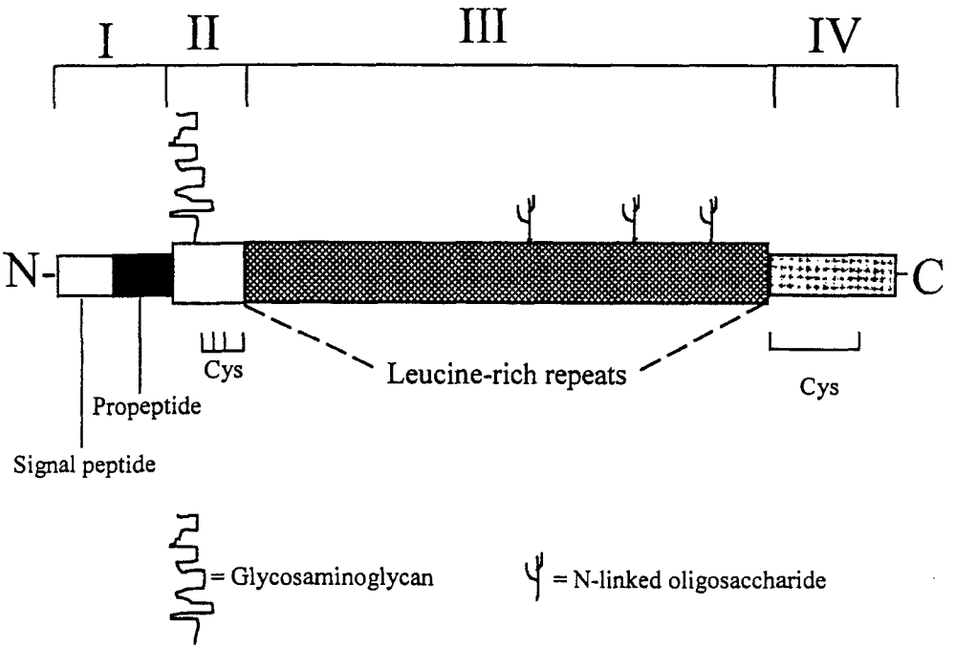


FIGURE 1. Structural features of decorin, the prototype member of the small leucine-rich proteoglycans. The four domains are marked by roman numerals. For additional details, see the text and Table 1.

(Krusius and Ruoslahti, 1986). The initial 30 residues, however, are not detected in the mature, secreted proteoglycan and likely represent a 16-amino acid signal peptide and a 14-amino acid propeptide. These features are shared also by biglycan, which contains a 16-residue signal peptide and a 21-residue propeptide (Fisher et al., 1989). The signal peptide targets the nascent core protein to the rough endoplasmic reticulum (Silbert and Sugumaran, 1995) and, in general, is cleaved cotranslationally and thus is absent in the mature protein. The amino acid sequence of the propeptide is highly conserved across species (Torok et al., 1993; Dreher et al., 1990; Scholzen et al., 1994) and contains a series of acidic amino acids that have been proposed to regulate the attachment of GAG chains (Ruoslahti, 1988). The propeptide, therefore, may function as a recognition/attachment site for xylosyltransferase (Sawhney et al., 1991), the first enzyme in the GAG biosynthetic cascade. The presence of the propeptide would help in positioning this enzyme in a strategic location, thereby allowing the addition of xylose to Ser^4 (Sawhney et al., 1991). In support of this view, transfection experiments using constructs harboring various deletions of the decorin propeptide lead to the secretion of proteoglycans substituted with shorter GAG chains (Oldberg et al., 1996). These deletions may affect the synthesis of the GAG linkage region by lowering the affinity between the protein substrate and the xylosyltransferase (Oldberg et al., 1996), and perhaps other glycosyltransferases, and/or by inducing a faster transition through the Golgi apparatus. Indeed, chinese hamster ovary (CHO) cell lines harboring a five-residue deletion in the propeptide secrete decorin proteoglycans at a rate of ~30% faster than wild-type cells (Oldberg et al., 1996). It is also

likely that a similar situation occurs in the biglycan because expression of a biglycan mutant vector lacking the propeptide results in secretion of a core protein devoid of both GAG chains (Hocking and McQuillan, 1996).

Immunohistochemical studies using an antiserum directed against the propeptide of biglycan suggested that probiglycan is absent from all matrix sites of biglycan deposition, indicating that the mature secreted product has lost this region by proteolytic processing before secretion into the pericellular space (Bianco et al., 1993). In contrast, keratinocytes were markedly stained with the antiprobiglycan antiserum, suggesting that retention of the propeptide may be related to biglycan routing and association with the cell surface. Analogously, probiglycan was localized within endothelial and smooth muscle cells of aorta but not in the extracellular spaces (Yeo et al., 1995). However, in cultured smooth muscle cells, biglycan can be secreted with the propeptide intact (Marcum and Thompson, 1991), and decorin is synthesized by skin fibroblasts or by transfected COS or CHO cells with unprocessed propeptide (Oldberg et al., 1996). This may also occur in tissues, insofar as such proforms of decorin and biglycan are found to be particularly abundant in adult human cartilage but not in juvenile cartilage and other connective tissues where both proteoglycans are highly expressed (Roughley et al., 1996b). Additional studies need to be done to determine whether other SLRP members may also utilize the propeptide for xylosylation or cellular sorting. Of note, class II members of SLRPs, such as fibromodulin, lumican, and keratocan, are not proteolytically processed following removal of their signal peptide, that is, the initially processed species corresponds to that detected in the extracellular space (Plaas et al., 1990; Funderburgh et al., 1993).

B. Domain II: A Negatively Charged Domain Carrying Sulfated GAG or Sulfotyrosine

In the mature core proteins of decorin, biglycan, and epiphycan, the GAG attachment sites are located in the amino terminal end, proximal to the cysteine-rich region (Figure 1). Using the exodipeptidase cathepsin C, it was demonstrated that a bovine skin proteoglycan, later shown to be decorin, carried only one GAG chain linked to the fourth amino acid residue in the sequence Asp-Glu-Ala-Ser-Gly- (Chopra et al., 1985). However, in chicken (Li et al., 1992) and pigeon (Register et al., 1993) decorin, the sequence is Asp-Glu-Ala-Gly-Ser, so that the GAG is attached to Ser^5 rather than Ser^4. Hence, it has been proposed that avian xyolsyltransferase activity differs from the mammalian enzyme activity (Li et al., 1992). In biglycan, GAG chains are attached at both Ser^5 and Ser^{11} (Neame et al., 1989), whereas in epiphycan, potential GAG attachment sites are also located at Ser^{70} and Ser^{75} in the avian species (Shinomura and Kimata, 1992) and at Ser^{64}, Ser^{96}, and Ser^{320} in the murine species (Kurita et al., 1996). The following consensus sequence for the GAG attachment site has been derived: (acidic)-(acidic)-X-Ser-Gly-(hydrophobic) (Neame et al., 1989). It also appears that there must be at least one acidic residue prior to the Ser-Gly dipeptide, but it is not necessary to have both. Studies using site-directed mutagenesis of the Ser residue have conclusively demonstrated the requirement for a specific amino acid context that would direct proper O-glycosylation of the protein core of decorin (Mann et al., 1990). Neame and co-workers (1989) found that ~10% of the two serine residues in biglycan corresponding to the two potential GAG attachment sites were unsubstituted, thus suggesting that biglycan could also exist in a monoglycanated form. Of note, a recent

investigation of avian decorin has shown that decorin can exist as both a mono- and a biglycanated proteoglycan carrying, in the latter case, a second dermatan sulfate chain at Ser^{16} (Blaschke et al., 1996). This biglycanated form of decorin was detected in adult chicken cornea and tendon (see footnote 2 in Blaschke et al., 1996). However, a monoclonal antibody directed toward avian decorin revealed only the monoglycanated species in embryonic skeletal muscle tissues (Lennon et al., 1991). Thus, further studies are needed to establish if there are tissue-specific controls for assigning one or two dermatan sulfate chains, or whether the biglycanated species of decorin may preferentially occur in adult life. The conjecture that decorin with two GAG chains may represent the avian equivalent of biglycan (Blaschke et al., 1996) is interesting but requires experimental proof.

Glycanated and nonglycanated species of decorin and biglycan have been isolated from adult cartilage (Sampaio et al., 1988; Johnstone et al., 1993). In the latter case, there is a proteolytic cleavage of the amino terminal peptide, with the consequent elimination of the GAG attachment domain. Indeed, it has been shown before that mild proteolysis of purified decorin with *Staphylococcus aureus* V8 protease releases the amino terminal GAG peptide (Vogel and Fisher, 1986). Because this GAG region is not required for collagen binding (Vogel et al., 1987), it is conceivable that partly processed forms may bind to and regulate collagen fibrillogenesis. The steady increase in cartilaginous decorin content with age in the first 25 years of life and its subsequent decline may be due to various rates of deposition and maintenance of the collagen fibril network (Sampaio et al., 1988).

With the exception of PRELP, all the members of class II and III (Table 1) contain consensus sequences for tyrosine sulfation, that is, they contain at least one tyrosine residue followed by an acidic amino acid [Asp or Glu] and thus are candidates for sulfation by tyrosyl sulfotransferase. However, only in the case of fibromodulin has it been formally demonstrated that several tyrosine residues are substituted with sulfate (Antonsson et al., 1991). It remains to be shown whether the other members will carry sulfotyrosine in domain II. The sulfation of amino-terminal tyrosine residues appears to be a widespread process because it was found in fibroblasts from tendon and sclera as well as in chondrocytes and transfected CHO cells. This domain would provide a net negative charge, either as sulfated GAG or tyrosine, to all the members of the SLRP gene family. This region would likely interact with cationic domains expressed at the cell surface or in extracellular matrix proteins.

Of note, the amino-terminal region of PRELP is quite different from any other SLRP members. Following the 20-amino-acid-residue signal peptide is a stretch of ~50 residues highly enriched in Pro and Arg, which together account for over half of the residues in this region (Bengtsson et al., 1995; Grover et al., 1996). This domain, which lacks a consensus sequence for either GAG attachment or tyrosine sulfation, is reminiscent of three turns of an extended collagen-type helix, and it might form an extended structure or a hairpin (Bengtsson et al., 1995). It has been proposed that this basic region may interact with GAGs insofar as it contains a consensus sequence for heparin binding (Bengtsson et al., 1995).

Domain II contains a cluster of highly conserved cysteine residues with a general consensus $Cx_{2-3}CxCx_{6-9}C$, where x is any amino acid and the subscripts denote the number of intervening residues (Table 2). Differences in the consensus cysteine-rich region are discussed below. Chemical analysis of bovine decorin has shown the pres-

TABLE 2
Alignment of the Amino Terminal Cysteine-Rich Region in the Small Leucine-Rich Proteoglycans

Proteoglycan	Sequence[a]	Ref.
Class I		
Decorin	CPFRCQCHLRVVQ...CSD	Krusius and Ruoslahti, 1986
Biglycan	CPFGCHCHLRVVQ...CSD	Fisher et al., 1989
Class II		
Fibromodulin	CPQECDCPPNFPTAMYCDN	Oldberg et al., 1989
Lumican	CAPECNCPESYPSAMYCDL	Grover et al., 1995
Keratocan	CPRECFCPPSFPTALYCEN	Corpuz et al., 1996
PRELP	CPRECYCPPDFPSALYCDS	Grover et al., 1996
Class III		
Epiphycan	CLL.CTCISTTV...YCDD	Kurita et al., 1996
Osteoglycin	CLL.CVCLSGSV...YCEE	Madisen et al., 1990

[a] All the sequences are human with the exception of epiphycan, which is murine. No sequence was available for osteoadherin; however, the structure of the cysteine-rich region appears to be similar to class II SLRPs.

ence of three disulfides and no free cysteine (Scott et al., 1986), thus leading to the conclusion that all four amino-terminal cysteine residues are bridged by disulfide bonds as well as the two carboxyl cysteine residues (see below). However, in bovine biglycan, only a disulfide bond between the first and the fourth cysteine could be demonstrated unequivocally (Neame et al., 1989).

C. Domain III: The Interacting Domain Harboring the Tandem Leucine-Rich Repeats

The central domain of most SLRPs, which can account for 60 to 80% of the total amino acids, comprises ten tandem leucine repeats, with the exception of epiphycan and osteoglycin, which contain only six repeats (Table 1). The number of LRRs could conceivably increase in each member if less stringent criteria for the number of conserved residues in the LRR unit are utilized. For simplicity, only the LRRs flanked by the two cysteine-rich regions are considered. Most of the leucine residues are in conserved positions (Patthy, 1987) with a general consensus sequence of LxxLxLxxNxLSxL, where L is leucine, isoleucine, or valine, and S is serine or threonine (Neame et al., 1989). Minor variations exist among the SLRPs (Kobe and Deisenhofer, 1994); however, the vast majority of the leucine and asparagine residues are highly conserved, with leucine residues located at positions 1, 4, 6, 11, and 14 in the LRR segments. A three-dimensional model structure of decorin, based on the ribonuclease inhibitor, is discussed in detail below. The functional properties of protein:protein interaction appear to be a common feature of the LRRs. Independent results from three laboratories indicate that this central domain, and particularly the region comprising LRR3-6, is responsible for binding collagen type I (Spiro et al., 1994; Svensson et al., 1995; Schönherr et al., 1995).

In human decorin, there are three potential sites for N-linked oligosaccharide sub-

stitution (Asn-X-Ser/Thr). In contrast, biglycan has only two sites. Variability in the number and complexity of these oligosaccharides, which are generally small (2 to 3 kDa), is a typical feature in SLRPs. Most of the oligosaccharides are of the complex type, but high-mannose-type oligosaccharides can also be found. A proposed functional role of oligosaccharides *in vivo* is to retard self-aggregation, thereby favoring the interaction of SLRPs with extracellular matrix and cell surface proteins (Scott, 1993). This is in harmony with the fact that biglycan, which contains fewer N-linked oligosaccharides than decorin, tends to self-associate (Liu et al., 1994).

In class II (Table 1), one to several asparagine residues within the consensus sequence for N-glycosylation can be substituted with asparagine-linked keratan sulfate. In the case of bovine fibromodulin from articular cartilage, all four Asn residues in the leucine-rich region can serve as acceptors for keratan sulfate addition (Plaas et al., 1990). Further processing includes the addition of polylactosamine, essentially unsulfated keratan sulfate, to one or two of the N-linked oligosaccharide acceptors of both fibromodulin (Plaas and Wong-Palms, 1993) and keratocan (Corpuz et al., 1996). Even in members of the class I subgroup — decorin from adult avian cornea (Blochberger et al., 1992a) or cartilage (Blaschke et al., 1996) — these sites can be substituted with keratan sulfate. Keratan sulfate chains N-linked to fibromodulin from bovine tracheal cartilage and O-linked chains from nonarticular aggrecan appear to bear similar structures despite having different modes of linkage to different protein cores (Lauder et al., 1994). These data highlight the meaningfulness of tissue-specific expression of sialyl and fucosyl transferases in the biosynthesis of keratan sulfate (Plaas, 1992). Interestingly, in the adult human articular cartilage, fibromodulin does not possess either keratan sulfate or nonsulfated polylactosamine chains, but in the juvenile and young adult counterparts, fibromodulin is a fully glycosylated proteoglycan (Roughley et al., 1996a).

It is still unclear whether PRELP carries keratan sulfate side chains. Keratanase digestion shifted the electrophoretic mobility of the parent molecule to a lower M_r position (Bengtsson et al., 1995). However, more experiments are needed to determine whether there is any tissue-specific form of keratan sulfate PRELP.

Lumican is a keratan sulfate proteoglycan primarily, if not exclusively, in the cornea, while being a glycoprotein in the extraocular tissues such as skin, cartilage, intestine, and muscle (Grover et al., 1995; Blochberger et al., 1992b). In addition, lumican with polylactosamine is present in early stages of corneal development, while the appearance of the fully sulfated keratan sulfate proteoglycan is a later event (Cornuet et al., 1994). Thus, substitution with keratan sulfate is a tissue-specific and developmentally regulated process that also appears to be influenced by age.

D. Domain IV: The Carboxyl Terminal Domain

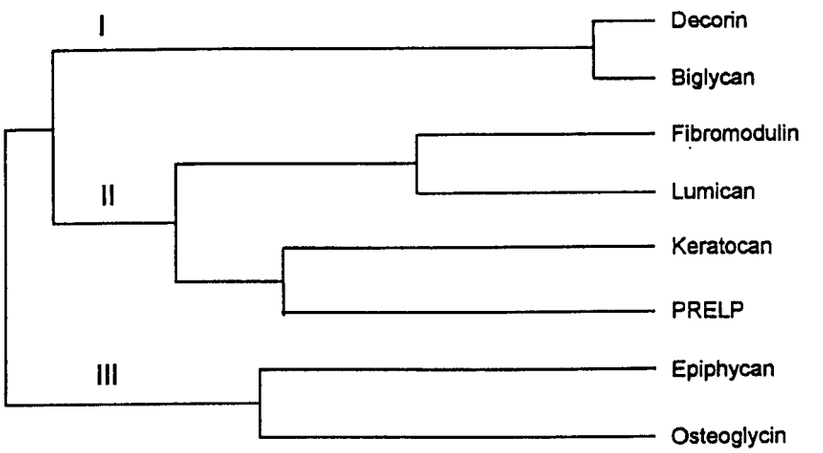
The carboxyl domain of the SLRPs is the least characterized so far. It comprises an ~50 amino acid domain with considerable similarities among family members. However, several stretches of residues differ among the three classes of SLRPs. This would contribute to the physical differences observed within the SLRP gene family members. Domain IV contains two cysteine residues that are positioned 32 residues apart in all cases, except keratocan and PRELP, where an insertion of seven and eight amino acids, respectively, has occurred. In bovine

biglycan, a disulfide bond has been demonstrated in this region (Neame et al., 1989). Hence, a large loop of 34 to 41 residues would be formed at this end of the molecule. Reduction and alkylation of bovine skin decorin (Scott et al., 1986) and bovine corneal lumican (Rada et al., 1993) results in total abrogation of their functional properties, exemplified by a loss of collagen fibrillogenesis-controlling activity. Thus, the presence of disulfide bridges in decorin and lumican at both the amino and carboxyl ends of the molecules may play a fundamental role in the ability of these two proteoglycans to interact with collagen. We can presume that a similar concept would also apply to fibromodulin, which also interacts with fibrillar collagens (Hedbom and Heinegård, 1989, 1993; Hedlund et al., 1994).

III. THREE DISTINCT SUBFAMILIES

The SLRPs show extensive protein sequence homology that increases if the amino terminal hypervariable region is excluded; however, they represent distinct gene products with an often nonoverlapping tissue distribution (Heinegård et al., 1985; Kresse et al., 1993). An analysis by multiple sequence alignment (Higgins and Sharp, 1988) of the evolutionary relationships among the eight published amino acid sequences encoding SLRPs identifies three distinct subfamilies (Figure 2). Class I comprises decorin (Krusius and Ruoslahti, 1986) and biglycan (Fisher et al., 1989; Neame et al., 1989), which show the highest internal homology in amino acid sequence (~57%). Class II comprises fibromodulin (Oldberg et al., 1989), lumican (Blochberger et al., 1992b), keratocan (Funderburgh et al., 1996a), and PRELP (Bengtsson et al., 1995; Grover et al., 1996). Two subclasses can also be seen (Figure 2), with fibromodulin and lumican comprising the first, and keratocan and PRELP comprising the second subclass. Osteoadherin appears to be a novel member of this subgroup; it contains Tyr-sulfate in the amino-terminal portion and harbors keratan sulfate (N-linked) in the variant found in human bone. It has a carboxyl terminal extension that is considerably larger than the other described members of this family and is expressed primarily, if not exclusively, in bone (Heinegård, D., personal communication). Class III comprises epiphycan (Deere et al., 1996; Johnson et al., 1977), previously named PG-Lb (Shinomura and Kimata, 1992; Kurita et al., 1996), and osteoglycin, formerly known as osteoinductive factor (Madisen et al., 1990). I prefer the term epiphycan over the nondescriptive PG-Lb because it reminds us of its highly selective tissue distribution (i.e., the epiphyseal cartilage). This would imply that epiphycan may play a role in a region of cartilage (i.e., the epiphysis) that is not associated with calcification, perhaps by delaying the onset of calcification or by contributing to the remodeling steps that occur before calcification (Johnson et al., 1977). Indeed, human epiphycan contains a potential calcium-binding domain and has been proposed to participate in osteogenic processes (Deere et al., 1996). Osteoglycin has been recently shown to be a keratan sulfate proteoglycan in bovine cornea (Funderburgh et al., 1996a). On the basis of the plot shown in Figure 2, the divergence of class III proteoglycans from the common precursors of class I and II gene products predated the presumed gene duplication and independent evolution of the latter two subgroups.

A close analysis of the structural characteristics and key motifs in the SLRPs reveals that these three classes can be separated from each other by additional



	I		II				III	
	Decorin	Biglycan	Fibromodulin	Lumican	Keratocan	PRELP	Epiphycan	Osteoglycin
Decorin	**100**	57	15	26	20	24	19	23
Biglycan		**100**	14	26	21	25	20	24
Fibromodulin			**100**	48	18	37	19	17
Lumican				**100**	33	36	16	23
Keratocan					**100**	55	13	18
PRELP						**100**	19	24
Epiphycan							**100**	40
Osteoglycin								**100**

FIGURE 2. Sequence-based evolutionary tree (top) and percent identity (bottom) of members of the SLRP gene family. Branch lengths (horizontal lines) are proportional to evolutionary distances. The dendogram was obtained using the program CLUSTAL in the PCGENE package (Higgins and Sharp, 1988). In the bottom panel, the numbers correspond to the percentage of identical amino acid residues as aligned using the ALIGN program. See Tables 1 and 2 for additional details.

attributes. First, class I members are apparently the only ones to have a propeptide. The presence of a functional propetide located just proximal to the GAG attachment sites may modulate the initial glycosylation of the protein core and perhaps regulate the size of the GAG chain, as discussed above. The reason why class II and III do not have a functional propeptide is, perhaps, because they lack the GAG attachment site (class II) or because the GAG attachment site is located distally (class III).

Class II and III members can be distinguished by the presence of consensus sequences for tyrosine sulfation, except PRELP (Table 1). The presence of sulfated tyrosine in the amino terminal end may provide a signal for the posttranslational addition of keratan sulfate chains (Corpuz et al., 1996). This hypothesis could be tested by

generating chimeric proteoglycans harboring the amino-terminal end of class II and the central and distal domains of class I SLRPs.

An additional distinguishing feature of the SLRP subfamilies is the spacing of the cysteine residues in domain II (Table 2). In class I, the four cysteine residues are spaced by 3, 1, and 6 residues; in class II, the spacing follows a 3, 1, 9 pattern, whereas class III follows a 2, 1, 6 pattern. Not only the spacing of the cysteine residues, but also the overall nature of the intervening amino acids is quite different among the three groups. The variation in disulfide bonding might also contribute to diversification of function among the various subfamilies of SLRPs.

Class III members can be further distinguished by the presence of only six LRRs, unlike the other members that all have at least ten LRRs (Table 1). On the basis of this, I predict that the three-dimensional structure of epiphycan/osteoglycin members would be much more open than those with a larger number of LRRs (see below).

There is a modest amount of information available regarding the genomic organization, chromosomal mapping (Table 1), and transcriptional regulation of various SLRPs. However, some general patterns are emerging. For example, the similarities at the protein core levels of individual members of the class I or II SLRPs may also extend, at least partially, to their genomic organization. Class I (decorin and biglycan) are composed of eight distinct exons, while class II (fibromodulin, lumican, and PRELP) genes are encoded by three exons. Human decorin (Danielson et al., 1993) and biglycan (Fisher et al., 1991) genes show a considerable structural homology with highly conserved intron/exon boundaries. However, because of the larger intron size in both human and murine decorin (Danielson et al., 1993; Scholzen et al., 1994), when compared with the human and murine biglycan (Fisher et al., 1991; Wegrowski et al., 1995b), the overall size of the decorin gene is about three to five times bigger than that of biglycan. An additional distinguishing feature is the presence of two alternatively spliced leader exons (Ia and Ib) that encode a 5′ untranslated region in the human decorin gene (Danielson et al., 1993). Interestingly, sequences with high homology to exon Ia are also found in the 5′ untranslated regions of avian (Li et al., 1992) and murine (Scholzen et al., 1994; Abramson and Woessner, 1992) decorin, whereas the bovine decorin gene contains exon Ib-like sequences (Day et al., 1987). Conservation of these exons suggests that they may be evolutionarily meaningful for the expression of decorin. In the vast majority of cases, genes with multiple 5′ untranslated regions express these exons in either a developmental- or tissue-specific fashion. Surprisingly, we found no corresponding exon Ib or adjacent 5′ region in the mouse (Scholzen et al., 1994). This may suggest that exon Ib was either deleted in the murine species or that this region underwent recombination during evolution.

At least for three members of class II genes — human fibromodulin (Antonsson et al., 1993), human lumican (Grover et al., 1995), and human PRELP (Grover et al., 1996) — there is evidence of a tri-exonic organization, with the central exon encoding all ten LRRs. In all of these genes, the two introns reside prior to the translation initiation and termination codons, respectively.

Although there is no available published information concerning the genomic organization of class III members, a partial sequence of the murine osteoglycin gene has shown the presence of a relatively small exon of 159 bp encoding the cysteine-rich region and the beginning of the LRRs (Ujita et al., 1995). These findings suggest that

members of class III do not have a triexonic organization. Furthermore, because the size and location of the osteoglycin exon does not fit any of the above-mentioned genomic organizations, it is likely that class III genes will harbor intron/exon junctions distinct from class I or II SLRPs.

V. REGULATION OF GENE EXPRESSION

To date, only the promoters of decorin (Santra et al., 1994; Mauviel et al., 1995; Mauviel et al., 1996) and biglycan (Wegrowski et al., 1995b; Ungefroren and Krull, 1996) genes have been characterized and tested for functional activity in transient cell transfection assays. The differential regulation by transforming growth factor-β (TGF-β) (Kähäri et al., 1991) and the divergent tissue expression (Bianco et al., 1990) of these two SLRPs would predict that the structural arrangement of their control regions would also be distinct. Indeed, this is the case. The promoter of decorin differs substantially from that of biglycan not only in general organization, but also in the arrays of cognate *cis*-acting elements that would drive its tissue-specific expression. The promoter of human decorin, the 5′ flanking region to exon Ib, can be subdivided into two distinct regions: a proximal region of ~188 bp and a distal region of ~800 bp (Santra et al., 1994). The proximal promoter contains a CAAT box and two closely spaced TATA boxes in the vicinity of the major transcription start site. *In vitro* transcription analysis generated two transcripts of the predicted length *vis á vis* the position of the two TATA boxes (Santra et al., 1994), indicating that the two TATA boxes are operational *in vivo* in the initiation of decorin gene transcription. The proximal promoter region also contains two tumor necrosis factor-α (TNF-α)-responsive elements, one residing within the 5′ untranslated region (exon Ib) and one between residues −188 and −140 (Mauviel et al., 1995). The downregulation of decorin gene expression can be mediated by TNF-α -inducible nuclear proteins that recognize and bind to the TNF-α responsive elements within this proximal region. Furthermore, a 48-bp region, between -188 and -140 relative to the major transcription start site, contains a canonical and functional AP-1 binding site. This site is a bimodal regulator of decorin gene expression and allows both its repression by TNF-α and its induction by interleukin-1 (IL-1β) (Mauviel et al., 1996). These findings are in agreement with the upregulation of decorin mRNA and protein levels in fibroblasts by either IL-1 (Heino et al., 1988) or IL-4 (Wegrowski et al., 1995a). Overexpression of c-*jun* inhibits decorin promoter activity, whereas blockage of c-*jun* expression by cotransfection with an antisense c-*jun* vector leads to an enhanced response to IL-1β and reversed responsiveness to TNF-α (Mauviel et al., 1996).

The distal promoter region of decorin contains numerous *cis*-acting elements, including an additional AP-1, one AP-5, two NF*k*B motifs, several direct repeats of undetermined function, and a TGF-β-negative element. The latter has been found in a variety of proteases that are downregulated by TGF-β (Iozzo and Cohen, 1993) and could potentially function to suppress decorin transcription. The possibility of an autoregulating decorin-TGF-β loop should be entertained, given the established interaction and affinity between these two gene products (Yamaguchi et al., 1990). The most distal promoter region of decorin harbors an ~150-bp segment composed of homopyrimidine residues in the coding strand

with high sensitivity to single-strand S1 endonuclease. This region could adopt an intramolecular hairpin triplex structure and could play a role in the chromatin organization of the decorin gene. Indeed, we showed that this region can upregulate a minimal heterologous promoter in transient cell transfection assays and, hence, could act as an upstream enhancer of the decorin gene (Santra et al., 1994). Of note, these features are not conserved in the mouse, where the region contained between exon Ia and II has no functional promoter activity (Scholzen et al., 1994). Perhaps the region 5′ to exon Ia is the murine promoter. However, this needs to be formally demonstrated in functional assays.

In contrast to the decorin gene, the biglycan 5′ flanking region does not possess CAAT or TATA boxes (Fisher et al., 1991), but is located in a region with an overall GC content of ~60% and with peaks reaching 85% (Wegrowski et al., 1995b). In addition, the biglycan promoter harbors multiple binding sites for the transcription factor Sp1 and several transcription start sites, overall features that are typically observed in housekeeping and growth-controlling genes. An additional distinguishing feature from the decorin gene is that the promoter region of the human biglycan gene is highly conserved in the mouse, with an overall homology of ~90% in the proximal 220 bp. In particular, two AP-2 and two Sp1 sites are perfectly conserved. The human promoter contains six IL-6-responsive elements and is trascriptionally induced after IL-6 treatment (Ungefroren and Krull, 1996). Similar to decorin, however, biglycan expression is suppressed by TNF-α (Mauviel et al., 1995; Ungefroren and Krull, 1996). In both the human and mouse biglycan promoter, there are several motifs that could bind members of the *Ets* family of oncogenes, such as PU-boxes and PEA3 motifs, which are known to confer transcriptional activation to B cells and macrophages (Klemsz et al., 1990).

Another striking difference between decorin and biglycan, a difference corroborated by the distinctive promoter features discussed above, is that their transcription is differentially regulated by cytokines and growth factors. When investigated, nanomolar concentrations of TGF-β inhibit decorin but invariably upregulate biglycan mRNA levels and proteoglycan biosynthesis (Bassols and Massagué, 1988; Breuer et al., 1990; Kähäri et al., 1991; Westergren-Thorsson et al., 1991; Romaris et al., 1991; Roughley et al., 1994; Vogel and Hernandez, 1992). Dexamethasone exerts a similar effect and further prevents the effects induced by TGF-α (Kähäri et al., 1995). In contrast, retinoic acid has opposite effects in the same cellular system (Kähäri et al., 1995). However, tissue-specific transcriptional mechanisms must exist insofar as in chondrocytes, retinoic acid downregulates biglycan but markedly upregulates decorin (Pearson and Sasse, 1992). Further regulatory influences may derive from the action of other genes. This would explain the intriguing finding that biglycan, although located on the X chromosome, behaves more like a pseudoautosomal gene, that is, it does not show the usual correlation between expression and gene dosage (Geerkens et al., 1995). The pseudoautosomal expression of biglycan could be attributed to a gene or genes that escape X chromosomal inactivation, thereby controlling the transcriptional activity of biglycan (Geerkens et al., 1995).

Notable differences in the regulation of expression of SLRP genes are also exemplified by the different tissue-specific patterns of decorin and biglycan mRNA expression in developing mouse (Scholzen et al., 1994) and human (Bianco et al., 1990) tissues, respectively. Overall, the expression and tissue localization of these two SLRPs appears to be substantially divergent and oc-

casionally mutually exclusive (Bianco et al., 1990). A detailed *in situ* hybridization analysis of decorin expression during murine ontogeny shows that the first detectable site of expression at day 11.5 postconception is the floor plate region, which corresponds to the unpaired ventral zone that forms the floor of the neural tube (Scholzen et al., 1994). With progressive maturation, the expression of decorin increases, and it concentrates to the major organ-lining layers, including the meninges, pericardium, pleura, peritoneum, and the capsules of parenchymal organs. Almost invariably, decorin was localized to the subepithelial layers of developing and adult tissues (Scholzen et al., 1994). The overall distribution of decorin by immunohistochemistry in a variety of species (Poole et al., 1986; Lennon et al., 1991; Voss et al., 1986) is compatible with the *in situ* hybridization analyses. For example, avian decorin is localized to the fibrous connective tissue septa of developing skeletal and cardiac muscles as well as to the periosteally deposited bony collar (Lennon et al., 1991). A similar distribution is also found in developing human muscle and bone (Bianco et al., 1990). Decorin is the major dermatan sulfate proteoglycan in bovine perimysium and endomysium (Eaggen et al., 1994), and its expression increases during differentiation of myotubes *in vitro* (Brandan et al., 1991). The intracellular concentration of decorin epitopes near and around the nuclear membrane of myotubes raises the intriguing possibility that this proteoglycan or protein core may interact within the cell with other proteins regulating myogenesis. The striking expression of decorin in the mesenchyme and in the capsules and organ-lining layers suggests that decorin may play a role in regulating the size and shape of a specific organ.

Biglycan displays a spatial and temporal pattern that is quite different from that of decorin (Bianco et al., 1990). Furthermore, the expression of biglycan in human chondrocytes decreases with the age of the donor, whereas that of decorin increases (Roughley et al., 1994). Biglycan appears to be essentially absent from typical connective tissue matrices, such as dermis, tendon, and cornea, and is expressed by a variety of cells not directly involved in the synthesis and assembly of fibrillar collagen (Bianco et al., 1990). For instance, biglycan is often localized to cellular elements, such as epithelial (maturing keratinocytes and renal tubular epithelial cells) and endothelial cells, as well as myocytes and myofibroblasts (Bianco et al., 1990). In such epithelial cells, biglycan appears to be a cell surface or surface-associated proteoglycan. Whether specific sets of integrins mediate this cell-binding property of biglycan needs to be elucidated.

VI. REGULATION OF COLLAGEN FIBRILLOGENESIS: THREE-DIMENSIONAL MODEL STRUCTURE OF DECORIN AND INTERACTION WITH FIBRILLAR COLLAGEN

It has long been established that collagen molecules are capable of self-assembly into fibrils when incubated at 37°C in physiologic solutions (Kadler et al., 1996). Nearly 3 decades ago it was shown that the interaction of dermatan sulfate proteoglycans with collagen gives rise to fibers with increased stability and changes in solubility (Toole and Lowther, 1968b; Toole, 1969). On this basis, it was hypothesized that the major role of dermatan sulfate proteoglycans was in the formation and orientation of collagen fibers. Indeed, the similarities between the distribution of decorin in collagen fibrils assembled *in vitro* (Scott et al., 1986; Lan et al., 1993) and those observed *in vivo* (Scott

and Orford, 1981) are notable. The predominant orientation of dermatan sulfate chains in the former is outward, away from the collagen fibrils. This orthogonal disposition will facilitate proper spacing of the collagen fibrils during axial growth or lateral fusion (Scott, 1995). A major function of collagen-associated SLRPs is to confer a net negative charge, a necessary requirement to prevent anomalous fusion of the fibrils and segregation of extrafibrillar molecules. When decorin binds to the surface of collagen fibrils, the edgewise assembly of individual triple helical molecules is noticeably delayed (Toole and Lowther, 1968a; Vogel et al., 1984) and the final diameter of the collagen fibrils is thinner than that generated in the absence of decorin (Vogel and Trotter, 1987). Corneal and scleral proteoglycans, which include several members of SLRP gene family, also decrease the extent of fibril formation and the width of the fibrils (Chandrasekhar et al., 1984). These effects can also be reproduced *in vitro* with the protein cores of the three major fibril-regulating SLRPs — decorin (Vogel et al., 1984), fibromodulin (Hedbom and Heinegård, 1989), and lumican (Rada et al., 1993) — but not with isolated GAGs. A logical conclusion is that the protein moiety of decorin, fibromodulin, and lumican carry all the information necessary to regulate this crucial biological process.

Recently, we generated a three-dimensional model of human decorin (Weber et al., 1996) based on the crystal structure of the porcine ribonuclease inhibitor (Kobe and Deisenhofer, 1993). The entire ribonuclease inhibitor molecule is composed of 15 LRRs with alternating short β-strands and α-helices parallel to a common axis and forming an open, nonglobular protein with a horseshoe-like configuration (Kobe and Deisenhofer, 1993) that allows ample interaction with ribonuclease (Kobe and Deisenhofer, 1995). The model of decorin depicts an arch-shaped structure (Figure 3) with the inner concave surface lined by the curved β-strands and the outer convex surface formed by the α-helices. This model of decorin forms a more open structure than the horseshoe-shaped ribonuclease inhibitor. This will allow a greater access of interactive proteins to the inner concave face and would permit the formation of favorable contact points with other proteins. The overall dimension of the decorin model are 6.5 nm (the distance between the two arms) ×4.5 nm (the distance between the apex and the base of the arch) ×3 nm (thickness). The overall structure and dimensions are similar to those reported for corneal SLRPs following visualization by rotary shadowing electron microscopy (Scott, 1996). Hence, it is likely that other members of the SLRP gene family will fold according to the prototype ribonuclease inhibitor. An interesting feature of the decorin model is that all the carbohydrate moieties lay on one side of the arch-shaped molecule. The single GAG chain at Ser^7 is relatively free to project away from the protein core, a fact that is also in agreement with cytochemical studies of SLRPs associated with fibrillar collagen (Scott, 1980).

The size of the internal cavity of the decorin molecule (~2.5 nm) immediately suggests that only one triple helical collagen molecule (~1.5 nm thick) could possibly fit into the arch. The inner concave surface of the arch harbors a series of polar and charged amino acid residues that would favor an interaction with a charged region of the collagen triple helix (Weber et al., 1996). Recently, it was shown that two topographically distinct regions in isolated collagen triple helices can bind specifically to decorin (Yu et al., 1993). A major contact site is located at ~0.8 D (1 D = 67 nm, or 234 residues) corresponding to the d band of collagen, and a minor site is positioned at ~1.6 D from the amino terminal end of col-

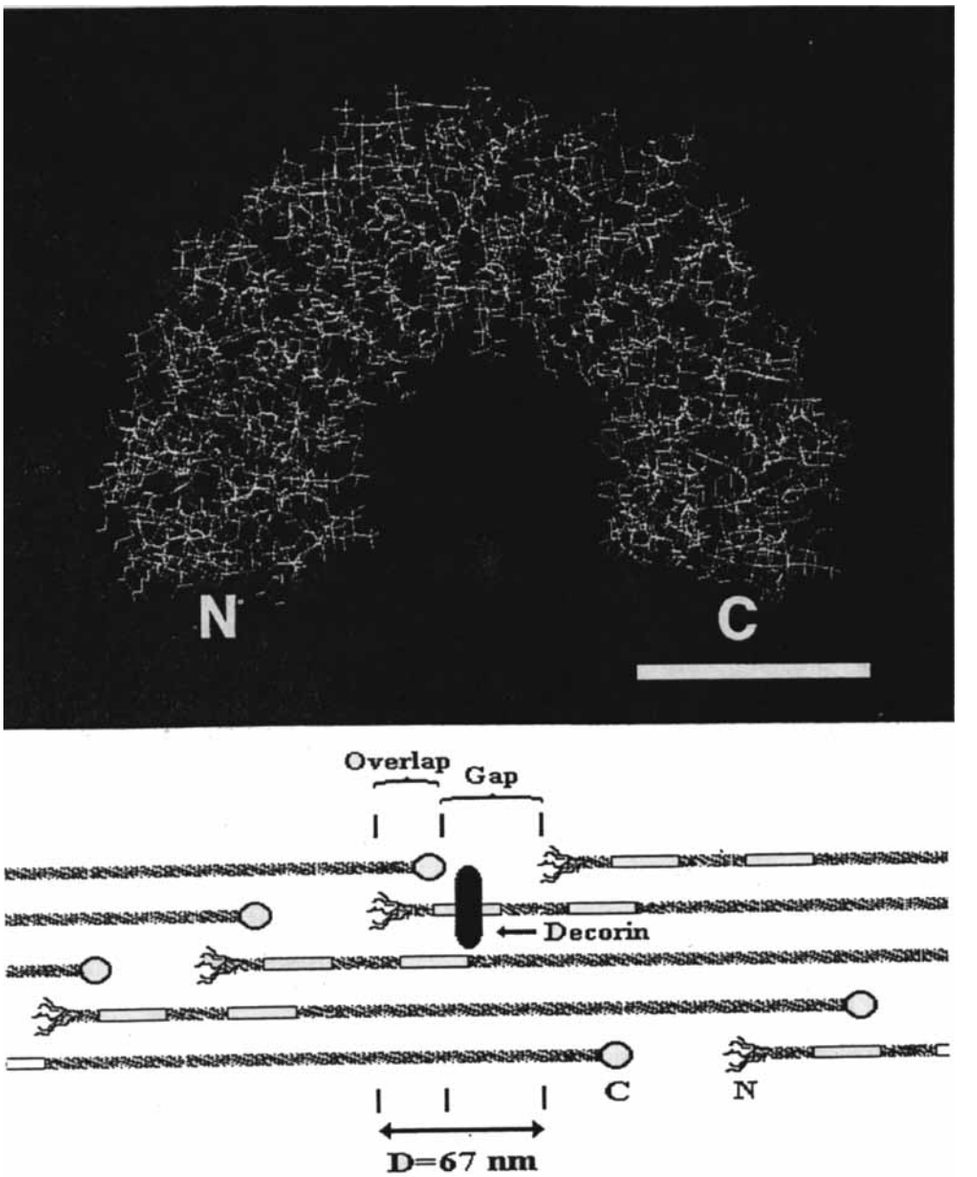


FIGURE 3. Three-dimensional model structure of human decorin (top) and schematic representation of the interaction between decorin and fibrillar collagen (bottom). The arch-shaped model of decorin was generated using the AMMP program (Harrison, 1993), as previously described (Weber et al., 1996). This is a model based on the crystal structure of ribonuclease inhibitor, and the details of the structure of decorin would likely differ, particularly in the less homologous amino and carboxyl ends of the molecule. One triple helix of collagen can fit into the decorin cavity. For clarity, the size of the decorin molecule in the bottom panel is larger (not in scale) than the individual collagen molecules. The unfilled rectangles in the collagen fibrils designate the two proposed binding sites for decorin (Yu et al., 1993). Bar in top panel, 2 nm; C, carboxyl end; N, amino end.

lagen type I (Yu et al., 1993). Close analysis of the major binding sequence revealed a stretch of 13 amino acids with charge complementarity to the inner surface of decorin. The model thus predicts binding of one collagen triple helix to the inner surface of the decorin arch, most closely to the third or fourth LRR. This is in agreement with

biochemical data utilizing recombinant decorin proteins (Spiro et al., 1994; Svensson et al., 1995; Schönherr et al., 1995). Of note, a monoclonal antibody directed against the protein core of bovine decorin recognizes a small peptide (^{146}QMIVVE^{151}) that is located in LRR5, right in the concave surface of the decorin model (Scott et al., 1993). This region is predicted to be highly hydrophobic, rigid, and inaccessible, and, thus, unfavorable for antigenicity. However, it was demonstrated that the antibody reacted with the native protein, thereby indicating that the epitope cannot be masked (Scott et al., 1993).

Although different proteins harboring the LRRs often bind unrelated ligands (Kobe and Deisenhofer, 1994), the basic principles derived from the crystallographic resolution of the complex between ribonuclease A and ribonuclease inhibitor may apply to other proteins with LRRs. Indeed, a salient feature of the decorin model is that it can provide a plausible molecular explanation for the central role of decorin, and perhaps other SLRPs, in regulating collagen fibrillogenesis. As shown in Figure 3 (bottom panel), collagen molecules aggregate in parallel into fibrils in a quarter-staggered array such that adjacent molecules (~4.5 D) overlap about three quarters of their length. This overlapping results in the characteristic banding pattern of collagen fibrils observed at the ultrastructural level in which each D-period can be further subdivided into an overlap zone of ~0.4 D and a more loosely packed gap zone of ~0.6 D (Piez, 1984). We propose that one decorin molecule encloses one triple helix of collagen in the gap region at the d band of fibrillar collagen. This model could also allow an additional interaction of decorin (perhaps via the GAG-free, carboxyl end of the molecule) with the secondary binding site. Our model differs somewhat from that of Scott (1996), who proposes decorin as a bidentate ligand attached to two parallel collagen molecules. However, both models predict a close interaction between decorin and collagen that would help stabilize fibrils and orient fibrillogenesis. The presence of decorin at these strategic locations would prevent lateral fusion of the collagen fibrils and promote correct formation of the fibrils during development.

VII. A LESSON FROM THE DECORIN KNOCKOUT MOUSE

The seminal observations that small dermatan sulfate proteoglycans modulate the energy-independent process of collagen fibrillogenesis (Toole and Lowther, 1968b; Vogel et al., 1984) via a specific association with the d band of collagen *in vivo* (Scott, 1980) stimulated us to generate animals with a targeted disruption of the decorin gene. Following the complete cloning of the murine decorin gene from a 129Sv genomic library (Scholzen et al., 1994), we were successful in breeding animals with both decorin alleles blocked by the presence of a neomycin-resistance cassette in exon II (Danielson et al., 1997). Although the animals grew to adulthood without any overt pathology, a close analysis revealed a phenotype typified by skin fragility. The syndrome caused by the lack of decorin includes an overall thinning of the skin, accumulation of loose connective tissue in the subdermal layer, and abrupt fracture and dissection of the skin between the deeper dermis and the fascia. Such skin fragility is often associated with skin laxity. Biomechanical measurements showed a significant reduction in the tensile strength of the affected, freshly isolated skin that exhibited a premature failure at ~7 N of force, while control animals failed at ~27 N. Ultrastructural analysis of dermal collagen showed aberrant organization of collagen

fibrils with abnormal packing and a great variability in average diameter (Figure 4A) when compared with age-matched wild-type controls (Figure 4B). Quantitative data showed that, although the average cross-sectional diameter of the collagen fibrils did not appreciably vary between normal and decorin –/– animals, the latter exhibited a wider range, with profiles ranging between 40 and 180 nm. Similar changes were also noted in tendon collagen. Moreover, these structural anomalies were associated with

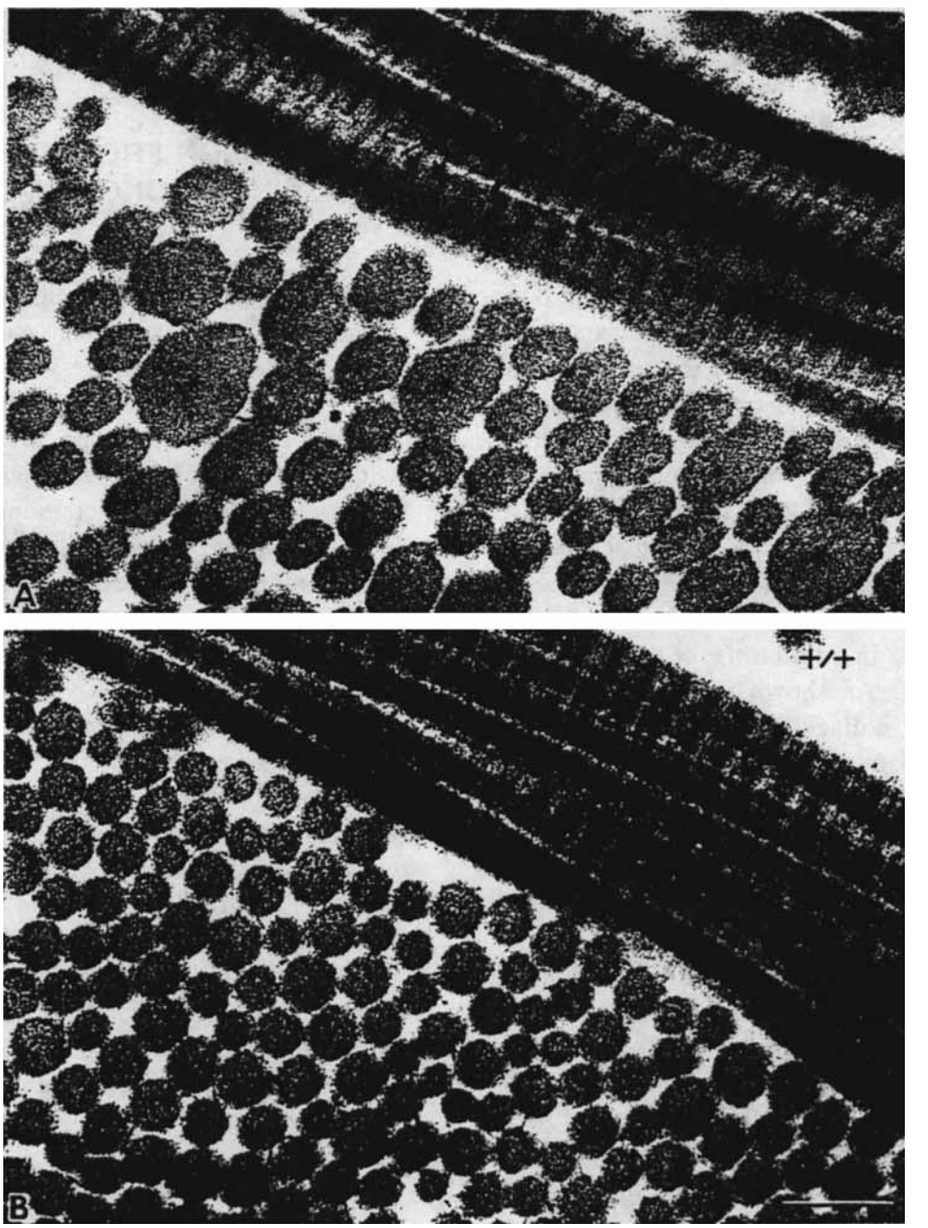


FIGURE 4. Ultrastructural analysis of skin from decorin-deficient animals reveals abnormal collagen fibrils (A) when compared with age-matched wild-type animals (B). Notice the irregular cross-sectional profiles (A, asterisks) and the increased variability in average sizes of collagen fibrils. Both pictures were taken at the same magnification. Bar, 0.2 μm.

an overall decrease in collagen-associated proteoglycans, although the typical periodicity of fibrillar collagen was preserved. The presence of variable-size, cross-sectional profiles of collagen could be interpreted as having several populations of collagen fibrils varying in overall diameter. However, scanning transmission electron microscopy revealed that the collagen fibrils isolated from the null animals exhibited a considerable nonuniformity along their axes when compared with the wild-type fibrils. In contrast to the wild-type fibrils where the most relatively uniform shafts were the larger-diameter fibrils, the fibrils from the decorin –/– animals exhibited more unevenness with increased diameter (i.e., with increased mass per unit length). These results strongly indicate that the larger and irregular fibrils observed by transmission electron microscopy (Figure 4A) are due to deregulated lateral fusion producing a fibril whose morphology changes continuously along its axial length (Danielson et al., 1997). The absence of decorin during the development of collagenous matrices would permit more extensive cross-linking between collagen fibrils, thereby generating the bizarre profiles observed in the tendons of the decorin null animals (Danielson et al., 1997). Moreover, the purported binding of collagen type XIV (Font et al., 1993) and type XII (Font et al., 1996) to the dermatan sulfate side chain of decorin, or that of type V (Whinna et al., 1993; Ramamurthy et al., 1996) and type VI (Bidanset et al., 1992; Hering et al., 1996) to the core protein of decorin, would be missing in the decorin null animals. The absence of these interactions may further contribute to abnormal collagen fiber formation. The structural changes of the decorin knockout animal provide the first genetic evidence that decorin is a key regulator of collagen fibrillogenesis: decorin prevents lateral fusion of collagen fibrils. Therefore, decorin would play a pivotal role in regulating the orderly assembly and growth of collagen fibrils, which in turn would affect the tensile strength of the skin and, perhaps, of other connective tissues. These data further predict the existence of a human skin fragility syndrome — which should be mapped to chromosome 12q23, perhaps one of the uncharacterized Ehlers-Danlos syndromes — caused by either a recessive deletion or point mutations of the collagen-binding domain of the decorin gene.

VIII. CONTROL OF CORNEAL TRANSPARENCY

The transparency of the cornea is the result of an unique architectural organization of fibrillar collagens and proteoglycans in the stroma, a highly hydrated meshwork regulated in part by overlying endothelial cells. A critical function of SLRPs is clearly demonstrated in the cornea, where the diameter of the collagen fibrils and the interfibrillary gaps must be maintained invariable to prevent corneal opacity. In this tissue, the relationships between SLRPs and collagen fibrils are consistent and quantitative (Scott, 1995). In general, the collagen fibrils have an average diameter of ~30 nm and are separated by a constant distance of ~66 nm (center to center). At least five members of the SLRP gene family have been isolated from corneal tissue. These include lumican (Blochberger et al., 1992b; Funderburgh et al., 1993), decorin (Blochberger et al., 1992a), keratocan (Corpuz et al., 1996), fibromodulin (Oldberg et al., 1989), and osteoglycin (Funderburgh et al., 1996a). These SLRPs primarily carry keratan sulfate side chains. Furthermore, PRELP was originally detected in bovine cornea by radioimmunoassay (Heinegård et al., 1986). In the adult cornea, a portion of decorin also receives keratan sulfate side chains

(Blochberger et al., 1992a). However, even though fibromodulin is also present in cornea, it does not appear to be one of the major keratan sulfate proteoglycans identified from sequencing tryptic digests of bovine cornea (Funderburgh et al., 1993). This finding suggests that fibromodulin may have a lower degree of keratan sulfate substitution than other cornea SLRPs. The recent establishment of a culture system of bovine keratocytes will be a useful tool for analyzing the molecular mechanisms that control keratan sulfate proteoglycan biosynthesis and metabolism (Funderburgh et al., 1996b)

The binding sites for keratan sulfate SLRPs in the cornea have been mapped to the a and c bands of collagen, in contrast to the dermatan sulfate SLRPs that, as discussed above, bind to the d and e bands of collagen (Scott, 1995). These locations have been confirmed at the immunolectron microscopic level in collagens from various tissue sources, including pericardium (Simionescu et al., 1989) and skin (Pringle and Dodd, 1990). Moreover, in rabbit cornea, both dermatan and keratan sulfate SLRPs associate with type VI collagen (Takahashi et al., 1993). The presence of multiple SLRPs in the transparent cornea may explain why there is no apparent abnormality in the cornea of the decorin null mice (Danielson et al., 1997) and suggests that maintenance of the interfibrillar spacing and fibrillar diameter is under multiple, and perhaps redundant, control mechanisms involving not only decorin, as in the skin, but also other members of the SLRP gene family. Indeed, lateral growth of collagen segments isolated from bovine (Rada et al., 1993) or avian (Birk et al., 1996) corneas can be efficiently inhibited by either exogenous decorin or lumican (Birk et al., 1996) extracted from their respective corneal stromas. Treatment of developing avian corneas with β-D-xyloside reduced the amount of proteoglycans (essentially dermatan sulfate but not keratan sulfate SLRPs) without altering the collagen fibril diameter (Hahn and Birk, 1992). Ergo, in the cornea, dermatan sulfate proteoglycans may not be involved in the regulation of fibril diameter but might be important in maintaining the interfibrillary spacing necessary for transparency.

At day 9 of cornea development, there is a marked induction of lumican biosynthesis to levels that are ~80-fold higher than those of decorin (Cornuet et al., 1994). This is associated with the production of a lumican species enriched in polylactosamine (unsulfated keratan sulfate). The fully sulfated form of lumican is not abundant before day 15 of development. Thus, it appears that there is a window of about a week in corneal development during which the polylactosamine form of lumican accumulates in the stroma. These findings suggest that sulfation of polylactosamine may play an active role in the acquisition of corneal transparency (Cornuet et al., 1994). This interpretation is supported by the finding that in macular corneal dystrophy, an inherited human disorder characterized by stromal opacities, the polylactosamine species of lumican is primarily expressed (Nakazawa et al., 1984). Hence, the persistence of an "embryonic" form of lumican could impair the vision in such patients. A recent study using synchrotron X-ray diffraction has shown a unique 4.6-Å periodicity in corneas from patients with macular cornea dystrophy (Quantock et al., 1996). This reflection was diminished by treatment with either chondroitinase or N-glycanase, suggesting that the 4.6-Å periodicity in this disease resides not exclusively in unsulfated lumican molecules, but also in dermatan-sulfated SLRPs or in a region of hybrid molecular aggregates containing various SLRPs.

Interestingly, a recent study investigating the interaction between corneal keratan

sulfate-proteoglycans and macrophages has demonstrated that removal of the sulfated side chains promotes macrophage adhesion (Funderburgh et al., 1997). Thus, the low-sulfate lumican known to be present in various pathological processes may function to target macrophages in regions of corneal inflammation.

IX. CONTROL OF CELL GROWTH

The control of cell division is regulated by a finely balanced mixture of signals that are in part derived from the specific extracellular matrix context (Wight et al., 1992; Iozzo, 1988). When vascular endothelial cells, which do not constitutively synthesize type I collagen or decorin (Järveläinen et al., 1991), initiate the formation of tubes and cords ("sprouting"), they begin the synthesis of both collagen type I and decorin (Järveläinen et al., 1992). Thus, one member of the SLRP gene family is directly implicated in the transition of endothelial cells, from a polygonal monolayer to a sprouting phenotype. In contrast, when endothelial cells are wounded *in vitro*, there is an increased expression of biglycan at the surface front of the migrating endothelial cells and this process is apparently mediated by release of endogenous basic fibroblast growth factor (Kinsella et al., 1997). Hence, decorin and biglycan may serve diverse functions when cells are migrating to form new blood vessels or to cover a denuded blood vessel wall.

Decorin, when present on the substrate with vitronectin, is also capable of affecting the remodeling of the extracellular matrix via induction of the matrix metalloproteinase collagenase (MMP-1) (Huttenlocher et al., 1995). This decorin-mediated induction of MMP-1 activity is also observed when the 120-kDa cell-binding domain of fibronectin is present but not when either intact fibronectin or type I collagen is present (Huttenlocher et al., 1995). Because both vitronectin and decorin are seen in areas of active tissue remodeling (wounds, atherosclerotic lesions, and tumors), these molecules could play a biological role at these sites. The interaction of decorin and biglycan with mediators of inflammation such as the C1q subcomponent of the C1 complex (Krumdieck et al., 1992; Hocking et al., 1996) and heparin cofactor II (Whinna et al., 1993), and the affinity of SLRPs for a variety of extracellular matrix proteins involved in tissue remodeling, such as collagen type III (Thieszen and Rosenquist, 1994), collagen type V (Whinna et al., 1993; Ramamurthy et al., 1996), collagen type VI (Bidanset et al., 1992), fibronectin (Schmidt et al., 1991), and thrombospondin (Winnemöller et al., 1992), strongly suggest that these molecules would play a role in such fundamental repair processes and consequently would affect the proliferation and growth of connective tissue cells.

When carcinomas, the most common forms of human tumors, expand at their primary organ sites, two major events occur: (1) disruption of the natural barriers, such as basement membranes and immunological surveillance, and (2) establishment of a tumor stroma that includes the development of an adequate blood supply (angiogenesis) and the synthesis of a tumor matrix that favors the growth of malignant cells. The active interplay between actively dividing cells and the host stromal elements leads to the formation of a growth factor-enriched milieu, a highly hydrated and unique tissue, the so-called tumor stroma, that is enriched in sulfated proteoglycans (Iozzo, 1988). Members of the SLRP gene family are, in general, markedly upregulated (Iozzo, 1995; Hunzelmann et al., 1995) and have thereby been implicated in the control of cell pro-

liferation either indirectly, by blocking or interacting with growth factors and cytokines (Ruoslahti and Yamaguchi, 1991), or directly, by acting on the cell cycle machinery (Iozzo and Murdoch, 1996). Below, I discuss a few examples of how members of the SLRP gene family may be directly involved in affecting the cellular growth of normal and malignant cells in a variety of contexts.

A. Interaction with TGF-β

Interactions between members of the SLRPs gene family and TGF-β is intricate, given the fact that they not only bind to but are also differentially regulated by the growth factor (Iozzo and Murdoch, 1996). A great deal of notoriety for decorin derives from the observation that *de novo* expression in CHO cells, which do not constitutively express this SLRP, leads to growth suppression (Yamaguchi and Ruoslahti, 1988). The decorin-expressing clones became quiescent at a low saturation density and occupied nearly three times the surface areas as wild-type cells, a phenotypic change that was proportional to the amount of synthesized decorin (Yamaguchi and Ruoslahti, 1988). Subsequently, it was shown that this growth inhibition was modulated by a specific binding of decorin, and also of biglycan, to TGF-β, thereby inactivating it (Yamaguchi et al., 1990). Because CHO cells require TGF-β for their growth, it was concluded that decorin-induced growth suppression and morphologic changes were secondary to the inhibition of this potent growth factor (Yamaguchi et al., 1990). Indeed, it appears that at least three members of the SLRP gene family, including decorin, biglycan, and fibromodulin, bind TGF-β, albeit with distinct affinities (Hildebrand et al., 1994). It should be pointed out, however, that these studies were carried out with recombinant prokaryotic proteins that may not be properly folded. Indeed, eukaryotically expressed biglycan (Hocking et al., 1996) and decorin (Ramamurthy et al., 1996) proteoglycans show extensive secondary structures, and disruption of their native configuration has profound and selective consequences on their affinities for other proteins. However, the evidence for a high-affinity interaction of decorin with TGF-β is overwhelming. Of note, the same SLRPs that bind TGF-β are also involved in binding collagen fibrils and regulating fibrillogenesis (Iozzo and Murdoch, 1996). These SLRPs, linked to either collagenous or cellular surfaces, could function as effector molecules in modulating growth factor activities. The reversibility of this interaction could provide additional flexibility for the utilization of the complexes and for the generation of a growth factor-enriched milieu.

Augmented TGF-β production appears to be a characteristic feature of a variety of fibrotic diseases, including cirrhosis, pulmonary fibrosis, and glomerular sclerosis. The parenteral administration of recombinant decorin or decorin purified from bovine tissues purportedly prevents glomerular sclerosis in a rat model of glomerulonephritis based on the intravenous injection of antithymocyte antiserum (Border et al., 1992). Because humans are not injected with heterologous sera, the results of the studies utilizing this animal model should be taken with caution and should not be extrapolated to the human situation. Nonetheless, a recent report by the same group of investigators has shown amelioration of glomerular sclerosis in rats transfected with a decorin-expressing vector directly into skeletal muscle (Isaka et al., 1996). This study, however, did not identify the mechanism of decorin's therapeu-

tic action and thus precludes any meaningful interpretation of the results. Utilization of the decorin-deficient animals should shed some light on the role decorin may play in regulating fibrogenesis.

Despite the aforementioned evidence implicating decorin in blocking the action of TGF-β, mounting documentation suggests a "lack" of inhibitory activity. For example, in quiescent fibroblasts that produce 20 to 40 times more decorin than cells in the logarithmic phase of growth, nanogram amounts of recombinant TGF-β are still capable of upregulating biglycan transcription (Mauviel et al., 1995). Intuitively, the large amount of native decorin should have blocked the cytokine. However, it is possible that fibrillar collagens may have rapidly sequestered decorin, thereby preventing further interaction with TGF-β. Furthermore, in the presence of a 10,000-fold molar excess of exogenous decorin over TGF-β, the cytokine effects on the human monocytic cell line U937 remained essentially unchanged (Kresse et al., 1994). Also, in MG-63 human osteosarcoma cells, the effects of a 500-fold molar excess of decorin only partially abolished TGF-β activities (Kresse et al., 1994). Osteoblastic cells exhibit high-affinity TGF-β-binding sites, with a K_d of ~0.3 and 5 n*M*; surprisingly, the addition of decorin enhanced the binding of iodinated TGF-β to its receptor(s) (Takeuchi et al., 1994). Thus, it appears that for certain cells, the binding of decorin to TGF-β augments the bioactivity of the cytokine rather than blocking it. These findings suggest that decorin/TGF-β complexes may still be capable of interacting with at least one signaling pathway, and in certain conditions, these complexes may activate rather than repress the cytokine activity. Additional studies are needed to address these conflicting reports, although the central role of SLRPs in regulating TGF-β activity is a fact.

B. Decorin as a Growth-Suppressor Protein: Induction of p21, a Universal Inhibitor of Cyclin-Dependent Kinases

In addition to the growth factor-modulating abilities of decorin described above, there is mounting evidence that this member of the SLRP gene family is directly implicated in the control of cell proliferation. Using cross-hybridization protocols, decorin was identified as one of eight genes, named quiescins, whose expression was specifically upregulated >tenfold in quiescent human lung fibroblasts when compared with cells in the logarithmic phase of growth (Coppock et al., 1993). The expression of decorin was markedly suppressed following transformation with SV40, and this inhibition was even greater than that observed in rapidly proliferating cells (Coppock et al., 1993). We were able to reproduce the same results in primary cultures of human skin fibroblasts, and further demonstrated that quiescence-dependent induction of decorin gene expression is transcriptionally regulated and long lasting (Mauviel et al., 1995). Elevation of decorin mRNA steady-state levels was maintained for up to 16 d postconfluence, and both transient cell transfection and *in vitro* transcription assays revealed a marked transcriptional enhancement of the decorin gene that correlated with growth arrest. In addition, induction of decorin promoter-CAT constructs could also be reproduced in HeLa cells, a carcinoma cell line, following serum starvation (Mauviel et al., 1995). That this regulation crosses cell types and species is demonstrated by the fact that in rat vascular smooth muscle cells there is also a 6- to 7-fold increase in decorin mRNA levels in density-arrested postconfluent cultures (Asundi and Dreher, 1992). These results are in sharp contrast to biglycan, whose transcript levels do not appear to vary as a function of cell

density in either human fibroblasts (Mauviel et al., 1995) or rat smooth muscle cells (Dreher et al., 1990).

Decorin is rarely expressed by malignant epithelial cells from a wide variety of human tumors, including colon, pancreas, prostate, and breast carcinomas (Iozzo and Cohen, 1993). However, in the tumor stroma of colon cancer, both the amount of chondroitin sulfate (Iozzo et al., 1982) and decorin proteoglycan (Adany et al., 1990) are markedly induced through a process that involves hypomethylation of the decorin gene (Adany and Iozzo, 1991) as well as induction of this gene product via tumor-secreted cytokines (Iozzo, 1985). Collectively, these observations stimulated us to address experimentally the question as to whether decorin influences cellular growth directly, via a TGF-β-independent pathway. To test this hypothesis, we transfected a CMV-driven, decorin-expressing vector into colon carcinoma cells. These cells are unique because they do not constitutively synthesize decorin, are unresponsive to TGF-β, and harbor a mutated p53 gene, thereby eliminating this growth factor and tumor suppressor gene, respectively, in modulating growth changes. The decorin-synthesizing colon carcinoma cells exhibited a marked reduction of their growth capacity in the absence or presence of TGF-β (Santra et al., 1995; De Luca et al., 1996). Several stably transfected clones were arrested in G_1 and could reenter the cell cycle after abrogation of decorin expression by antisense oligodeoxynucleotide treatment (Santra et al., 1995). We extended these original observations and discovered a link between *de novo* decorin synthesis and inhibition of the cell cycle machinery. Specifically, we discovered that the decorin-induced growth arrest was linked to a marked upregulation of p21 (De Luca et al., 1996). We further demonstrated that the decorin-expressing cells contained p21 within their nuclei and that p21 was in a multimeric complex with cyclin and cyclin-dependent kinases (CDK). Indeed, p21 is a potent inhibitor of CDK (Harper et al., 1993), which can be transcriptionally induced by p53 (El-Deiry et al., 1993) and whose expression is elevated in senescent fibroblasts (Noda et al., 1994). Of note, p21 mRNA levels increase severalfold in fibroblasts rendered quiescent by serum deprivation or in non-dividing, contact-inhibited cells (Noda et al., 1994). The concurrent upregulation of p21 and decorin when cells attain growth arrest status and the findings in transfected colon carcinoma cells suggest that decorin may be a mediator molecule of a negative feedback loop that "directly" affects the cell cycle machinery via either an autocrine pathway (in fibroblasts and perhaps smooth muscle cells) or a paracrine pathway (in epithelial/mesenchymal cell interactions). Recently, we found that *de novo* expression of decorin in several mammalian tumor cell lines of various histogenetic origins leads to comparable growth suppression and that p21 is a universal downstream target for decorin (unpublished observations). Thus, we conclude that decorin is a potent growth suppressor acting extracellularly, because in all transfected cells decorin is rapidly and efficiently released into the extracellular space as a fully glycosylated chondroitin/dermatan sulfate proteoglycan. The elevated concentrations of this gene product around the invasive carcinoma cells may thus represent a form of defensive paracrine mechanism devised by the host stromal cells to offset the growth of malignant cells at the invasive front of solid tumors. Future studies are needed to determine the signal transducing pathway(s) through which decorin and perhaps other SLRPs could modulate cellular growth.

C. Biglycan and the Stimulation of Hemopoiesis

In addition to various interleukins and granulocyte-macrophage colony stimulating factor (GM-CSF), hemopoiesis is controlled by the local microenvironment composed primarily of stromal cells and their products. For instance, the local milieu is accountable for inducing tissue-specific monocytic lineage cells such as Kupffer cells of the liver sinusoid, microglial cells in the brain, or monocytic cells in the thymus. In this regard, a factor with potent monocytic stimulatory activity isolated from thymic myoid cells was identified as biglycan (Kamo et al., 1993). Biglycan induced formation of aggregated colonies from nonadherent thymic and bone marrow cells cultured in semisolid media, and, similar to GM-CSF, colony-forming cells induced by biglycan were predominantly phagocytic and carried macrophage markers (Kamo et al., 1993). About 90% of this active biglycan species was present as mature proteoglycan (i.e., without the signal and propeptide regions), whereas in the remaining 10%, a propeptide of 14 amino acid residues was identified (Kamo et al., 1993). A recent investigation employing a new cloning strategy designed to recognize molecules that interact with precursors of B lymphocytes found that biglycan was one of eight interactive products (Oritani and Kincade, 1996). Of note, the 91-residue amino terminal portion of biglycan was incapable of stimulating the growth of myeloid cells, whereas it markedly enhanced the cloning of IL-7-responsive precursors in semisolid agar (Oritani and Kincade, 1996). The availability of biglycan at the surface of endothelial cells may play a role in hemopoiesis. Moreover, the homology of biglycan with platelet glycoprotein Ib (Fisher et al., 1989) may indicate potential interaction with matrix glycoproteins such as von Willebrand factor (Couchman and Woods, 1993). Further studies are needed to unravel the functional roles of biglycan *in vivo* and, specifically, to establish whether biglycan is directly involved in presenting growth factors to competent blood elements in the bone marrow microenvironment.

X. CONCLUSIONS AND PROSPECTS

The versatility of the decorin-binding activity suggests that this prototype proteoglycan and, perhaps, molecules with comparable structural architecture may function as modulators of fundamental biological processes. Several conceptual advances, however, need to be achieved. First, the identification of specific receptors for SLRPs should explain the molecular mechanism of SLRP-mediated signal transduction. One objective of future studies should be to place SLRPs in a broader context involving gene regulation, cytokine induction, and cell signaling events that underscore vital biological processes. Second, the design of peptides that closely mimic the structure of the interactive protein sites within the SLRPs would further our understanding of the biology of these proteoglycans and should explain the diversity of functions *vis á vis* their protein differences. Third, site-directed mutagenesis of SLRP protein cores should establish which specific subdomain(s) of the SLRP protein core modulate functions. Fourth, it should now be feasible to assess function by targeted disruption of each of the SLRP genes in animals. This could aid in the discovery of human genetic diseases linked to a specific SLRP, such as in the case of the decorin-null animals, which may foretell a similar pathogenetic mechanism

for a human skin fragility syndrome. The field is ripe for major discoveries.

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