Non-proteoglycan forms of biglycan increase with age in human articular cartilage

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Polyclonal anti-peptide antibodies were raised to the C-terminal regions of human biglycan and decorin. These antibodies were used in immunoblotting to study structural variations with age in the proteoglycan core proteins present in extracts of human articular cartilage and intervertebral disc. Three forms of the biglycan core protein were identified. The largest form was detected only after chondroitinase treatment and represents the proteoglycan form of the molecule from which the glycosaminoglycan chains have been removed. However, chondroitinase treatment did not alter the electrophoretic mobility of the two smaller proteins, which appear to represent non-proteoglycan forms of the molecule, resulting either from a failure to substitute the intact proteoglycan core protein with glycosaminoglycan chains during its synthesis or from proteolytic processing of the intact proteoglycan causing removal of the N-terminal region bearing the glycosaminoglycan chains. The non-proteoglycan forms constituted a minor proportion of biglycan in the newborn, but were the major components in the adult. A similar trend was seen in both articular cartilage and intervertebral disc. In comparison, decorin appears to exist predominantly as a proteoglycan at all ages, with two core protein sizes being present after chondroitinase treatment. Non-proteoglycan forms were detected in the adult, but they were always a minor constituent.

INTRODUCTION

Articular cartilage contains two dermatan sulphate proteoglycans, biglycan and decorin, which bear the glycosaminoglycan chains in their N-terminal regions [1,2]. The major structural difference between the two molecules is the presence of two sites for dermatan sulphate attachment in biglycan, but only one site in decorin [3]. In other respects the core proteins of biglycan and decorin share considerable structural similarity, as they are of similar length and both possess small C- and N-terminal disulphide-bonded loops separated by a region of leucine-rich repeats [4,5]. The structure of the dermatan sulphate chains appears to be similar at a given site, although there is considerable variation with age and between different connective tissues [6]. In addition, the relative abundance of the two proteoglycans varies with both age and tissue type [7,8].

Biglycan and decorin also differ in their functional properties. Decorin is able to interact with the fibril-forming collagens and influence the process of fibrilogenesis [9]; an interaction which appears to involve the decorin core protein rather than its dermatan sulphate chains [10]. Decorin remains bound at the surface of mature collagen fibrils, and has been postulated to play a role in fibril interaction. Such a role would be compatible with the self-association properties of dermatan sulphate [11]. In contrast, biglycan does not show a similar type of interaction, and at present its functional role is unclear. Immunolocalization has revealed that biglycan is preferentially located in the pericellular matrix [12], a site rich in type VI collagen [13]. It is therefore possible that biglycan is also a collagen-binding molecule, but that its preference for collagen type is distinct from that of decorin. The extracellular difference in decorin and biglycan localization also extends to the genome, with the human biglycan gene residing on the X chromosome whereas the decorin gene is on chromosome 12 [14]. The two genes respond differently to transforming growth factor-β, with decorin expression being enhanced and that of biglycan suppressed [15,16]; this would again support distinct functional roles for the two molecules.

In human articular cartilage extracts, immunoassay of core protein epitopes has revealed that the proportion of decorin relative to biglycan increases with age [17]. This appears to be due mainly to variations in decorin levels, as biglycan epitopes show little change. However, in purified proteoglycan preparations, decorin and biglycan appear to be of similar abundance in the newborn, whereas biglycan is difficult to detect in the adult [18]. One explanation for this apparent discrepancy is that biglycan might exist in a glycosaminoglycan-free form in the adult, such that it is amenable to detection by immunoassay but not to preparative techniques that depend on the properties of a sulphated glycosaminoglycan chain. Recently it has been reported that non-proteoglycan forms of decorin and biglycan do exist in adult human cartilage [19]. The purpose of the present work was to determine whether the abundance of such molecules increases with age.

MATERIALS AND METHODS

Preparation of tissue extracts

Both intervertebral discs from the lumbar spine and articular cartilage from the femoral condyles of the same individuals were obtained at autopsy within 20 h *post mortem*. Only tissue judged to be macroscopically normal was taken. Bovine articular cartilage was obtained at a local slaughter house. The tissue was finely diced, and in the case of human specimens was then cut into 20 μm sections using a cryostat to achieve maximal extraction of proteoglycan [20]. Samples were extracted with 10 vol. of 4 M guanidinium chloride, 100 mM sodium acetate,

Abbreviations used: Fmoc, 9-fluorenylmethoxycarbonyl-; TBST (Tris-Buffered Saline and Tween), 10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20.

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Decorin															
Peptide			C	G	Y	V	R	S	A	I	Q	L	G	N	Y	K	
Human...	F	R	C	V	Y	V	R	S	A	I	Q	L	G	N	Y	K	
Bovine...	F	R	C	V	Y	V	R	A	A	V	Q	L	G	N	Y	K	
Biglycan																	
Peptide			C	G	T	D	R	L	A	I	Q	F	G	N	Y	K	K
Human...	F	R	C	V	T	D	R	L	A	I	Q	F	G	N	Y	K	K
Bovine...	F	R	C	V	T	D	R	L	A	I	Q	F	G	N	Y	Y	

Figure 1 Sequences of the synthetic peptides used for immunization and of the C-terminal regions of human and bovine decorin and biglycan

pH 6.0, containing proteinase inhibitors [21], and then dialysed into the appropriate buffer.

Anti-peptide antibodies

Peptides (Figure 1) were synthesized at a 0.25 mmol scale, using standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry on an Applied Biosystems model 431 A solid-phase peptide synthesizer. Crude peptides were purified by reverse-phase chromatography (Prep-10 Aquapore C8 column; Applied Biosystems) using an acetonitrile gradient in 0.1% trifluoroacetic acid. Peptides contained a penultimate spacer amino acid, glycine, preceded by an N-terminal cysteine residue that was used as the coupling site for preparation of peptide–protein conjugates. The bifunctional reagent, *N*-hydroxysuccinimidyl bromoacetate, was synthesized as described by Bernatowicz and Matsueda [22]. Coupling to ovalbumin using this reagent was carried out as described previously [23], and the success of the coupling reaction was determined by observation of a decrease in electrophoretic mobility of the peptide–ovalbumin conjugate on SDS/PAGE analysis, relative to a cysteine–ovalbumin conjugate prepared in the same manner.

Female rabbits were immunized with the peptide conjugate homogenized in Freund's complete adjuvant. Subsequent boosts after 14, 28, 42 and 98 days were in incomplete adjuvant. The animals were bled out 20 days after the last boost. All injections contained 500 μg of conjugate in 250 μl of a 1:1 mixture with adjuvant and were by the intramuscular route.

SDS/PAGE and immunoblotting

Cartilage and disc samples (40 μl) were analysed on 10% polyacrylamide slab gels (5 cm × 5 cm × 1 mm) in SDS, using procedures described by Laemmli [24]. After electrophoresis, the fractionated proteins were electrophoretically transferred to nitrocellulose membranes [25]. Electroblotting was performed in 20% (v/v) methanol/25 mM Tris/190 mM glycine, pH 8.3, at 100 V for 4 h in a Bio-Rad Mini Trans-Blot apparatus. Control experiments demonstrated that the various proteoglycan components under study were transferred to the nitrocellulose membranes in a quantitative manner under these conditions. The transfer sheet was incubated in blocking solution [3% (w/v) BSA in 10 mM sodium potassium phosphate, pH 7.2, 0.145 M NaCl, 0.05% sodium azide] overnight, then incubated with a 1:50 dilution of anti-decorin or anti-biglycan serum in TBST (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 30 min at room temperature. After three washes with TBST buffer, the nitrocellulose sheets were incubated for 30 min at room temperature with a 1:7500 dilution of an alkaline phosphatase-conjugated goat anti-rabbit second antibody (Pro-Mega) in TBST buffer. The nitrocellulose was thoroughly washed in TBST buffer and then in one change of alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris/HCl, pH 9.5) before addition of freshly prepared alkaline phosphatase substrate solution [66 μl of Nitro Blue Tetrazolium (50 mg/ml in 70% dimethylformamide) and 33 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethylformamide) in 10 ml of alkaline phosphatase buffer]. In general, immunoblots were incubated for 10–30 min at room temperature under subdued light to achieve optimum colour development.

Chondroitinase ABC and N-Glycanase F digestions

Cartilage or disc extracts were dialysed into 0.1 M Tris/HCl, pH 7.3, 0.1 M NaCl, and then incubated with 0.1 unit/ml chondroitinase ABC (Sigma) for 4 h at 40 °C before SDS/PAGE immunoblot analysis. Control experiments demonstrated that this concentration of chondroitinase is in excess of that required to convert all biglycan and decorin to their limit degradation products. For digestion with N-Glycanase F, samples were dialysed into 0.1 M Tris/HCl, pH 6.8, containing 0.1% SDS, then incubated in a boiling water bath for 3 min. An equal volume of 0.125 M Tris/HCl, pH 6.8, containing 0.5% Nonidet P40, 0.1% SDS and 10 units/ml N-Glycanase F (Boehringer Mannheim) was added and the mixture was incubated at 37 °C overnight.

Specificity studies

Blots of chondroitinase-digested human and bovine articular cartilage extracts were probed as above, except in some cases antisera were preincubated with the synthetic peptides used for immunization (1.5 mg/ml of serum) for 24 h before use for immunoblot development.

RESULTS

In order to study biglycan and decorin core protein heterogeneity, whole-tissue extracts were examined by SDS/PAGE followed by immunoblotting. Polyclonal antisera were used for detection of the core proteins, and in both cases these were raised to peptides based on the core protein sequence following the C-terminal disulphide-bonded loop. As no prior proteoglycan preparation was involved, this procedure is independent of the presence of glycosaminoglycan chains, and will detect any core protein synthesized without dermatan sulphate or any free core protein derived from a proteoglycan by proteolysis, providing that the C-terminal epitope is present. Under these circumstances three core protein species were detected for biglycan, following chondroitinase ABC treatment in adult cartilage extracts (Figure 2a). Only the largest of these had a mobility that was dependent upon chondroitinase treatment and therefore represented a proteoglycan with previously attached chondroitin sulphate or dermatan sulphate chains. This component migrated at the same position as the reference molecular mass standard ovalbumin, as reported previously for purified biglycan [2]. Under the conditions used the intact proteoglycan was not detectable by immunoblotting prior to chondroitinase treatment. The other two components represent non-proteoglycan forms of biglycan lacking chondroitin sulphate chains, the mobility of which cannot be altered by chondroitinase treatment. Chondroitinase ABC treatment revealed two core proteins for decorin which were not

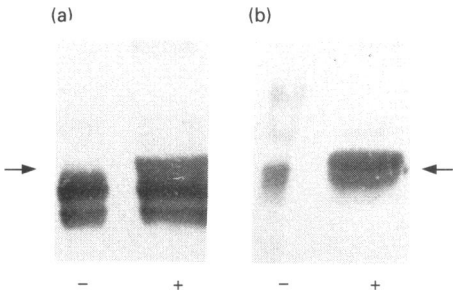

Figure 2 Identification of decorin and biglycan core proteins in extracts of adult human articular cartilage

Proteoglycan core proteins were analysed by SDS/PAGE and immunoblotting using antisera specific for biglycan (**a**) or decorin (**b**). Analysis was performed with (+) or without (−) chondroitinase ABC pretreatment. Arrows indicate the migration position of ovalbumin.

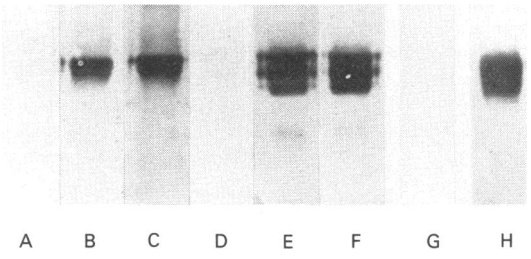

Figure 3 Specificity of anti-decorin and anti-biglycan antisera

Guanidinium chloride extracts of either adult (77-year-old) human (lanes A–F) or bovine (lanes G and H) articular cartilage were digested with chondroitinase ABC and subjected to SDS/PAGE and electroblotting. The blots were then probed with: lane A, anti-decorin preincubated with the immunizing peptide; (B), anti-decorin preincubated with the biglycan peptide; C, anti-decorin; D, anti-biglycan preincubated with the immunizing peptide; E, anti-biglycan preincubated with the decorin peptide; F, anti-biglycan; G, anti-decorin; H, anti-biglycan.

apparent before treatment, and must therefore represent proteoglycan forms of the molecule (Figure 2b). As reported previously for purified decorin, the reference molecular mass standard ovalbumin bisected these components in its migration position. In the absence of chondroitinase treatment, minor amounts of free core proteins were evident, indicating that decorin may also exist as non-proteoglycan forms.

One concern with such analysis is the specificity of the antisera used for detection. In this case specificity was demonstrated by preincubating the antisera with the peptides against which they were raised (Figure 3). When the biglycan peptide was used, binding of the anti-biglycan antiserum was prevented but interaction of the anti-decorin antiserum was unchanged. The reverse was true for the decorin peptide. Thus there is no cross-reactivity of the anti-biglycan antiserum with decorin or of the anti-decorin antiserum with biglycan. The C-terminal peptides of decorin and biglycan do show some similarity, having identical amino acids at 10 out of 15 sites [4], yet the antibodies produced are specific. It is also of interest that bovine biglycan differs from human biglycan only in its C-terminal amino acid [5], and that the anti-biglycan antiserum shows species cross-reactivity, whereas there is no species cross-reactivity for the anti-decorin antiserum in the case of bovine decorin, where two internal amino acids are distinct [4,26] (Figure 1 and 3).

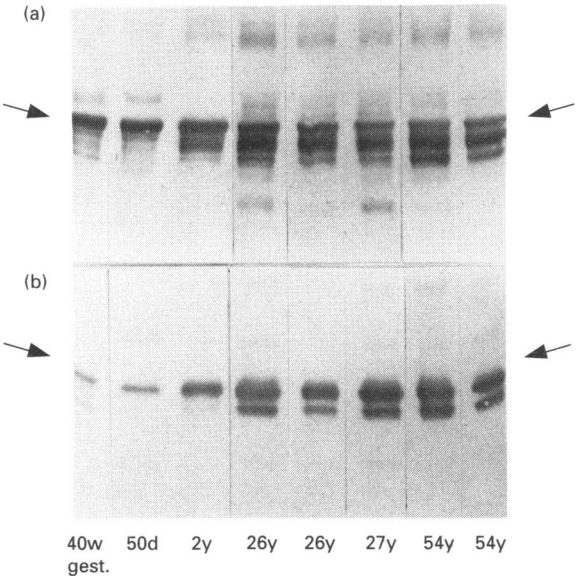

Figure 4 Age-related changes in biglycan in human articular cartilage

Equal volumes of cartilage extracts from a fetus of 40 weeks' gestation (40w gest.) and from individuals of age 50 days (50d) and 2–54 years as indicated (y = year) were analysed with (**a**) or without (**b**) chondroitinase ABC pretreatment. Arrows indicate the migration position of ovalbumin.

Variation in the non-proteoglycan forms of biglycan with age in human articular cartilage was tested by analysis of extracts from individuals ranging in age from newborn to mature adult (Figure 4). Chondroitinase treatment of the samples indicated that, while the non-proteoglycan forms of bigylcan were abundant in the adult, they were low in the newborn. Indeed, in the adult the majority of biglycan exists in a non-proteoglycan form, whereas in the newborn the proteoglycan form predominates. The proteoglycan form of biglycan appears to be present at a similar abundance throughout life, whereas the non-proteoglycan form increases in abundance during juvenile development. This change in abundance of the non-proteoglycan form is also coupled with a change in structure, as the young juvenile specimens exhibited a single non-proteoglycan core protein, whereas the adult also had an additional smaller component. Thus the generation of non-proteoglycan forms of biglycan is age-dependent in both its degree and heterogeneity.

It was of interest to determine whether other connective tissues exhibited a similar phenomenon or whether this property of biglycan was selective to cartilage. To this end, extracts of intervertebral disc were examined in a similar manner. Analysis of nucleus pulposus samples showed a similar trend to that described above for the autologous articular cartilage (Figure 5), with core protein components of identical mobility being detected. A similar trend was also observed in the annulus fibrosus (results not shown). As with articular cartilage, the two non-proteoglycan core proteins predominated in the adult specimens, whereas in the youngest specimens a single non-proteoglycan form was present as a minor component. The only observable differences appeared to be in the degree of the non-proteoglycan forms observed in the adult. The discs from young adults exhibited a greater proportion of the non-proteoglycan core protein than did autologous articular cartilage samples, and in

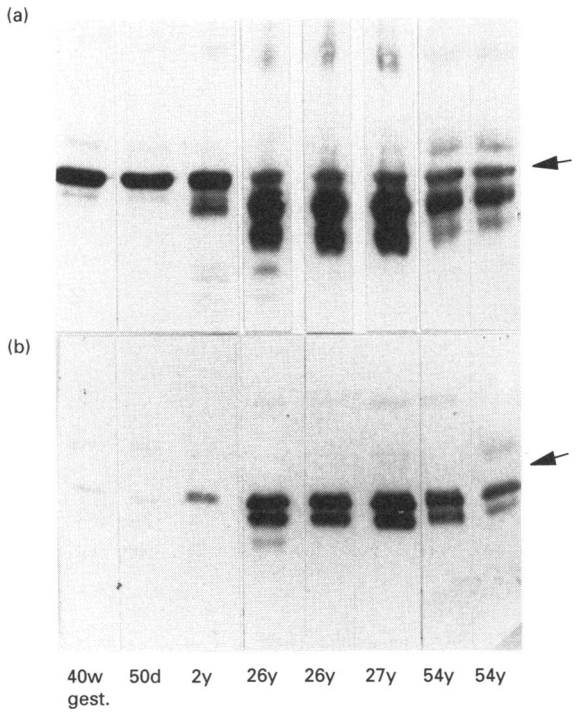

Figure 5 Age-related changes in biglycan in the nucleus pulposus of human intervertebral discs

Equal volumes of tissue extracts from a fetus of 40 weeks' gestatation (40w gest.) and individuals of age 50 days (50d) and 2–54 years as indicated (y = years) were analysed with (**a**) or without (**b**) chondroitinase ABC pretreatment. Arrows indicate the migration position of ovalbumin.

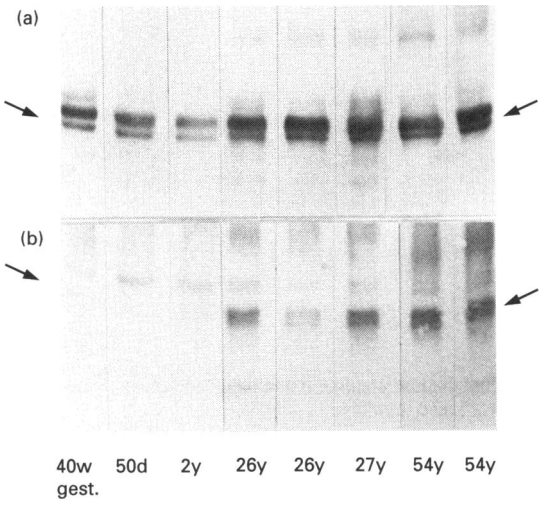

Figure 6 Age-related changes in decorin in human articular cartilage

Equal volumes of cartilage extracts from a fetus of 40 weeks' gestation (40w gest.) and from individuals of ages 50 days (50d) and 2–54 years as indicated (y = years) were analysed with (**a**) or without (**b**) chondroitinase ABC pretreatment. Arrows indicate the migration position of ovalbumin.

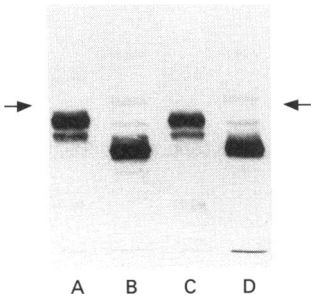

Figure 7 Effect of N-Glycanase F treatment on intervertebral disc and cartilage biglycan

Extracts of adult intervertebral disc annulus fibrosus (lanes A and B) and articular cartilage (lanes C and D) were analysed before (lanes A and C) and after (lanes B and D) N-Glycanase F treatment. Arrows indicate the migration position of ovalbumin.

the more mature adults there was a decrease in the abundance of all core proteins. Thus the generation of non-proteoglycan forms of biglycan with age probably occurs in a similar manner in different connective tissues, though the relative abundance of the non-proteoglycan forms can vary.

In contrast to the analysis with the anti-biglycan antiserum, use of the anti-decorin antiserum did not show a marked age-related trend. At all ages the articular cartilage extracts exhibited two predominant decorin core proteins following chondroitinase ABC treatment, the mobility of which was constant in samples from all ages (Figure 6). Both core proteins represented proteoglycan forms of decorin, as their mobility decreased without enzyme digestion. In the absence of chondroitinase treatment, no non-proteoglycan forms of decorin were observed in the young specimens, but two components of similar electrophoretic mobilities to the proteoglycan core proteins were observed in all adult specimens. These non-proteoglycan forms of decorin were, however, minor components relative to the proteoglycan forms at all ages. The similar electrophoretic mobilities and low abundances of these non-proteoglycan forms rendered them indistinguishable from the proteoglycan forms in chondroitinase-treated samples. Thus, unlike biglycan, decorin appears to exist in a predominant proteoglycan form throughout life. This observation is not unique to articular cartilage, as a similar decorin core protein pattern was observed in autologous samples of nucleus pulposus and annulus fibrosus from the intervertebral disc (results not shown).

The existence of two non-proteoglycan forms of biglycan could be due to different oligosaccharide glycosylation patterns or to proteolytic cleavage giving different core protein sizes. To distinguish between these possibilities, the cartilage extracts were subjected to treatment with N-Glycanase prior to electrophoresis and immunoblotting analysis (Figure 7). Under these conditions the two non-proteoglycan biglycan proteins in an adult extract migrated as a single component of increased mobility. This would suggest that the two non-proteoglycan forms differ principally in their degree or type of N-linked oligosaccharide substitution rather than being derived from the proteoglycan form by cleavage at different proteolytic sites.

DISCUSSION

In human articular cartilage and intervertebral disc, both decorin and biglycan may exist in non-proteoglycan forms, and in both cases such forms increase in their abundance in the adult relative

to the juvenile. However, the non-proteoglycan forms of decorin are always a minor component relative to the proteoglycan forms, whereas in the case of biglycan the non-proteoglycan forms predominate in the adult. Adult human patellar cartilage has also been reported to possess non-proteoglycan forms of decorin and biglycan, with the non-proteoglycan forms of biglycan being in greater abundance [19]. Such observations would explain why immunoassays of biglycan using core protein epitopes show a fairly constant level throughout life [17], yet analysis of proteoglycan preparations from adult cartilage extracts appear to be depleted in biglycan relative to decorin [18]. The appearance of analogous products in the intervertebral disc suggests that this phenomenon may occur in connective tissues in general and that a common mechanism may be involved.

This observation raises two obvious questions. First, how do the non-proteoglycan forms relate in structure to the proteoglycan forms, and secondly, do the proteoglycan forms have different functional properties from the non-proteoglycan forms? There are two possible origins for the glycosaminoglycan-free biglycan and decorin proteins. They may be synthesized as such, or alternatively they may be proteolytic products of initially produced proteoglycans. As the dermatan sulphate chains reside in the immediate N-terminal region [2], such proteolysis need not result in a large decrease in core protein size. A precedent for such a proteolytic scenario already exists in articular cartilage [27] and intervertebral disc [28], where processing of link protein has been shown to result in an increased matrix accumulation of proteolytically modified forms with age [29]. The biglycan and decorin components also exhibit a decreased abundance in the more mature adult disc in a manner analogous to link protein, and such a loss of matrix macromolecules has been associated with proteolysis accompanying disc degeneration with age [28].

Thus proteolysis with age and accumulation of the glycosaminoglycan-free core proteins would appear to be a logical explanation for the current data. It was therefore somewhat surprising to find that the two forms of non-proteoglycan biglycan did not appear to represent different core protein sizes, but rather differences in N-linked oligosaccharide substitution, even though the proteoglycan form of biglycan has been reported to show no heterogeneity in its degree of oligosaccharide substitution [5]. This is in contrast with decorin, in which variable degrees of oligosaccharide substitution have been reported [30]. If proteolysis does account for the non-proteoglycan forms of biglycan, then the two forms are derived by cleavage at the same site and do not represent a precursor/production relationship. It cannot be excluded, however, that the non-proteoglycan forms of biglycan are synthesized as such. In this respect it is of interest to note that in organ cultures of human articular cartilage, the proteoglycan form of biglycan is abundantly produced in the newborn but not in the adult [8]. Indeed, relative to decorin, synthesis of $^{35}SO_4$-labelled biglycan by adult cartilage is barely detectable in this system. As the tissue content of immunoreactive biglycan does not decrease with age, these data could be viewed as being supportive of the direct synthesis of non-proteoglycan forms of biglycan in the adult. Alternatively, there is little synthesis of biglycan in the adult but, relative to decorin, biglycan turns over slowly and therefore accumulates in the matrix. A precedent for the synthesis of matrix proteoglycans in non-glycosaminoglycan-substituted forms is to be found with fibromodulin, which may bear either N-linked oligosaccharides or keratan sulphate chains [31].

As the various biglycan proteins were visualized by immunological means, it is possible that not all degradation products would be detected if processing could occur at both the N- and C-termini. Antibody detection in the present work requires the presence of an intact C-terminus. While one cannot categorically state that there is no processing at this terminus, there are a number of reasons why processing would be much more likely to occur at the N-terminus, where the distance to the neighbouring disulphide-bonded loop is greater and where the extremely hydrophilic glycosaminoglycan chains reside. In addition, C-terminal processing would be expected to produce multiple core protein sizes for the proteoglycan forms of the molecule, particularly in the adult. Such processing was not observed in dermatan sulphate proteoglycans purified from the adult human meniscus [32]. There is no evidence in the present work for proteolytic processing within the central region of the proteoglycans, which appears to occur within the cartilage of the adult human patellar [19]. Indeed, if proteolytic processing does occur in the femoral condylar cartilage, then it is likely to be confined to the N-terminal region of the proteoglycans.

Finally, it is important to address the question of whether non-proteoglycan forms of biglycan or decorin would have an altered functional role. The answer of course would be yes if a dermatan sulphate chain is essential for function. If self-association of decorin molecules via dermatan sulphate chains is important in the interaction of collagen fibrils, then the absence of such chains would be expected to be detrimental to tissue integrity. The predominance of the proteoglycan form of decorin throughout life would support a need for the dermatan sulphate chains. This type of argument would then lead one to suggest that the dermatan sulphate chains of biglycan may not be essential in the normal adult. However, until the precise function of biglycan is established, it is impossible to verify the validity of such a statement.

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