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The link proteins

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Abstract. Aggregates of chondroitin-keratan sulfate proteoglycan (aggrecan) and hyaluronic acid (hyaluronan) are the major space-filling components of cartilage. A glycoprotein, link protein (LP; 40–48 kDa) stabilizes the aggregate by binding to both hyaluronic acid and aggrecan. In the absence of LP, aggregates are smaller (as estimated by rotary shadowing of electron micrographs) and less stable (they dissociate at pH 5) than they are in the presence of LP. The proteoglycan aggregate, including LP, is dissociated in the presence of chaotropes such as 4 M guanidine hydrochloride. On removal of the chaotrope, the complex will reassociate. This forms the basis of the isolation of LP from cartilage and has been described in detail elsewhere. Tryptic digestion of the proteoglycan aggregates results in a high molecular weight product that consists of hyaluronic acid to which is bound LP and the N-terminal globular domain of aggrecan (hyaluronic acid binding region; HABR) in a 1:1 stoichiometry. The amino acid sequences of LP and HABR are surprisingly similar. The amino acid sequence can be divided into three domains; an N-terminal domain that falls into the immunoglobulin super-family and two C-terminal domains that are similar to each other. The DNA structure echoes this similarity, in that the major domains are reflected in three separate exons in both LP and HABR. The two C-terminal domains are largely responsible for the association with HA and are related to two recently described hyaluronate-binding proteins, CD44 and TSG-6. A variety of approaches, including analysis of the forms of LP found in vivo, rotary shadowing and analysis of the sequence in the immunoglobulin-like domain, have shed considerable light on the structure-function relationships of LP. This review describes the structure and function of LP in detail, focusing on what can be inferred from the similarity of LP, HABR and related molecules such as immunoglobulins and lymphocyte HA-receptors.
Key words. Cartilage; proteoglycan; link protein; hyaluronic acid; extracellular matrix.

Introduction

The bulk of cartilage consists of proteoglycan aggregates constrained within a network of collagen fibrils. The aggregates consist of hyaluronic acid (HA), chondroitin-keratan sulfate proteoglycan (aggrecan) monomers and a glycoprotein, link protein (LP). The link protein stabilizes the aggregate and may also have a role in protecting the part of the proteoglycan (PG) monomer which binds to HA (the G1 domain) from proteolytic degradation. There are approximately 100 proteoglycan monomers per aggregate and, it is thought, a 1:1 ratio of link protein with the monomers. The large size of the proteoglycan aggregate (an approximately cylindrical structure as long as 4 μm and with a diameter as great as 0.8 μm[68]) and its polyanionic characteristics enables cartilage to absorb transient and long-term compressive loads, as well as providing a reservoir of lubricating fluid at the joint surface. In structural cartilages, such as nasal or auricular cartilage, the aggregate provides stiffness without the high degree of rigidity associated with bone.

Proteoglycan aggregates, isolated as a protein-polysaccharide complex from bovine nasal cartilage, were known to require a disulfide bond-containing element for their formation as early as 1969[71]. A low buoyant density glycoprotein fraction that aided the formation of proteoglycan aggregates was subsequently found.[28] Three discrete components appeared to be necessary for maximal aggregation[23], and these were shown to include hyaluronic acid[25] and link protein[33].

Purification

Link protein is usually purified from cartilage by using its affinity for hyaluronate and aggrecan. Typically, cartilage is extracted with dissociative solvents, the extract dialyzed to re-associate the proteoglycan aggregates and the aggregate isolated by density gradient centrifugation on a cesium chloride gradient. The aggregate is then dissociated once more and re-spun on a cesium chloride gradient to give a high density (A1D1) fraction and a low density (A1D4) fraction. The A1D4 fraction is rich in glycoprotein and, on a molar basis, contains mostly link protein (LP).

Link protein is rather heterogeneous[47]. Cartilage from different species or different age groups within the same species contains different amounts of three closely related glycoproteins, LP1 (the highest molecular weight form), LP2 and LP3. LP1 and LP2 have identical protein cores, while LP3 has been truncated at the N-terminal[38]. LP1 and LP2 have been separated prepar-

atively by electrophoresis[5], and wheat germ agglutinin affinity chromatography[11]. An extensive discussion of the purification of LP has been published elsewhere[2]. Link protein represents about 0.05% of the net weight of cartilage[18]. This represents a concentration of approximately 10 μM.

Link protein subspecies and nomenclature

It was noticed fairly early on that link protein existed in a number of forms, differing in molecular weight and charge. These differing forms result from differing levels of glycosylation and from loss of a portion of the N-terminal. This loss is either a result of proteolytic cleavage[52] or possibly a result of attack by free radicals[66]. Various terminologies have been used to describe LP subspecies; the one generally used is that the largest form (with two N-linked oligosaccharides) is LP1, the smaller form (with one N-linked oligosaccharide at residue 41) is LP2 and the form that is smaller than both of these, and which accumulates with age, is LP3. A form of LP3 which is ostensibly similar to that occurring in vivo, but which has a different N-terminal (LLVE . . .), is obtained by digestion of bovine or rat chondrosarcoma PG aggregate with trypsin.

The apparent difference in molecular weight of LPs from different species can also be explained in this way. Thus chicken LP is usually glycosylated completely at both sites, bovine and human LPs are partially glycosylated at residue 6 and rat chondrosarcoma LP does not have an N-glycoslyation site at residue 6. In biosynthetic studies, there appears to be only one protein produced, both in rat[30] and bovine chondrocytes[31]. LP1 is thus a doubly glycosylated LP species, LP2 is a singly glycosylated species and LP3 is a product of degradative modifications in the first 18 residues. LP3 is therefore singly glycosylated (at least in all LP forms examined to-date). Only one gene for LP has been detected in chromosomal mapping[54].

Function

The only function of LP found to date is to stabilize the proteoglycan aggregate. It achieves this by binding to both the proteoglycan monomer and hyaluronate, as clearly visualized in glycerol spraying/rotary shadowing studies[46]. In the absence of LP, the N-terminal globular domain of aggrecan (G1), isolated as the tryptic fragment, hyaluronate binding region (HABR), is somewhat dispersed along the hyaluronate chain. Adjacent G1 domains are spaced (center to center) about 12 nm apart, even when HA is limiting. There is space between protein domains along the HA chain. When LP is present, the globular LP and HABR molecules form a solid array along the HA chain. LP appears to induce co-operativity, such that the protein domains do not become dispersed, even when excess HA is present. Proteoglycan aggregates extracted from Swarm rat chondrosarcoma without dissociation show this especially clearly; regions of HA which do not have protein associated can separate sections of HA with densely packed LP and PG[44]. Aggrecan associated with hyaluronate is rather unstable at pH 5; in the presence of LP, the aggregate is much more stable and is larger[11, 24]. Interestingly, a more stable aggregate is formed in the presence of both LP1 and LP2 than is formed in the presence of LP1 alone[11]. In the same analysis, it was found that aggregates that were formed in the presence of LP1 alone were larger than those formed in the presence of LP1 and LP2. LP associated with hyaluronate in the absence of the proteoglycan monomer is stable at pH 5[69] indicating that at least part of the LP-derived stabilization effect is a result of the different sensitivities of LP-HA and aggrecan-HA interactions to pH changes.

LP is capable of self-association; its insolubility as a result of this has hindered many physical studies. Rosenberg et al. have found that the solubility of LP in non-dissociative, physiological buffers is considerably enhanced in the presence of EDTA[67], implicating trace amounts of divalent cations in LP insolubility. In free solution and in the absence of divalent cations, LP forms a monomer-hexamer equilibrium mixture. On addition of HA_{10-12}, the predominant form of LP is a dimer. LP binds 2 nmol of Zn^{2+} at a separate site from the HA-binding site[67]. LP can also bind to other divalent cations (Ni^{2+}, Co^{2+}). In the presence of Zn^{2+}, LP becomes insoluble. When HA is present, LP is more soluble, but can still be precipitated as an HA-LP dimer-Zn^{2+} complex. Other divalent cations (Mg^{2+}, Co^{2+}, Ca^{2+}) have little or no effect on LP solubility. The physiological relevance of this interaction is unclear, however it could have profound effects on the structure of proteoglycan aggregates. Taken together with the rotary shadowing studies described above, it implies that in PG aggregates with HA and LP, the LP may be able to bind to an adjacent LP molecule.

There is some evidence that link protein can bind to the triple helices of collagen types I and III[10]. The interaction is saturable, although as the molar ratio of native collagen: LP is 7–13:1, there do not appear to be many LP binding sites on collagen fibrils. It is not clear what role this would play in vivo, as these collagens are not typically found in cartilagenous tissues. As discussed below, LP is found in other tissues than cartilage.

Structure: protein

The complete amino acid sequence has been determined for rat chondrosarcoma LP[49], and, derived from cDNA sequence, for chick LP[13], pig LP[17], and human LP[54]. Partial sequences for bovine LP[6] and a shark LP (Neame, unpublished) have also been determined. All

Percent interspecies variation of the four exon-derived domains of link protein

	N-term	Loop A	Loop B	Loop B′
Mammalian	33	9	1	4
Avian	55	24	6	3
Shark		43 (98%)	29 (98%)	32 (77%)
Aggrecan		73	54	56
Versican		68	53	55

The percent variation of the domains of mammalian link proteins (rat[49], human[54], pig[17] and bovine[6] for loop A) are shown compared with the percent variation of chicken link protein[13]. The actual similarity is greater than these numbers suggest, as many of the changes are conserved changes, representing minor alterations in the properties of the amino acid side chain. Partial sequences are available for shark link protein (Neame, unpublished). While not complete, the shark data emphasize the different rates of change in the various domains. The numbers in parentheses indicate the amount sequenced in each domain. The percent changes in aggrecan and versican are also shown. Human link protein is used as a reference.

mammalian LPs sequenced to-date contain 339 amino acids, while chick LP contains 340 amino acids. There are 10 cysteine residues which define 5 disulfide bonds[49]. The degree of conservation of protein sequence from LPs of different species is striking (table, fig. 1). Partial sequence of shark cartilage LP (Neame, unpublished) indicates that this sequence conservation is still dramatic even across large evolutionary distances.

LP contains three disulfide-bonded loops (fig. 2)[49]. The most N-terminal of these, loop A (using the nomenclature of Doege et al.[14]), contains a single disulfide bond. The two C-terminal loops, loops B and B′, are similar to each other and contain two disulfide bonds each. These two loops are known as the proteoglycan tandem repeat, or PTR[60]. A striking feature of the structure of LP is its close resemblance to the structure of the N-terminal globular domains (G1) of the aggregating chondroitin-keratan sulfate proteoglycan, aggrecan[6, 15, 16, 50, 51] and of the fibroblast-derived chondroitin sulfate proteoglycan, versican[77]. Both of these proteoglycans contain the same group of three motifs at the N-terminal; the two domains comprising the PTR (loops B and B′), in particular, are very similar to those in LP.

The N-terminal 18 amino acids, coded for by exon 2, are the most variable. As they can be removed in vivo during the normal course of aging, it is unclear whether they have a function. However the presence or absence of the N-linked oligosaccharide at residue 6 affects aggregate properties[11] as described below.

Loop A

The N-terminal region of LP (and, by analogy, G1 from aggrecan and versican), loop A, is a member of the immunoglobulin superfamily[6, 32, 60]. Specifically, it is similar to the IgG V_L-region. By comparing loop A with related IgG light chain variable regions which have been crystallized, it is possible to estimate the extent of the 7 β-sheets which comprise this folding motif[60]. The β-sheets can be seen as conserved hydrophobic residues when these regions of LP, aggrecan and versican are aligned. The immunoglobulin folds that have been crystallized to-date form two layers of anti-parallel beta sheets with a disulfide bond stabilizing the structure[12]. The residues which form the inside of the two layers of beta sheets are hydrophobic; thus alternating amino acid side chains tend to be hydrophobic. Figure 2 shows a highly speculative diagram of the structure of this part of LP. In the absence of physical data (X-ray diffraction patterns or NMR) it is not possible to say what the exact relationships of the β-sheets are to each other.

Loops B and B′

The two C-terminal loops are strikingly similar to each other. Of the separate exon-derived structures in LP, these loops vary the least between species. The table summarizes the rates of change of these domains relative to the N-terminal and IgG superfamily-related domains. It has been suggested that as loop B is more conserved between LP and aggrecan, it is more likely to be involved in binding to HA[16]. However, comparing the sequences of the putative HA-binding domains in CD44[22, 76] and TSG-6[36] with the PTR sequences in LP does not indicate that loop B is more likely to bind to HA than loop B′.

The main source of evolutionary variability in the PTR domain will be in the amino acid side chains which associate with solvent. Internally-pointing residues, or those involved in binding to HA, will be relatively invariant. The most variable region in both loop B and in loop B′ is the section between the third and fourth cysteines. This sequence contains an N-linked oligosaccharide attachment site in all versions of non-LP PTR loops, except aggrecan G2 loop B and versican G1 loop B. The most conserved region is between cysteines 1 and 3; it is reasonable to assume that this region is critically important for either the folding of these loops and/or in their HA-binding function.

A species-non-specific, LP-specific monoclonal antibody, 8A4[9] binds to the PTR loops. Sepharose-8A4 affinity chromatography of peptides derived from chymotryptic and and tryptic digests of LP has shown that there is an epitope for this antibody on both loop B and on loop B′[51]. As this antibody does not bind to other, ostensibly very similar, structures (aggrecan G1 or G2, versican G1) it is possible to derive a minimal sequence for the epitope: LXDGSVXYPIobP. The lower case letters refer to hydroxylated (o) and basic amino (b) acids respectively. The underlined glycine is probably critical, as it does not occur in aggrecan, although much of the rest of the epitope does. While this antibody recognizes LP-HA-HABR complex, it does not

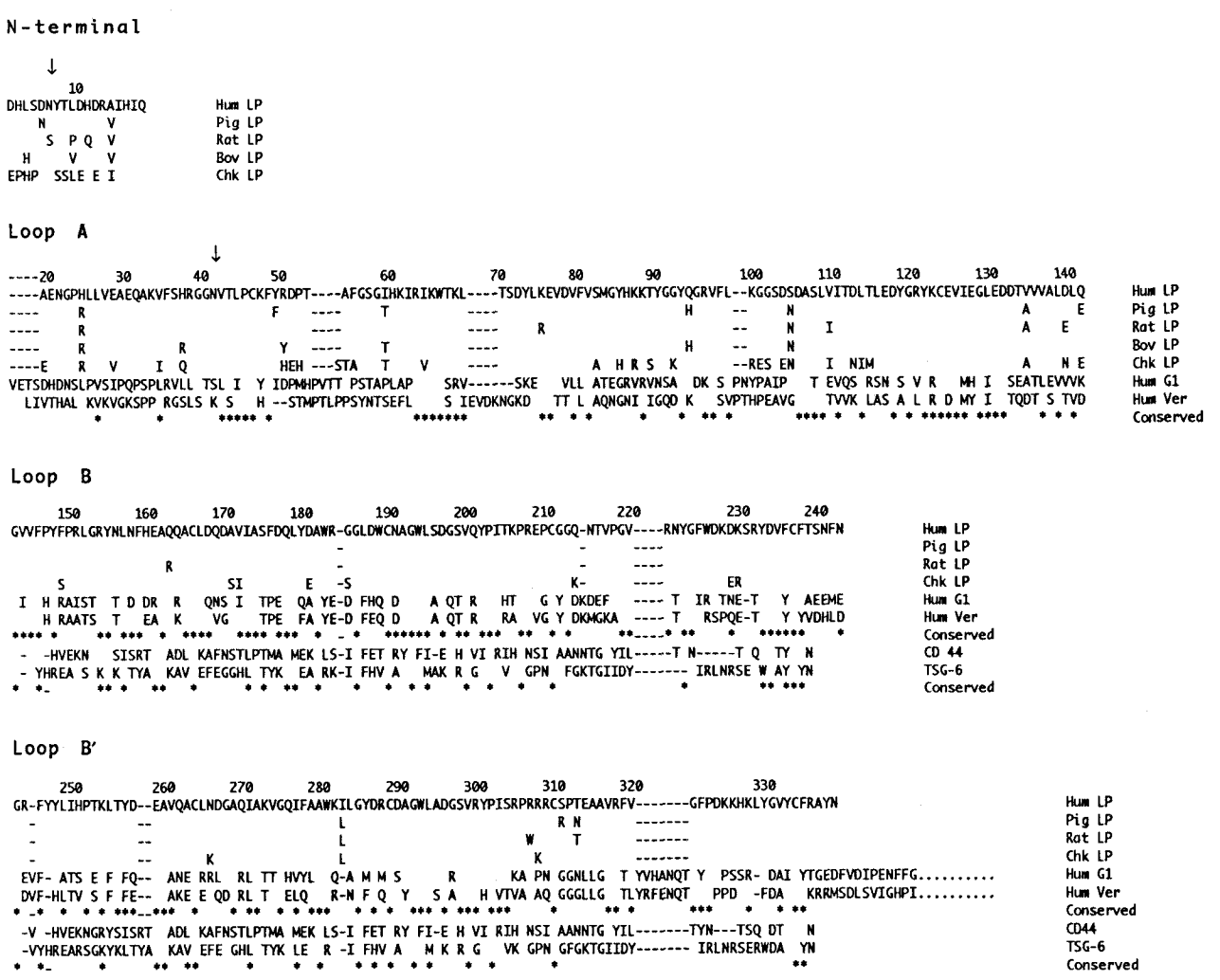

Figure 1. Alignment of human link protein with other link proteins and with related, HA-binding, molecules. The amino acid sequence of human link protein is used for comparison with other link proteins and with aggrecan, versican and the HA receptors CD44[76] and TSG-6[36]. Only residues which differ from those in human LP are shown. Human LP (Hum LP[54]), pig LP[17], rat LP[49], partial bovine LP (Bov LP[6], c.f. ref. 40), chicken LP (Chk LP[13]), human aggrecan G1 (Hum G1[16]), human versican (Hum Ver[77]), ↓, N-glycoslyation site; *, conserved residue.

recognize native proteoglycan aggregates[9], implying that there is some structural difference between a laboratory-generated ternary complex and the complex which assembles in vivo.

Structure: carbohydrate

With some exceptions (for example, rat) LP has two N-linked oligosaccharides. One is at amino acid 6 and the other is at amino acid 41. Rat LP does not have the most N-terminal carbohydrate. Indeed, substitution at this site seems to be rather variable. Human and chick LPs are usually N-glycosylated at this position, while bovine LP is rather more variable.

The N-linked oligosaccharide at position 41 has, in addition to N-acetyl glucosamine, some N-acetyl galactosamine. This site is always glycosylated; the glycosylation site at residue 6 may be missing as a result of protease treatment[38] or as a result of catabolic processing[47], or as a result of inefficient N-glycosylation.

N-linked oligosaccharides in bovine articular LP are of the complex or hybrid type (resistant to endoglycosidases H and F). LP1 increases in size and heterogeneity as it goes through the Golgi. LP2 does not, although both become more endoglycosidase H resistant[31]. Thus the most processing and the most heterogeneity is found on the N-linked oligosaccharide at residue 6.

At least in bovine LP, the N-linked oligosaccharide at residue 6 can be sulfated, resulting in considerable charge heterogeneity[31]. The presence or absence of N-linked oligosaccharides does not affect the transport of LP to the extracellular matrix, indicating that the function of these substituents is not in intracellular trafficking[41]. The carbohydrate in LP is also variably substituted with sialic acid, and this accounts for some of the heterogeneity[70].

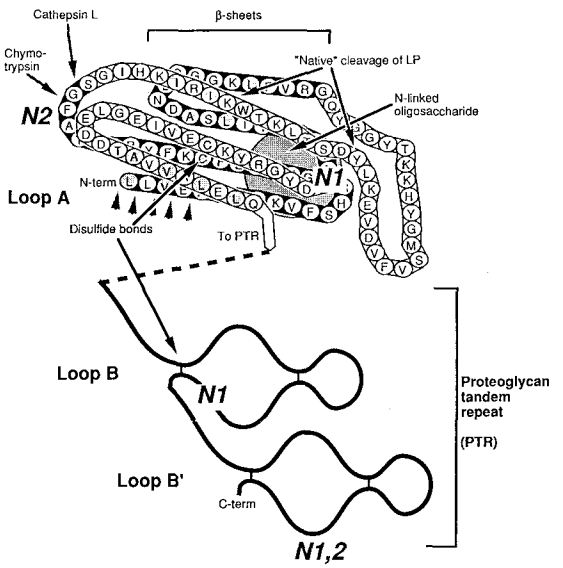

Figure 2. A diagram showing the major regions of LP3 (the tryptic fragment of LP obtained after digestion of proteoglycan aggregate). Loop A, which is a member of the immunoglobulin fold superfamily, is shown in some detail based on the putative locations of its β-sheets. The extents of the β-sheets are not known exactly, so it is not possible to estimate the relative positions of individual amino acids. Shaded residues are highly likely to have their side-chains oriented towards the interior of the structure, based on the fact that they are hydrophobic and on the geometry of a β-sheet. The shaded circle is the location of the N-linked oligosaccharide at residue 41 in LP. The approximate positions of N-linked oligosaccharides in related structures are shown in italics for aggrecan G1 (N1), and versican G1 (N2). In IgG, the loop of amino acids on the right hand side is usually folded over the top of the three uppermost β-sheets to form a groove. Likewise, in an immunoglobulin, the three short loops of sequence on the left would form the complementarity-determining regions. Attached to loop A by a dashed line is the proteoglycan tandem repeat (PTR), which consists of two closely related structures (loops B and B'). We cannot speculate what the structure of this region actually is. In the G1 domain of aggrecan, both of these loops have attached N-linked oligosaccharides. Sites of proteoglytic attack when LP is in an aggregate with HA and PG are shown by gray arrows. Multiple enzymes will cleave loop A in the region of the first β-sheet (5 short gray arrows).

Glycosylation is not the only source of LP microheterogeneity. LP3 from human articular cartilage has an N-terminal resulting from a cleavage between His 16 and Ile 17[53], effectively removing the most N-terminal oligosaccharide, as shown in figure 3.

Structure-function relationships

Based on neutron and synchrotron X-ray scattering, LP is a somewhat oblate molecule of 10 × 2.5 × 3 nm[61]. In a ternary complex of LP with HA and the tryptic fragment of aggrecan which still binds to LP and HA (HABR), scattering and interaction studies suggest that the LP-HABR complex is 12 × 3 × 6 nm[62]. This would be a side-by-side arrangement of LP and HABR and would minimize the exposed surfaces of both molecules, thus explaining the protease resistance of the complex[29] and its reduced ability to bind antibodies[9,63].

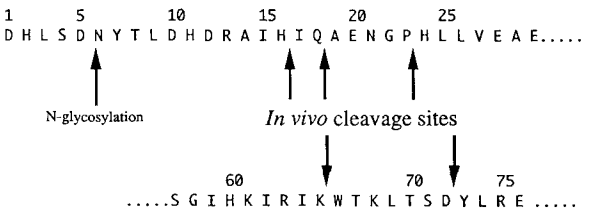

Figure 3. Cleavage sites in the N-terminal region and loop A of human LP. There are 5 cleavage sites observed in LP isolated from human articular cartilage. These are all in loop A and are indicated by the arrows pointing at the peptide bonds between residues 16–17, 18–19, 23–24, 65–66 and 72–73. These sites are somewhat similar to those observed when proteoglycan aggregates are digested with various proteases in vitro[52].

Link protein stabilizes proteoglycan aggregates. To achieve this, it has two functions; it binds to HA with a dissociation constant of $1-4 \times 10^{-8}$ M[43] and it also binds to the HABR region of the proteoglycan monomer[37]. Tryptic digestion of LP bound to HA releases a fragment of LP corresponding to the two C-terminal loops, B and B'[59]. This region, the PTR, remains bound to hyaluronate, providing conclusive evidence for the involvement of this domain in the interaction with HA. The actual cleavage site is 3 residues in front of the beginning of the loop B (as defined by the exon structure of the gene). This cleavage site is, in fact, a chymotrypsin-type cleavage site. If high purity trypsin is used, then a fragment which includes the disulfide bond of loop A remains bound to the HA (Neame, unpublished).

Within loop A, the most conserved regions between LP and aggrecan G1 are those which include the two cysteines of the disulfide bond, and the likely β-sheet between residues 60 and 70 (figs 1 and 2). The two upper and rearmost β-sheets in figure 2 (residues 93–110) are also quite conserved, although the sequence between the sheets is not. Part of the loop on the right hand side of figure 2 (residues 74–81) is also conserved. While other β-sheets are highly conserved as sheets (in other words, every other amino acid is conserved), the residues which would be likely to be exposed to the solvent are less conserved. For example, in the last β-sheet before the PTR is reached in figure 2, the equivalent residue in versican and aggrecan to LP residue Val_{136} is Thr; Ala_{138} in LP is Glu in aggrecan, Ser in versican.

Mild tryptic digestion of a complex of LP and the HABR from the aggregating proteoglycan yielded a fragment of LP corresponding approximately to loop A, indicating that this structure is largely responsible for the interaction between LP and aggrecan[59]. To-date, it has not been possible to map the site of interaction between HABR and LP in more detail. However, it cannot be at the sites in loop A attacked by proteases, as these would be protected in aggregates. It cannot be ruled out that LP does not bind exclusively to loop A in HABR, but also interacts with the PTR. However, the

structural similarity between G1 and LP makes this an unattractive possibility.

It has been speculated that the interaction of LP with HA is mediated by basic amino acids. This derives partly from the fact that derivatization with succinic anhydride (which modifies lysine) or with 2–3 butanedione (which modifies arginine) reduces the ability of either LP[42] or G1[26] to bind to HA. The shortest oligosaccharide inhibitor of LP-HA binding is 10 monosaccharides long[25], corresponding to a binding site 5 nm long. This would be quite large, wider than LP (see above) and equivalent to half its length. Four synthetic peptides representing different parts of loops B and B′ all inhibited LP's ability to bind to HA equally[21]. This would represent half the length of LP and would indicate that the binding site is quite complex.

To-date, X-ray crystallography and NMR spectrophotometry have not been used to define the structure of LP. However, two structural inferences can be made from available data. Clearly the region around LP residues 17–24 is available to solvent in proteoglycan aggregates, as this region can be attacked by protease while leaving the rest of the molecule intact. Secondly, a monoclonal antibody, 6A1, which is specific for rat chondrosarcoma LP, does not react with LP from which the N-terminal 24 amino acids have been removed and does not react with LP from which the N-linked oligosaccharide has been removed[9]. The antibody is therefore reacting with both of these regions, in spite of the fact that they are remote from each other in the amino acid sequence. Therefore, the N-terminal region may be folded back under the diagram of loop A shown in figure 2.

While the function of the N-terminal region is unclear, the presence or absence of the N-linked oligosaccharide in this region has some effects on the structure of the proteoglycan aggregate, at least in bovine tissue. In the presence of LP1 alone, the proteoglycan aggregate is less stable than it is in the presence of both LP1 and LP2. However, the aggregate size is greater in the presence of LP1 alone[11]. This may be a result of closer packing of the LP-aggrecan complex onto the HA when both LP forms are present. Further experiments need to be done in this area using defined-length HA to determine the packing density of aggrecan onto HA. Rotary shadowing studies indicate that the packing density of LP and HABR can be quite high[44]; superficially it appears to be close to saturation.

Efforts have been made to use predictive algorithms to define the locations of secondary structural motifs in the C-terminal, PTR domain of LP[60]. However, the results are not conclusive. As these structures form an entirely new class of molecules, and as the rules for predictive algorithms derive from a limited subset of proteins (i.e. those which were crystallized prior to about 1980), it is probably too much to expect useful information to be obtained by attempting to predict the structure of the PTR domain. Nevertheless, by comparing all the loop B and B′ sequences shown in figure 1, it is clear that each half of the PTR starts with hydrophobic residues, then has a tendency towards α-helix up to the first cysteine. The domain continues to be rather hydrophobic, until the last 20–30 amino acids are reached, where it becomes hydrophilic. On SDS-PAGE gels, the PTR (isolated as a V8-protease digestion-derived peptide) appears as an anomalously low molecular weight band unless it is reduced, indicating a very compact structure.

DNA analysis, exon organization and chromosome location

There are 5 exons in the rat LP[64] and chick LP[34] genes. These reflect the protein domains; exon 1 codes for the signal peptide, exon 2 codes for the N-terminal region and exons 3–5 code for loops A, B and B′. The chick gene is >80 kb and is present as a single copy[34]. The relative degree of similarity between the exons coding for loops B and B indicates that gene duplication occurred at some point in the past. As these exons are relatively conserved in mammals, birds and sharks, it is clear that the primordial gene appeared fairly early in higher animal evolution. The HA-receptors described above clearly represent another descendant of the primordial gene.

Alternative splicing can generate a large mRNA for rat LP, although this form represents less than 10% of the total mRNA for LP[64]. This form of LP has not been observed in proteoglycan aggregates. A larger form of LP has not been observed in studies analyzing the biosynthesis of LP. It is likely, therefore that this larger LP gene transcript is rapidly degraded if it is synthesized[30]. The 3′ untranslated region of link protein genes is also conserved to about 65% homology[54].

The promoter for the link protein gene does not have a great resemblance to other, cartilage derived, protein gene promoters. However, a 12bp segment in the 5′ flanking region of the gene with 83–91% homology between LP, cartilage matrix protein, type II collagen and fibronectin, may be significant[54]. The gene for human LP is found on chromosome 5 in the q13→q14.1 region[54]; it does not appear to correlate with any known genetic disease loci in this region[54].

Distribution

Characteristically, LP is thought of as a cartilage macromolecule. However, this may be largely due to its ease of isolation from this issue. Cartilage has the advantage that over 90% of the tissue is extracellular matrix; LP is thus extremely abundant. A fibroblast proteoglycan, versican, has an N-terminal domain strikingly similar to LP and to the N-terminal (G1) domain of cartilage aggrecan (fig. 1)[77]. LP, or a very similar molecule, might stabilize the aggregation of versican with hyaluronic

acid. LP has been isolated from aorta[20, 75] and appears to be very similar to that isolated from cartilage. Hyaluronic acid binding region and link protein epitopes have been found in brain tissue[65]. Protein sequence data[4, 55] indicates that a glial hyaluronate-binding protein is actually the N-terminal domain of versican. LP has also been found in the embryonic chick retina and in lens epithelium and fibers, based on DNA hybridization and immunostaining[74]. Transcripts for LP have been found in the chick mesonephros, although no concomitant transcript for aggrecan was present[73].

Thus, LP appears to be more widely distributed than the aggrecan core protein. In tissues where the aggrecan core protein is absent, it is possible that the link protein is associated with versican, although this remains to be proven. It is also possible that it is associated with collagen in these tissues[10].

Degradation

The most readily apparent degradation of LP results in the formation of LP3. A later event, seen in adult articular cartilage, results in smaller fragments which can be seen on SDS-PAGE gels after reduction of the LP. The cleavages that result in these fragments are between the first two cysteines[52]. By performing N-terminal sequence analysis of electrophoretically separated LP fragments isolated from mature human cartilage[52, 66], it has been possible to define the exposed regions of LP in proteoglycan aggregates.

The initial in vivo cleavage of LP occurs near the N-terminal and may be mediated by stromelysin[53], as shown in figure 3. This site is two amino acids removed from the tryptic site which produces the in vitro-derived form of LP3 (i.e. two residues before the N-terminal of loop A in fig. 2). Additional cleavages are labeled as 'native' cleavage of LP in figure 2. Other proteolytic cleavage sites, generated by treatment of the aggregate with various proteases, which are exposed in proteoglycan aggregates also occur in loop A and are summarized in Nguyen et al.[52] The structure of loop A can be approximated by comparing it to crystallized IgG structures; when this is done (fig. 2) it can be seen that the regions exposed to proteolytic attack (gray arrows) are in two areas. One susceptible site is at the N-terminal end of the first β-sheet, while the other is in the vicinity of the third β-sheet. In the absence of proteoglycan but the presence of HA, the whole of loop A can be digested away[59], unless trypsin free of chymotryptic activity is used, in which case a trypsin-resistant fragment can be obtained, held together by the disulfide bond (Neame, unpublished).

Comparison with similar structures: aggrecan and versican

A ternary complex of hyaluronic acid, part of the proteoglycan monomer and LP can be obtained by tryptic digestion of proteoglycan aggregates[29]. The part of the proteoglycan monomer obtained in this way is called the hyaluronate binding region (HABR) and derives from the first three sequence domains of aggrecan, together with part of the bridge between the first and second globular domains. Initially, there did not appear to be any evidence for significant similarity in internal sequence[57, 58] and there was no immunological cross-reactivity[9, 63]. The first indications that LP and the aggrecan monomer contained similar structures derived from sequence comparison of peptides from LP with the sequence of cyanogen bromide peptide from aggrecan[51]. Partial sequence data from the proteoglycan indicated that this similarity was extensive[56]. Subsequently, when the sequence of the HABR region of rat chondrosarcoma aggrecan was determined[15, 50], it was realized that this similarity included the whole molecule, although the C-terminal two-thirds was clearly most closely related (loops B and B′, fig. 2).

By virtue of their similar sequences, the interaction of LP with HA might be expected to be similar to that of HABR with HA. However, whereas the interaction of the proteoglycan with HA becomes weaker at lower pH[11, 24], the interaction between LP and HA stays the same and the complex actually forms faster at lower pH[69]. In addition, whereas LP does not have any N-linked oligosaccharides in the PTR, the G1 domain has one N-linked oligosaccharide in loop B and one in loop B′[3, 50]. Versican also has a likely N-linked oligosaccharide in loop B′. The effect of pH on the interaction between LP and HA versus the effect of pH on the interaction between G1 and HA may not be a direct response to differences in the HA binding site. It may be a result of conformational changes elsewhere in the molecule.

The G2 domain in aggrecan is similar to the two C-terminal loops in LP. However, isolated G2 does not bind to HA[19], even though this structure in LP and G1 appears to be primarily responsible for the association with HA. A key difference when comparing LP, G1 in aggrecan and G1 in versican with G2 in aggrecan is that an extra N-linked oligosaccharide seems likely to be in loop B′ in G2, 4 residues after the first cysteine. This might disrupt the folding of the domain. However, CD44, which binds HA[1], also has a likely N-linked oligosaccharide at this point.

Comparison with similar structures: HA-receptors

A single loop from the repeating sequence (the PTR) at the C-terminal of LP is similar to a sequence at the N-terminal of a lymphocyte receptor, CD44[76] or the Hermes antigen[22]. CD44 is capable of undergoing exon shuffling; one form is a cell surface heparan sulfate proteoglycan[8]. This single loop, or 'half-PTR', which is most closely related to loop B of the G2 domain of

aggrecan, was the first example of this motif outside the aggregating proteoglycan field. Another, more recently described, tumor necrosis factor and interleukin-1 inducible, secreted protein TSG-6, is even more closely related to loop B[36] in both PG and LP. These workers have provided evidence that the interaction of HA with these loop structures involves more than ionic interactions by precipitating the HA-TSG-6 with cetylpyridinium chloride, which binds to the acidic groups on HA. Complex interactions between proteins and oligosaccharides often involve aromatic amino acids, particularly tyrosine, as well as ionic interaction[7].

The presence of an HA-binding domain in CD44 does not automatically confer on lymphocytes an ability to bind HA. A cytoplasmic activating event also seems to be necessary[39]. This may indicate that the synergistic effect of another molecule is necessary. Surprisingly, an anti-CD44 monoclonal antibody can actually enhance HA-binding[39]. It is important to note that there are other HA receptors which do not resemble LP, for example a motility associated HA-receptor (RHAMM)[27]. It is probable that there are also other, as yet undiscovered, HA-binding proteins.

Developmental expression

In the developing chick limb bud, the LP gene is transcribed simultaneously with the aggrecan gene. It coincides with the histological detection of cartilage. This occurs some time after collagen type II transcription and before cartilage matrix protein[72]. However, this does not mean that LP and aggrecan are regulated together, as the LP transcription product, as well as LP, can be detected in the chick mesonephros (which functions as an embryonic kidney) at about the same time that LP appears in the wing bud[73]. However, aggrecan is not present in the mesonephros.

In the developing limb, the highest level of LP mRNA is found in the proliferating region and in the upper hypertrophic zone. In spite of the fact that LP has been found in aorta, in situ hybridization of LP cDNA in the developing human embryo only showed LP in cartilagenous structures[48]. This is in contrast to the chick embryo[73]. So far, no LP-associated developmental disorders have been detected. Given the extremely high degree of conservation of LP through evolution, and its critical role in cartilage, and therefore skeletal, development, it is probable that changes to LP structure would be deleterious and therefore catastrophic for the developing organism.

Conclusion

Link protein is the first member to be characterized of a family of HA-binding proteins which, to-date, also includes aggrecan, versican, CD44 and TSG-6. The most obvious directions to take to substantially increase our understanding of this family will require physical methods, X-ray crystallography or nuclear magnetic resonance. While crystallography is a suitable technique for detailed structural analyses of molecules of this size, the difficulty of obtaining crystals when somewhat heterogeneous oligosaccharides are present may make this overly difficult. A better approach will be to use expression systems to produce large amounts of individual domains. These are small enough to be analyzed by NMR using present technology. Free solution studies involving HA oligomers will also be possible and the exact binding site for HA defined. It will be particularly interesting to determine why the aggregating molecules have two HA-binding loops, whereas HA receptors only have one, yet still bind HA with high affinity.

Does link protein help to stabilize an aggreate of versican with hyaluronic acid? LP and LP gene transcripts have been detected in enough non-cartilagenous tissues to make this a compelling theory. Loop A of versican G1 is more closely related to aggrecan G1 than it is to LP (fig. 1). This would indicate that it performs a similar function to aggrecan G1. The differences between aggrecan G1 loop A and versican G1 loop A are comparable to the differences between loop B or loop B′ from these proteoglycans. Versican binds to HA[35]. The structure-function analysis of versican as compared to aggrecan and LP and its need or lack of need for LP for association with LP clearly require more work.

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Note added in proof: While this review was in press, the sequences of two further proteoglycans containing the same link protein-like structure were published. One is the sequence of chick aggrecan

(Chandrasekaran, L., and Tanzer, M., Molecular cloning of chicken aggrecan. Structural analyses. Biochem. J. *288* (1992) 903–910;

and the other is a versican-like proteoglycan

(Rauch, U., Karthikeyan, L., Maurel, P., Margolis, R. U., and Margolis, R. K., Cloning and primary structure of neurocan, a developmentally regulated, aggregating chondroitin sulfate proteoglycan of brain. J. biol. Chem. *267* (1992) 19536–19547).

These add further evidence of the relatively rapid rate of evolutionary change in the loop which falls into the immunoglobulin fold superfamily with respect to the PTR domain.

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