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Primary structure of a protein isolated from reef shark (*Carcharhinus springeri*) cartilage that is similar to the mammalian C-type lectin homolog, tetranectin

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Abstract

During the course of characterization of low molecular weight proteins in cartilage, we have isolated a protein from reef shark (*Carcharhinus springeri*) cartilage that bears a striking resemblance to the tetranectin monomer originally described by Clemmensen et al. (1986, *Eur. J. Biochem.* *156*, 327–333). The protein was isolated by extraction of neural arch cartilage with 4 M guanidine hydrochloride, dialysis of the extract to bring the guanidine to 0.4 M (reassociating proteoglycan aggregates), followed by cesium chloride density gradient removal of the proteoglycans. The amino acid sequence had 166 amino acids and a calculated molecular weight of 18,430. The shark protein was 45% identical to human tetranectin, indicating that it was in the family of mammalian C-type lectins and that it was likely to be a shark analog of human tetranectin. The function of tetranectin is unknown; it was originally isolated by virtue of its affinity for the kringle-4 domain of plasminogen. Sequence comparison of human tetranectin and the shark-derived protein gives clues to potentially important regions of the molecule.

Keywords: cartilage; lectin; sequence; shark

Cartilage, a relatively acellular tissue, consists predominantly of proteoglycan aggregates and collagen. However, it also contains a variety of other macromolecules, some of which appear to regulate the structure of the matrix (for a review, see Heinegård & Oldberg, 1989). For example, small leucine-rich proteoglycans appear to be involved in the control of collagen fibril diameter (Vogel et al., 1984), a 40-kDa glycoprotein, link protein, stabilizes proteoglycan aggregation with hyaluronic acid (Buckwalter et al., 1984), and growth factors are sequestered in the matrix by virtue of their ability to bind to the negatively charged glycosaminoglycans (Folkman et al., 1988). Other, lower molecular weight proteins have also been described; in the course of isolation of proteoglycans from extracts of bovine cartilage, we have previously isolated and described three proteins that are abundant in growing bovine cartilage (NBRF PIR numbers A33136, A33138, and A33139) (Neame et al., 1990a,b).

Shark cartilage does not calcify in the same way as cartilage from animals with a bony skeleton. In an attempt to define fundamental differences between shark cartilage and mammalian cartilage and whether some of the above proteins, or others, are altered significantly, we have subjected cartilage from shark neural arch to the same extraction procedures that we use for mammalian cartilage. The noncollagenous, nonproteoglycan-associated proteins of less than 50 kDa that we found were quite different from those that we have found in mammalian cartilage. One abundant protein was over 50% similar to the human protein, tetranectin, and is likely to be the shark equivalent of this protein. We report the primary structure of this protein and its disulfide bond pattern.

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Results

Dissociative extracts of shark cartilage, obtained by treatment of thin slices of tissue with 4 M guanidine hydrochloride, were separated into glycosaminoglycan-rich and glycosaminoglycan-poor fractions by cesium chloride density gradient ultracentrifugation. As expected, there was a substantial collagen-rich pellet at the top of the density gradient and proteoglycan aggregates at the bottom. Gel

filtration analysis of the low density fraction on a Sephacryl S-300 column equilibrated in dissociative buffer resulted in a prominent double peak in the size range of 10–20 kDa (Fig. 1).

Proteins in individual fractions from the gel filtration analysis were separated by reversed-phase high performance liquid chromatography (HPLC) (Fig. 2). Peaks were analyzed by amino acid analysis and Edman degradation. Two unique proteins were found to be major components in the size range 10–25 kDa. Both of these proteins were analyzed by direct protein sequencing of proteolytic digest-derived fragments. The higher molecular weight of these two proteins is described here.

The N-terminal of the higher molecular weight protein could be followed for 34 residues. The protein was initially digested in unreduced form. Subsequent digestions were of protein that had been reduced and S-carboxymethylated with iodoacetic acid. Alignment of peptides from cleavages at aspartate (Fig. 3), lysine (not shown), methionine (CNBr; not shown), glutamate (V8 protease; not shown), and lysine and arginine (trypsin) (not shown) resulted in a sequence of 166 amino acids. In some cases, particularly those where the native protein was used, digestion did not go to completion, which aided the alignment of peptides (Fig. 4; Table 1).

The majority of the sequencing and subsequent alignment was quite straightforward. The weakest area was around residue 54, a sequence that was obtained from a region near the C-terminal end of a long peptide. However, we feel that the sequence is correct, as we have sequenced through this area twice (peptides D-2 and K-3) and have confirmed sections with tryptic peptides. One amino acid (Cys-47) was not determined directly, as the sequence in this area was from peptides that had been reduced but not S-carboxymethylated. The assignment of this amino acid was deduced by the behavior and sequence analysis of peptides from digestions with endoprotease AspN and trypsin, as discussed below.

The C-terminal was defined as valine by sequence analysis of peptides isolated from digests with endoprotease Lys-C, trypsin, V8 protease, and endoprotease Asp-N, as shown in Table 1. The calculated molecular weight was 18,430 and the calculated pI was 9.4.

Analysis of peptides before and after reduction defined the disulfide bond pattern. A digest with endoprotease Asp-N (Fig. 3) had two peaks that moved on reduction. These were collected, reduced, and reanalyzed by reversed-phase HPLC and Edman degradation. One of these peptides, D-2, had a single N-terminal but, after reduction, it eluted later on the reversed-phase chromatogram. There were two undefined amino acids on sequence anal-

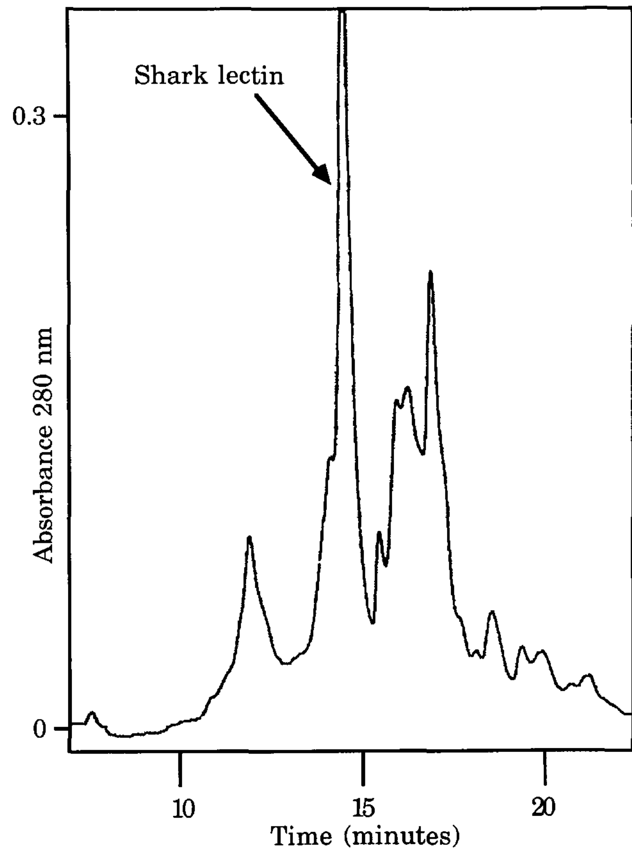

Fig. 2. Individual fractions from Figure 1 were analyzed by reversed-phase HPLC (Brownlee RP-300, 4.6 x 250 mm, acetonitrile gradient, 0.1% TFA). Shown is the HPLC profile of the fraction containing shark lectin in Figure 1 (arrow).

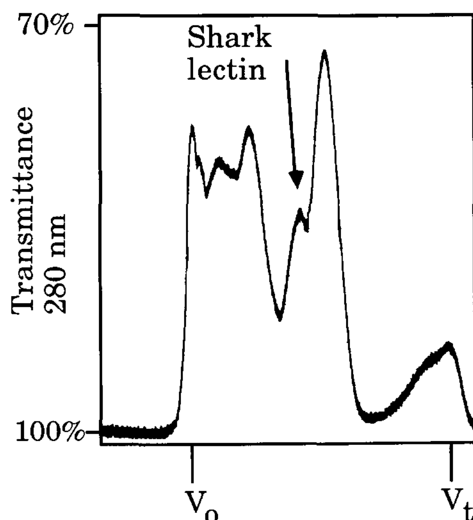

Fig. 1. Gel filtration analysis (Sephacryl S-300, 2.6 × 90 cm, equilibrated in 4 M guanidine HCl) of the low density fractions from a cesium chloride gradient of a guanidine-HCl extract of 20 g of shark neural arch cartilage. The peak pointed to is the shark lectin. The other, lower molecular weight protein has a unique sequence and is unrelated to any sequences in Genbank or the NBRF PIR database (Neame, Young, & Treep, unpubl.).

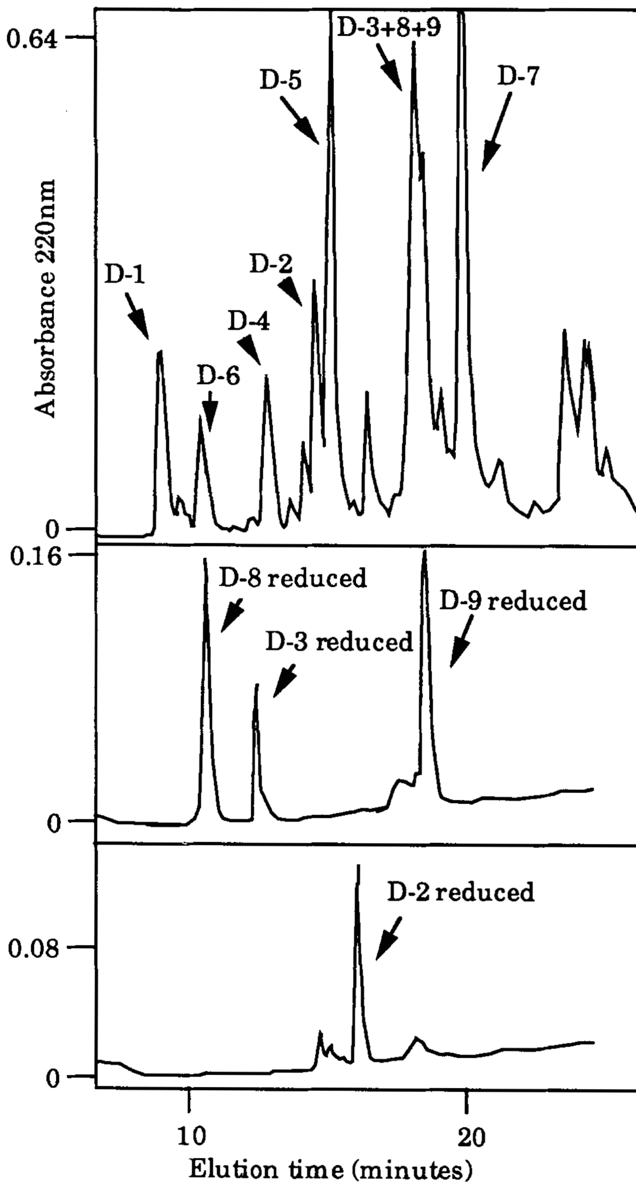

Fig. 3. Reversed-phase separation of peptides derived from an endoprotease Asp-N digest of 5 nmol shark lectin (upper panel). The column was a Brownlee RP-300 (30 x 2.1 mm). Elution was with a gradient of acetonitrile in 0.1% TFA (0–70% in 45 min, flow 300 μL/min). Peak D-3+8+9 was collected, reduced, and on reanalysis gave three peaks (middle panel). Peak D2 was collected and on reduction and reanalysis eluted slightly later but was otherwise unchanged (lower panel).

ysis of the reduced peptide; one of these (Cys-37) had been determined to be cysteine in a peptide (E-1) derived from V8 digestion of reduced and carboxymethylated protein. It seemed reasonable to suppose that the other amino acid ([Cys]-47) might also be cysteine. The other peptide that moved had three N-terminals (D-3, D-8, and D-9) and split into three peaks on collection, reduction, and reanalysis. A tryptic peptide (T-13/T-23, Table 1) was isolated, which defined a disulfide bond between Cys-64 and Cys-160. By elimination, the remaining disulfide bond holding D-3, D-8, and D-9 together must have been between Cys-136 and Cys-152. These disulfide bond assignments have been confirmed with tryptic peptides that had two N-terminals; T-21/T-22, T-13/T-23, and T-8/T-11 (Table 1). The latter peptides have not, however, been reduced to confirm that they are disulfide bonded together. However, when sequencing these pairs of peptides, a small peak eluting between phenylthiohydantoin (pth)-alanine and pth-tyrosine was observed at the cycle where the putative second cysteine in each pair would be expected (which would be cystine). This peak is in the location of a pth-cystine derivative obtained when cystine is subjected to Edman degradation in an ABI 477 sequencer. This is further evidence that the amino acid at position 47 is cysteine.

Comparison of the sequence with those in Genbank (release 68) and the PIR (release 28) databases indicated that this protein was in the C-type lectin family and was >50% similar to tetranectin (45% identity, 18% conserved changes) (Fig. 5). The disulfide bonds are in the positions that have been predicted by Fuhlendorff et al. (1987) for mammalian tetranectin and the C-terminal two disulfide bonds are also consistent with the consensus for the C-type lectin family (Drickamer, 1988), of which tetranectin is a member.

Discussion

We have isolated a unique protein from 4 M guanidine-HCl extracts of shark neural arch cartilage. Its high yield after CsCl gradient centrifugation and gel filtration indicates that on a molar basis, it is one of the main noncollagenous, nonproteoglycan components of the tissue. As

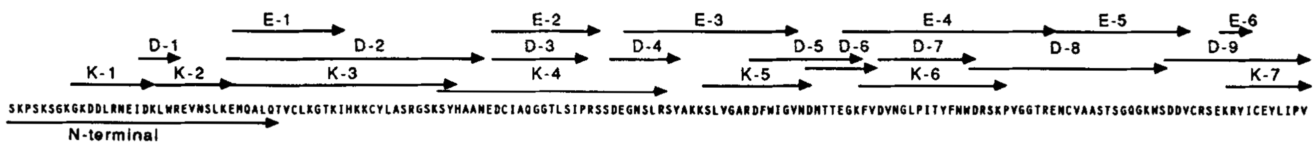

Fig. 4. Alignment of peptides from digests with endoprotease Lys-C (K-), endoprotease Glu-C (E-), and endoprotease Asp-N (D-), which define the sequence of the shark lectin-like protein. Peptides are numbered from the N-terminal and correspond to the peaks in Figure 3 and sequences in Table 1.

Table 1. *Yields, in pmol, of amino acids found in peptides from which the complete sequence was derived*[a]

	N-terminus	Lys-C			Asp-N			Trypsin	Glu-C	
1 Ser	135									
2 Lys	735									
3 Pro	651									
4 Ser	81									
5 Lys	453									
6 Ser	78									
7 Gly	390									
8 Lys	444	**K-1**								
9 Gly	330	21								
10 Lys	432	17								
11 Asp	391	5								
12 Asp	345	12								
13 Leu	369	14								
14 Arg	414	n.q.								
15 Asn	234	3								
16 Glu	189	4								
17 Ile	195	7				**D-1**				
18 Asp	201	3				105				
19 Lys	150	1				204		**T-6**		
20 Leu	270					177		188		
21 Trp	36					9		41		
22 Arg	21					52		12		
23 Glu	69									
24 Val	108									
25 Asn	96									
26 Ser	36									
27 Leu	72									
28 Lys	99			**K-3**	**D-2**		**D-2**	**T-8 &**		**rcm**
29 Glu	21			82	82		82	34	**E-1**	**E-1**
30 Met	72			148	146		146	107	31	51
31 Gln	93			100	98		98	44	21	90
32 Ala	21			73	71		71	160	34	69
33 Leu	30			120	114		114	112	33	99
34 Gln	45			80	75		75	64	17	72
35 Thr				95	92		92	25	7	33
36 Val				96	92		92	68	29	87
37 Cys				–	–		–	C-C?	–	54*
38 Leu				112	103		103	56	32	48
39 Lys				65	59		59	2	6	33
40 Gly				65	59		59		17	12
41 Thr				53	49		49		10	
42 Lys				38	38		38		3	
43 Ile				70	61		61		14	
44 His				10	5		5		n.d.	
45 Lys				40	34		34			
46 Lys				41	43		43	**T-11 &**		
47 (Cys)				–	–		–	–		
48 Tyr				28	23		23	62		
49 Leu				49	40		40	103		
50 Ala				16	13		13	160		
51 Ser				8	6		6	18		
52 Arg				12	59		59	4		
53 Gly				16	16		16			
54 Ser			**rcm**	3	3		3			
55 Lys		**K-4**	**K-4**	10	10		10	**T-13#**		
56 Ser		53	12	2	2		2	23		
57 Tyr		63	46	8	8		8	122		
58 His		7	8	n.q.	1		1	34		
59 Ala		71	42	3	3		3	136		
60 Ala		86	78	4	4		4	184		
61 Asn		61	8	3	3		3	18		
62 Glu		55	9		1	**D-3**	1	28	**E-2**	
63 Asp		35	3			31		18	10	

(*continued*)

Table 1. *Continued*

	N-terminus	Lys-C		Asp-N		Trypsin		Glu-C
64 Cys		-	4*	-			C-C?	-
65 Ile		52	6	16			55	53
66 Ala		52	8	15			36	37
67 Gln		34	2	16			18	21
68 Gly		40	2	23			24	62
69 Gly		49	6	26			23	56
70 Thr		19	n.q.	11			8	4
71 Leu		23	2	9			24	13
72 Ser		7	n.q.	2			3	2
73 Ile		16	n.q.	8			14	23
74 Pro		13		6			9	12
75 Arg		12		3				2
76 Ser		2		2				1
77 Ser		4		**D-4**				
78 Asp		4		60				1
79 Glu		6		45				**E-3**
80 Gly		3		36				40
81 Asn		3		33				18
82 Ser		1		6				9
83 Leu		1		42				41
84 Arg				42				9
85 Ser				5				8
86 Tyr				30				15
87 Ala				30				22
88 Lys				33				7
89 Lys		**K-5**		43				7
90 Ser		5						4
91 Leu		16						14
92 Val		28						12
93 Gly		24						9
94 Ala		17						8
95 Arg		19		**D-5**	**D-5**	**T-17**		2
96 Asp		10		144	56	56		3
97 Phe		15		138	101	101		3
98 Trp		4		27	44	44		2
99 Ile		11		87	130	130		2
100 Gly		10		48	81	81		3
101 Val		10		87	112	112		2
102 Asn		8		**D-6**	22	22		
103 Asp				634	22	22		
104 Met				589	90	90		
105 Thr				514	21	21		
106 Thr				485	16	16		
107 Glu				474	21	21		**E-4**
108 Gly				396	36	36		176
109 Lys		**K-6**		495	9	9	**T-18**	158
110 Phe		42		239			159	253
111 Val		41		86	**D-7**		157	233
112 Asp		31			89		46	93
113 Val		37			71		138	153
114 Asn		18			86		68	33
115 Gly		19			80		102	51
116 Leu		20			43		135	73
117 Pro		12			78		40	37
118 Ile		9			50		102	44
119 Thr		3			66		26	21
120 Tyr		6			56		41	24
121 Phe		7			51		65	33
122 Asn		6		**rcm**	53		52	12
123 Trp		1		**D-8**	12		39	5
124 Asp		3		38			18	1
125 Arg		5		22			4	3

(continued)

Table 1. *Continued*

	N-terminus	Lys-C	Asp-N	Trypsin		Glu-C
126 Ser		1	15			1
127 Lys		2	32			2
128 Pro			25			3
129 Val			18			2
130 Gly			21			5
131 Gly			24			11
132 Thr			18			1
133 Arg			12	**T-21@**		
134 Glu			13	26		**E-5**
135 Asn			12	43		114
136 Cys			*10	-		-
137 Val			10	148		233
138 Ala			13	106		169
139 Ala			15	110		183
140 Ser			4	14		20
141 Thr			8	30		52
142 Ser			1	15		10
143 Gly			5	42		68
144 Gln			4	20		23
145 Gly			4	34		51
146 Lys				5	**T-22@**	10
147 Trp			**rcm**		48	7
148 Ser			**D-9**		15	3
149 Asp			35		20	6
150 Asp			40		25	5
151 Val			43		98	7
152 Cys			*36		C-C?	-
153 Arg			47		4	n.q.
154 Ser			14			1
155 Glu		**rcm**	25			**E-6**
156 Lys		**K-7**	32			39
157 Arg		12	23		**T-23#**	3
158 Tyr		54	27		85	29
159 Ile		117	34		113	53
160 Cys		*30	*28		-	-
161 Glu		30	21		80	**E-7**
162 Tyr		29	21		187	229
163 Leu		71	6		78	82
164 Ile		40	3		63	65
165 Pro		8	2		27	14
166 Val		4	1		10	4

[a] The values here are background-corrected values from the ABI 477 sequencer. rcm, reduced and S-carboxymethylated; *, cysteine, determined as carboxymethyl cysteine; n.q., not quantitated; –, nothing detected; #, @, &, sequenced as two equimolar peptides disulfide bonded together; C–C?, location of a peak eluting between pth-Ala and pth-Tyr, thought to be a pth derivative of cystine.

much as 50 nmol could be isolated from 20 g of tissue. Sequence analysis indicated that it had 166 amino acids, three disulfide bonds, and was significantly similar to a mammalian protein, tetranectin.

A major component of cartilage, including shark cartilage, is the large aggregating proteoglycan, aggrecan. The C-terminal of this proteoglycan is in the C-type lectin family (Drickamer, 1988). We had expected to find catabolic products of aggrecan in the low density region of the preparative cesium chloride gradient and originally thought that the protein described here might derive from the C-terminal globular domain of aggrecan. However, the protein described here is 45% identical to human tetranectin, as opposed to 21% identical to the human aggrecan C-terminal domain (Fig. 5) and therefore is unlikely to derive from the proteoglycan that is present in shark cartilage.

Tetranectin is also in the C-type lectin family. It forms a tetrameric protein with four identical peptide chains (20 kDa) that are noncovalently associated with each other

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NGLQADLSSFKSQELNERNEASDLLERLREEVTKLRMELQVSSGFVCNTCPEKWINFQRKCYYFGKGTK   182
                                             .   :  :     .:::    :.:
EIESSSLLYSGEETHTVETATSPTDASIPASPEWKR  ESESTAADQEVCEEGWNKYQGHCYRHFPDRE 2207
             .    .     :    .   :          : : ::     :    ::
             SKPSKSGKGKDDLRNEIDKLWREVNSLKEMQALQTVC LKGTKIHKKCYLASRGSK    55
              :   . :  ..:.. .: : .::  ::: ::::::: :::::.: ::.::   .:
EPPTQKPKKIVNAKKDVVNTKMFEELKSRLDTLAQEVALLKEQQALQTVC LKGTKVHMKCFLAFTQTK    68

 QWVHARYACDDMEGQLVSIHS      PEEQDFLTKHASHTGSWIGLRNLDLKGEFIWVDGSHVDY     242
     :   :    : :                  .        :::. ..   : :.  .:  . :
 TWVDAERRCREQQSHLSSIVT      PEEQEFVNNNA QDYQWIGLNDRTIEGDFRWSDGHPMQF    2266
 ..  :.  :  :   ::                          :::.:: : :: :   .: :. .
 SYHAANEDCIAQGGTLSIPRSSDEGNSLRSYAKKSLVG ARDFWIGVNDMTTEGKFVDVNGLPITY     120
 ..: : ::::  ::::  : .  : . :  : . :.     . :.:.:::  ::  ::. :. : :
 TFHEASEDCISRGGTLGTPQTGSENDALYEYLRQSVGN EAEIWLGLNDMAAEGTWVDMTGARIAY     133

 SNWA  PGEPTSRSQGEDCVMM  RGSGRWNDAFCDRKLGAWVCDRLATCTPPASE.....  IgE Receptor
  ::             :.::..     : : :  :        :. :.
 ENWR PNQPDNFFAAGEDCVVMIWHEKGEWNDVPCNYHLP FTCKKGTATTYKRRL.....  Human Aggrecan
  ::             :.::..     : : :  :      . :
 FNWD   RSKPVGGTRENCVAASTSGQGKWSDDVCRSEKR YICEYLIPV             Shark lectin
  ::.      : ::  :::...:   .::: :  :: .   :::.. : .
 KNWETEITAQPDGGKTENCAVLSGAANGKWFDKRCRDQLP YICQFGIVL             Tetranectin
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Fig. 5. Alignment of the shark lectin-like protein described here with tetranectin (Fuhlendorff et al., 1987), part of the C-terminal domain of human aggrecan (Doege et al., 1991), and the sequence of human immunoglobulin receptor (Kikutani et al., 1986). Numbering is from the N-terminal in the case of shark lectin and tetranectin and from the beginning of the signal sequence in the case of aggrecan and IgE receptor. A colon (:) indicates identity with the shark-derived sequence; a single dot indicates a conserved change. Gaps have been inserted to improve the alignment.

(Clemmensen et al., 1986). It was originally isolated from serum by virtue of its ability to bind to the plasminogen kringle 4-domain.

The function of tetranectin is not known. It is capable of binding chondroitin sulfate and heparan sulfate proteoglycans and it has been suggested that it is involved in the packaging of molecules destined for exocytosis, for example, hormones or glycoproteins (Clemmensen, 1989). Tetranectin, or a tetranectin-like molecule, is found in the extracellular matrix of WI-38 embryonal fibroblasts (Clemmensen et al., 1991). We do not know what the function of tetranectin might be in shark cartilage. It is possible that it simply accumulates with time, binding to the negatively charged glycosaminoglycan chains. Its estimated pI is 9.4 and arginine and lysine represent 16% of the total amino acids, so this is a distinct possibility. If it is involved in packaging of glycoproteins for extracellular export, then it might aid in preventing glycosaminoglycans from becoming fully hydrated intracellularly, reducing their bulk in the process. As our isolation conditions involve extreme dissociative conditions, we do not, as yet, know whether the shark protein forms tetramers. To date, we have not found tetranectin in mammalian cartilage. However, it may be present at low levels.

A major difference between the sequence described here and the human sequence described by Fuhlendorff et al. (1987) is the presence of 13 additional amino acids at the N-terminal of the latter. If these were present at one stage in the shark protein, it is possible that these are removed by extracellular proteolytic processing. Most C-type lectins have additional structures at the N-terminal, either membrane-spanning segments in the receptor forms, or a collagen-like region in the case of mannose receptors or glycosaminoglycan-containing domains in versican and aggrecan (for a review, see Drickamer, 1988). The difference in the N-terminals of the shark and human tetranectins is strong evidence that they derive from a larger molecule.

Comparison of predicted secondary structure among C-type lectins using a consensus of Chou–Fasman and Robson–Garnier algorithms did not reveal any predominant features, except for a strong tendency toward alpha helix in the region prior to the first cysteine. This helix would have an amphipathic structure. The similarity between human tetranectin and the shark protein is particularly high in this region, indicating that it may be functionally important. It is noteworthy that the C-terminal half of this protein and tetranectin are very simi-

lar to C-type lectins, whereas the N-terminal half is not. It is likely that lectin-like activity is predominantly in the C-terminal 74 amino acids whereas the N-terminal is involved in a specific tetranectin function.

Materials and methods

Tissue extraction

Neural arch from a reef shark (*Carcharhinus springeri*) was cleaned of peripheral tissue and sliced into thin (<1-mm) slices. Extraction and purification of nonproteoglycan components was by standard methods as described previously (Neame et al., 1990a,b). Briefly, the slices were extracted with 4 M guanidine HCl + protease inhibitors (overnight) in 50 mM sodium acetate, pH 6.5. The extract was filtered on a Buchner funnel to remove the tissue slices and dialyzed against nine volumes of 50 mM sodium acetate to bring the guanidine concentration to 0.4 M, reassociating proteoglycan aggregates. Solid cesium chloride was added to bring the density to 1.5. The extract was centrifuged at 40,000 rpm for 40 h (10 °C) in a 50.2 Ti rotor (Beckman) to remove proteoglycans.

Protein purification

The density gradient tubes were sliced into four sections and the upper section (density <1.35) was used for the remainder of the preparation. This fraction was dialyzed against water overnight, concentrated approximately 10-fold on a Savant Speedvac, and adjusted to 4 M by the addition of solid guanidine hydrochloride. The solution was then applied to a Sephacryl S-300 column (2.6 × 90 cm) that had been equilibrated in 50 mM Tris-HCl, 4 M guanidine HCl, pH 6.5. Individual fractions were then further analyzed by application to a Brownlee RP-300 column (4.6 × 25 cm) equilibrated in 0.1% trifluoroacetic acid (TFA) and eluted with a gradient of acetonitrile in 0.1% TFA (flow rate 1 mL/min, 0–70% acetonitrile in 45 min).

Approximately 1 mg of purified protein could be obtained from 20 g of cartilage.

Sequence analysis

Protein was digested in its native state or after reduction with dithiothreitol and carboxymethylation with iodoacetic acid. Individual peaks were analyzed by Edman degradation and amino acid analysis. Sequencing of intact protein or of peptides derived from proteolysis of shark lectin was performed on either an Applied Biosystems 477A or a 473A instrument. Amino acid analysis was performed on an Applied Biosystems 420A-H automated hydrolysis and precolumn derivatization instrument.

Proteases (sequencing grade; trypsin [E.C. 3.4.21.4], endoprotease Asp-N, endoprotease Lys-C [E.C. 3.4.99.30], endoprotease Glu-C [E.C. 3.4.21.19]) were from Boehringer Mannheim and were used as described in the manufacturers' literature, at enzyme:substrate molar ratios between 1:25 and 1:50. Other reagents were from Sigma. The HPLC columns were Brownlee RP-300 (for protein preparation: 4.6 × 250 mm; for peptide separation: 2.1 × 30 mm). Gel filtration columns Sephacryl S-300, Superose 12) were from Pharmacia-LKB.

Acknowledgments

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