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THE INTERACTION OF LINK PROTEINS WITH PROTEOGLYCAN MONOMERS IN THE ABSENCE OF HYALURONIC ACID

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SUMMARY. Cartilage proteoglycan aggregates contain three classes of interacting components: proteoglycan monomers, hyaluronic acid and link proteins. Direct evidence is presented for a link protein to proteoglycan monomer association which hitherto has only been presumed to occur. Thus, when mixtures of purified link proteins and proteoglycan monomers were subjected to ultracentrifugation or gel chromatography under 'associative' conditions, link proteins were found to fractionate with proteoglycan.

INTRODUCTION. Cartilage proteoglycans associate with hyaluronic acid and link proteins to give aggregate structures which are believed to be their principle form *in vivo*. A model of such proteoglycan aggregate structure which fits the available data has been proposed (1). Features of the model are interactions between its three types of component. The interaction of many proteoglycan monomers with a single strand of hyaluronic acid (interaction A, Fig. 1) was first recognized by Hardingham and Muir (2). Since then, many features of this association have been documented (3-5). In contrast, evidence for the interaction between link proteins and hyaluronic acid (interaction C, Fig. 1) is limited (6,7) and for link proteins to proteoglycan monomers (interaction B, Fig. 1) is lacking.

In this paper, evidence that interactions between link proteins and proteoglycan monomers do occur is presented. This finding should stimulate efforts to demonstrate whether similar interactions are important *in vivo*.

METHODS. Uronic acid and protein were determined by automated carbazole and Lowry procedures, respectively (8). For assay of radioactivity, aqueous samples were diluted to 2 ml with water, 'Scintiverse' (Fisher Chemical Co.) (5 ml) added, and the resulting gels counted in a Packard Tricarb Liquid Scintillation Spectrometer.

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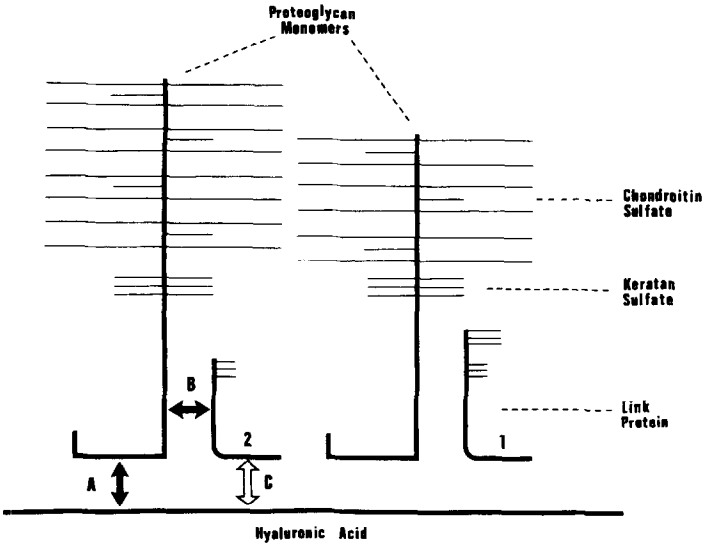

Figure 1
Model of Proteoglycan Aggregate Interactions. Interactions between A, proteoglycan monomer and hyaluronic acid; B, proteoglycan monomer and link protein; C, link protein and hyaluronic acid. 1, link protein 1; 2, Link protein 2.

Polyacrylamide gel electrophoresis in SDS was performed as described previously (9). Prior to scintillation counting, gels were sliced (1.0 mm thick) using a Gilson 'Aliquogel' Fractionator.

A column (1.0 x 100 cm) of Sepharose CL 6B was equilibrated with 4 M guanidine containing 0.05 M sodium acetate, pH 5.8. Another column of identical dimensions but containing Sepharose CL 2B was equilibrated with 0.5 M sodium acetate, pH 7.0. Flow rates in both cases were 3-4 ml/h and fractions were collected at 30 min. intervals.

Proteoglycans were extracted from bovine nasal cartilage by the procedure of Sajdera and Hascall (10) and employing the protease inhibitors recommended by Oegema et al. (11). Proteoglycan aggregate (A1), proteoglycan monomer (A1D1) and link protein (A1D4) preparations were isolated by equilibrium density gradient centrifugation as described previously (9) with the exception that centrifugations were in a Beckman Spinco 50.2 Ti rotor operated at 40,000 rpm. The proteoglycan monomer preparation was recovered at a buoyant density >1.70 and should therefore be free of hyaluronic acid which has a buoyant density of 1.46 in CsCl containing 4 M guanidine.

A sample (41.2 mg) of A1D4 was partially N-acetylated with 500 μCi of [1-^{14}C]-acetic anhydride (120 mCi/mmol, Amersham) according to the method of Riordan and Vallee (13). The [^{14}C]-A1D4 product had a specific

Abbreviation: SDS, sodium dodecyl sulfate. The abbreviations for proteoglycan aggregate (A1), proteoglycan monomer (A1D1) and 'link protein' preparation (A1D4) follow the notation suggested by Heinegård[12].

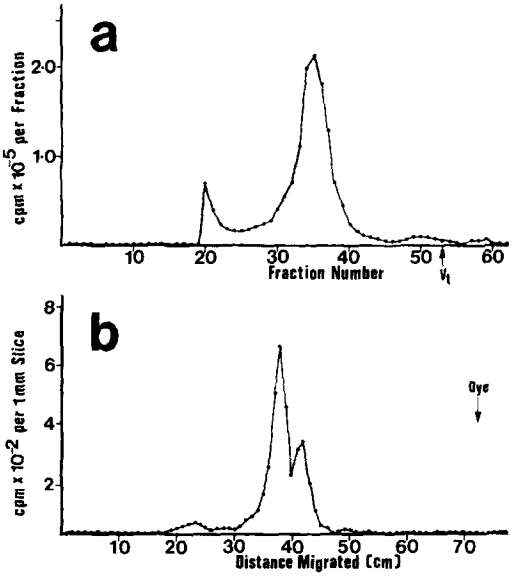

Figure 2
a. Fractionation of [^{14}C]-A1D4 on Sepharose CL 6B in 4 M guanidine hydrochloride, 0.05 M sodium acetate, pH 5.8.
b. Acrylamide gel electrophoresis in SDS of a sample (100 µl) from fraction 35, Figure 2a.

activity of 0.96 µCi/mg dry weight and it can be calculated that <4% of the constituent amino groups were acetylated. [^{14}C]-A1D4 (1.37 µCi) and unlabeled A1D4 (5 mg) were chromatographed on Sepharose CL 6B in 4 M guanidine containing 0.05 M sodium acetate, pH 5.8. Fractions collected from the column were assayed for radioactivity (Fig. 2a). Aliquots (100 µl) of each fraction were dialyzed, freeze dried and examined by polyacrylamide gel electrophoresis in SDS in order to locate the link proteins. The link proteins free of other proteins and protein rich proteoglycans were identified in fractions 33 - 37 which coincide with the major peak of radioactivity (Fig. 2a). Duplicate samples of fraction 35 were fractionated by gel electrophoresis and stained with Coomassie Blue or sliced and assayed for radioactivity (Fig. 2b). More than 95% of the radioactivity was coincident with the two major Coomassie Blue stained bands of link proteins 1 and 2 plus 3. Therefore, this sample of purified labeled link proteins was employed in experiments on proteoglycan monomer to link protein interactions reported below.

Hyaluronic acid from a mesothelioma was a gift from Dr. Lennart Roden. It was exhaustively digested with papain and fractionated by dissociative equilibrium density gradient centrifugation. The fraction recovered at a buoyant density between 1.45 and 1.50 contained <1.0% protein and had a glucosamine to galactosamine ratio >240:1.

RESULTS AND DISCUSSION. Proteoglycan monomer (A1D1, 4 mg) and [^{14}C]-link protein (36.3 µg) were mixed in 4.0 M guanidine, 0.05 M sodium

acetate, pH 5.8 (1.0 ml) at room temperature (ie. 'dissociative' conditions). Other mixtures involving [^{14}C]-link proteins, proteoglycan monomer and hyaluronic acid were prepared as detailed in the legend to Figure 3. In the ternary mixture, approximately physiological proportions of the components were employed, as the aim was to reconstitute proteoglycan aggregate. Both binary mixtures contained a relative excess of link proteins in order to achieve optimal binding of the link proteins. Mixtures were diluted with 0.05 M sodium acetate, pH 5.8 (9 vol.), adjusted to a density of 1.67 by addition of CsCl and then centrifuged (ie. under 'associative' conditions) in the 50.2 Ti rotor using 11.5 ml polyallomer tubes at 30,000 rpm for 64h at 15°. The tube contents were collected as ten fractions numbered 1 to 10 from bottom to top, respectively. From the proteoglycan-link protein mixture, all uronic acid (96% recovered) and therefore proteoglycan was confined to the bottom two fractions (Fig. 3c), which was also the finding when proteoglycan monomer alone was similarly centrifuged (Fig. 3f). Radioactivity (64% recovered) closely followed the uronic acid profile (Fig. 3c) indicating association between proteoglycan monomers and link protein. In the absence of proteoglycan, no radioactivity due to link proteins was recovered from the tube (Fig. 3d). As the link proteins are water insoluble under associative conditions, this result is to be expected. Indeed some radioactivity could be recovered from the tube cap by rinsing in 4 M guanidine. In the presence of hyaluronic acid and proteoglycan monomer, the link proteins again associate as judged by coincidence of uronic acid and radioactivity (100% and 96% recovered, respectively) in the bottom fractions (Fig. 3a). When hyaluronic acid was centrifuged in company with [^{14}C]-link proteins, some retention of radioactivity (30% recovered) resulted, although the radioactivity and uronic acid profiles were not closely matched (Fig. 3b).

It may be considered possible that the demonstrated association

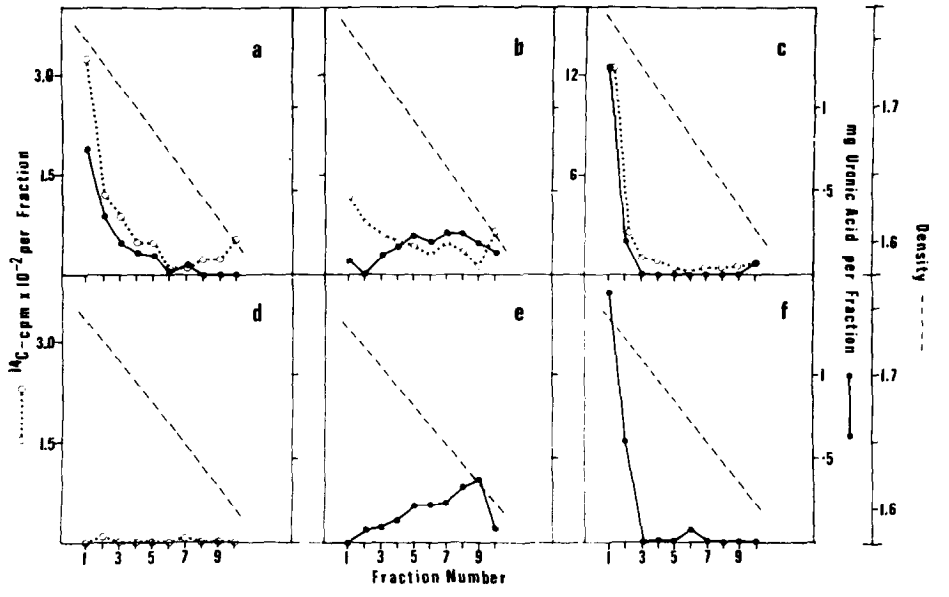

Figure 3
'Associative' Density Gradient Centrifugation Profiles
All mixtures were in 4 M guanidine hydrochloride, pH 5.8 (1 ml), before diluting with 9 volumes of 0.05 M sodium acetate, pH 5.8. CsCl was added to a density of 1.67 g/ml. Individual mixtures contained:

	AlDl	Hyaluronic Acid	[^{14}C]-Link Proteins
	mg	mg	µg
a	4.0	0.05	12.1
b	--	2.50	36.3
c	4.0	--	36.3
d	--	--	36.3
e	--	2.50	--
f	4.0	--	--

between AlDl and [^{14}C]-link proteins (Fig. 3c) was due to the presence of a small amount of hyaluronic acid in the AlDl preparation. To check this possibility, mixtures were also analyzed by gel chromatography. A mixture of AlDl and [^{14}C]-link proteins chromatographed on a column of Sepharose CL 2B in 0.5 M sodium acetate, pH 7.0 gave an elution profile as illustrated in Fig. 4d. Analysis for uronic acid revealed a typically broad included peak for proteoglycan monomer (cf. Fig. 4c) with which the majority of the recovered counts (recovery 72%) were associated.

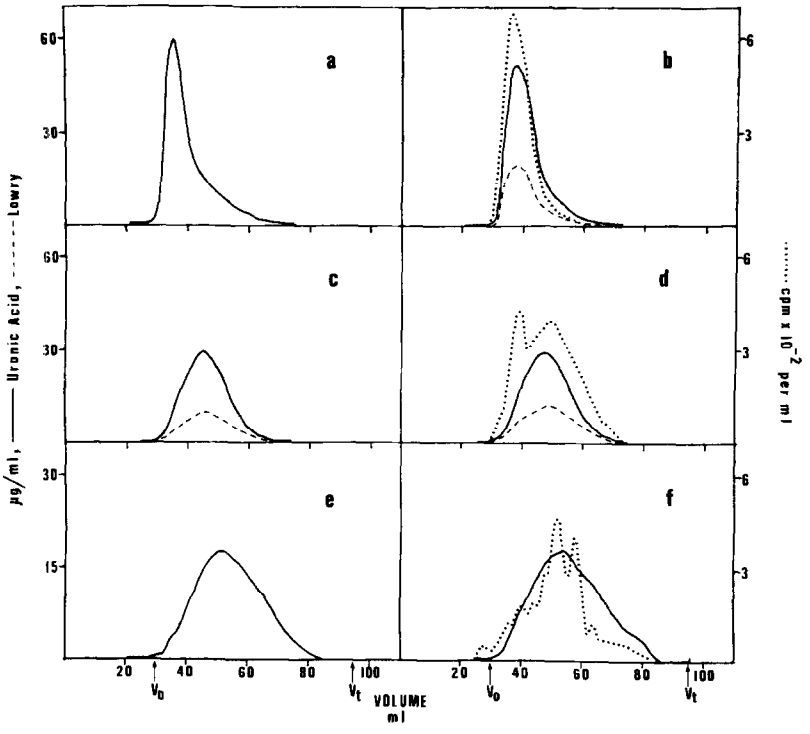

Figure 4

Chromatography on Sepharose CL 2B in 0.5 M sodium acetate, pH 7.0. Mixtures were prepared in 4 M guanidine hydrochloride, 0.05 M sodium acetate, pH. 5.8 (1 ml), before dialysing against 9 volumes of 0.05 M sodium acetate, pH 5.8. Individual mixtures contained:

	A1	A1D1	Hyaluronic Acid	[^{14}C]-Link Proteins
	mg	mg	mg	μg
a	4.0	--	--	--
b	--	4.0	0.05	23.5
c	--	4.0	--	--
d	--	4.0	--	70.0
e	--	--	2.50	--
f	--	--	2.50	70.0

Furthermore, an increase in the Lowry protein content of each fraction across the profile was evident. Therefore, under associative conditions, interaction between [^{14}C]-link proteins and proteoglycan monomers is clearly demonstrated. The sharp peak of radioactivity at K_{av}=0.05 may result from self association of link proteins, interaction of link proteins with a trace of hyaluronic acid, or the relatively greater

association of link proteins with proteoglycan monomers of larger size. With proteoglycan monomers and hyaluronic acid, [^{14}C]-link proteins associate as judged by a peak of radioactivity at K_{av}=0 (recovery of radioactivity, 88%), which is coincident with the uronic acid elution profile (Fig. 4b). Evidently proteoglycan aggregate has been reconstituted (cf. the elution profiles of A1 and proteoglycan monomer alone, Fig. 4a and 4c, respectively.) Again there is some evidence for association of [^{14}C]-link proteins with hyaluronic acid alone (Fig. 4f).

The presentation of experimental evidence for a link protein to proteoglycan monomer interaction serves to establish another presumed feature of the model of proteoglycan aggregate structure (1, and Fig 1). Indeed, it may be a particularly important interaction whereby the hyaluronic acid binding region of the proteoglycan can assume a conformation which favors its stable association with hyaluronic acid. Of course, many questions remain concerning the nature of the link protein to proteoglycan monomer interaction. Do both major link proteins interact similarly? As they share many compositional and structural characteristics (14), it is probable that they do. Further work is being undertaken to determine what specific structural features possessed by link proteins are required for interaction.

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