Vol. 106, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1982 Pages 158-163

CONCANAVALIN A-BINDING LINK PROTEIN IN THE PROTEOGLYCAN AGGREGATE OF HYALINE CARTILAGE

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Received March 24, 1982

Two link proteins derived from the proteoglycan aggregates formed *in vitro* after extraction and reassociation were recovered in SDS-electrophoresis. About 67 % of the link protein b of human rib cartilage, and 8 % of that of canine articular cartilage bound specifically to Concanavalin A-Sepharose 4B in the presence of 1 M NaCl at pH 7.2. The link proteins proved to be species specific, but both the Ouchterlony's double diffusion and solid-phase radioimmunoassay failed to reveal any difference in the antigenic profile of link proteins obtained from the species examined. The A_5 and A_6 fractions of cartilage extract (ie. the top fraction of the associative density gradient centrifugation) were also found to contain Concanavalin A-binding proteins, of which 56 % was estimated to be link protein.

INTRODUCTION

Proteoglycan monomers associate with hyaluronic acid to form large aggregates which are stabilized by glycoproteins (1, 2). The glycoproteins are referred to as link a and link b (3,4,5) can interact noncovalently with both the protein core of proteoglycan monomers and with hyaluronic acid (5,6,7). Soluble link proteins appear to contain similar or identical polypeptide subunits with distinct structures of oligosaccharide moieties (3,4,8), therefore they are immunologically indistinguishable from each other (9).

In this study a subfraction of link protein b was found to interact specifically with Concanavalin A (Con A), which interaction permits to separate it from the other components of the proteoglycan aggregate. In addition, comparative studies for the Con A-binding proteins of different species were also made.

MATERIALS AND METHODS

Link proteins were isolated by repeated equilibrium density gradient centrifugation under dissociative condition from human rib and canine articular cartilages, and the A_1D_5-$_6D_6$ fractions were subsequently purified on Sephacryl S-300 Superfine (Pharmacia) in the presence of 4 M guanidine-HCl and 0.15 M sodium acetate at pH 6.2 (4,10). The link-protein containing fractions were

0006-291X/82/090158-06$01.00/0

pooled, concentrated on Amicon (Minicon B 15) and then rechromatographed on Sephacryl S-300 in 4 M guanidine-HCl. The protein concentrations were determined by Lowry's method (11) using bovine serum albumin as a standard. Fractions from the density gradient centrifugation were also examined.

Link proteins obtained from proteoglycan aggregates were loaded on a Con A-Sepharose 4B (Pharmacia) column equilibrated in 1 M NaCl and 1 mM $MgCl_2$ buffered with 0.05 M Tris-HCl at pH 7.2. The proteins associating to Con A were desorbed by the linear concentration gradient of methyl-α-D-mannoside (Sigma). Fractions were collected, occasionally concentrated or exposed to enzymatic treatments, and were analysed both with SDS-electrophoresis and with immunological procedures.

Polyacrylamide gel electrophoresis was performed in the presence of 2 % sodium dodecyl sulfate and 0.15 M Tris-HCl at pH 8.0 (3,4). The gels were stained with Coomassie blue R 250 (Serva), densitometred and compared with molecular weight standards.

Monospecific antibodies against link proteins were obtained by immunosorbent procedure from the rabbit immunosera raised to the proteoglycan aggregate (10). Ouchterlony gel diffusion in agarose, and solid-phase radioimmunoassay (12,13) were performed to identify and quantify the antigenic profile of link proteins obtained by affinity chromatography.

RESULTS

The link protein fraction derived from the proteoglycan aggregate of either human rib or canine articular cartilages displayed two well separable bands (Fig. 1), with molecular weights ranging between 43,000 and 50,000 daltons. Pronase and pepsin completely hydrolized the link proteins, although, they proved to be relatively resistant to tryptic treatment in a four hour experiment. The link protein fraction containing both link a and link b formed a single precipitin line with specific antibodies (Fig. 2). Immune reactions failed to reveal any cross reactivity between the link proteins derived from hyaline cartilages of different species (Fig. 2).

A fraction of link protein bound to Con A-Sepharose, which could be desorbed with methyl-α-D-mannoside (Fig. 3). The interaction of link protein with Con A-Sepharose could be inhibited by methyl-α-D-mannoside, mannose, glucose and N-acetyl-D-glucosamine, but it was not influenced at 0.5 M concentrations of galactose, glucuronic acid, sialic acid and methyl-α-D-pentose derivatives.

About 92 % of the applied link protein was recovered by chromatography procedure (Table I). The Con A-binding protein proved to be link protein b (Fig.1), of which 67 % of the original quantity was able to bind to this lectin in triplicated experiments. It was a peculiar observation, however, that the total quantity of link protein a increased, in spite of the protein-loss that occurred

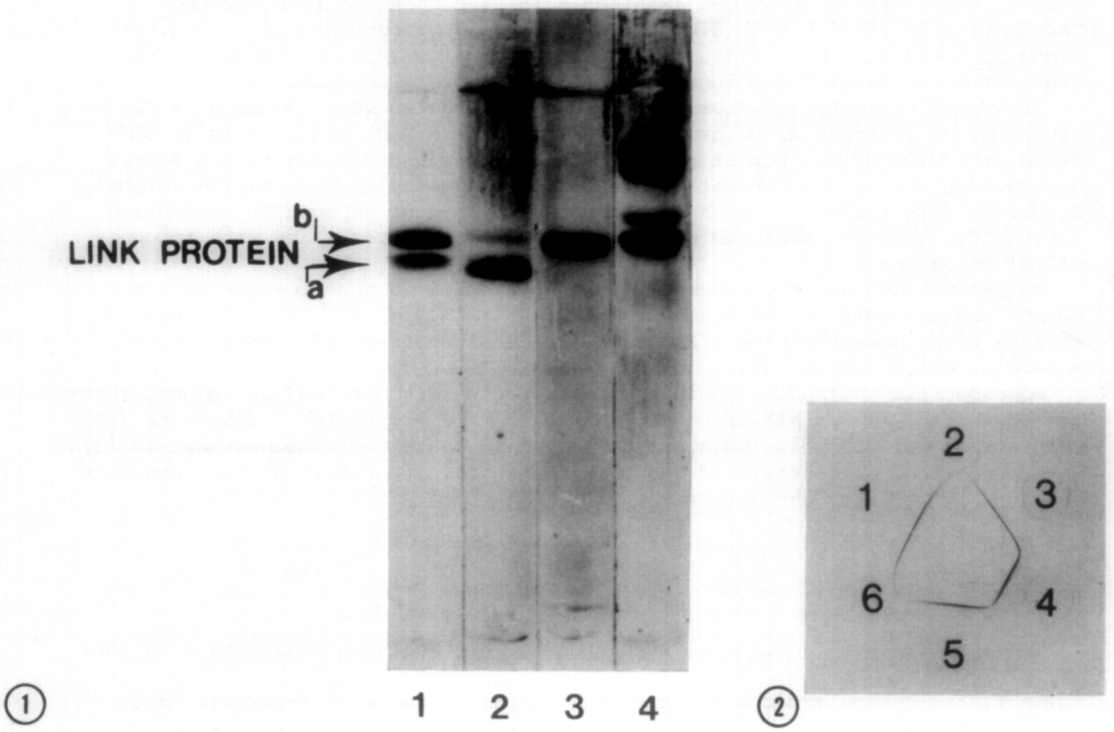

Fig. 1. SDS-electrophoresis (10 % polyacrylamide gel) of the fractions obtained from human rib cartilage. Link proteins before (1) and after (2) the Con A-Sepharose affinity chromatography. The Con A-binding link protein b (3), and the Con A-binding proteins of A_5 fraction obtained by equilibrium density gradient centrifugation under associative conditions (4) were desorbed with 0.3 M methyl-α-D-mannoside in 1 M NaCl and 1 mM $MgCl_2$. 20 µg, 15 µg, 15 µg and 50 µg proteins were applied on gels, respectively.

Fig. 2. Immunodiffusion in agarose gel. The central well contained monospecific antibodies against link protein fraction of human rib cartilage (75 µg IgG in 50 µl phosphate buffered saline, pH 7.4). The peripheral wells were filled with the following antigen solutions:

wells 1 and 4: 12.5 µg link protein of human rib cartilage,
well 2: 12.7 µg link protein of canine articular cartilage,
well 3: 18.3 µg Con A-binding link protein of human rib cartilage, obtained from the fraction 16 shown in Fig. 3A,
well 5: 12.5 µg link protein of human rib cartilage (as in wells 1 and 4) was digested with 62.5 mU DPCC-treated trypsin (Serva) in 0.01 M $CaCl_2$ and 0.05 M Tris-HCl, pH 8.0, at 37°C for 4 hours,
well 6: 12.5 µg link protein of human rib cartilage was digested with 7.5 PUK units of pronase (Serva) in 0.05 M Tris-HCl, pH 7.4, at 37°C for 4 hours.

in the chromatography (Table I). The proportion of Con A-binding link protein b was only 8 % in canine articular cartilage, and about 12-13 % in the whole link protein fraction of bovine nasal cartilage (10). The linear concentration gradient of methyl-α-D-mannoside revealed two species of Con A-binding protein

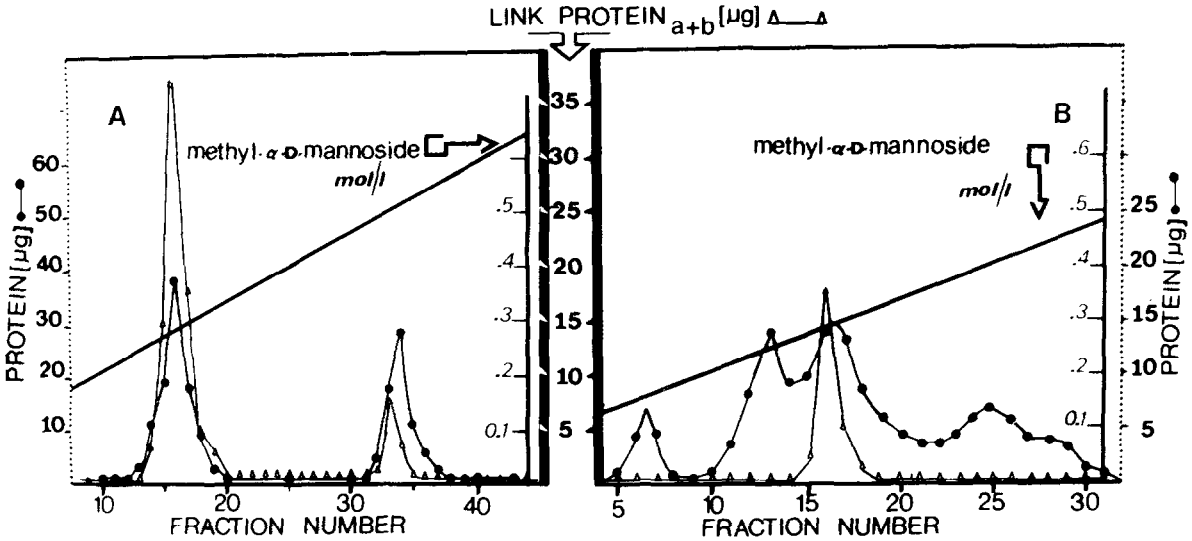

Fig. 3. Elution patterns of Con A-binding proteins. 150 µg link protein [A] or 150 µg protein of A5 fraction [B] obtained from human rib cartilage were loaded on Con A-Sepharose 4B column (0.4x3.6 cm) equilibrated in 1.0 M NaCl and 1 mM $MgCl_2$ buffered with Tris-HCl at pH 7.2. The Con A-binding proteins were desorbed by linear concentration gradient of methyl-α-D-mannoside indicated in both figures in mol/l.

(Fig. 3) derived from human rib cartilage. These subfractions, however, were indistinguishable in SDS-electrophoresis or by immunochemical procedures. In a series of control experiments, broad elution pattern as shown in Figure 3 was obtained when the A_5 or A_6 fractions of human rib cartilage containing nucleic acids, extraneous proteins, collagen peptides, cathepsins, hyaluronic acid etc. (14,15), were loaded on the Con A-Sepharose column. Analytical SDS-

Table I

Subfractions of link proteins obtained from proteoglycan aggregates

Link protein fraction	link protein "a"	link protein "b"	Con A-binding link protein
Human rib cartilage:			
before chromatography	37	63	
after chromatography	43	7	42
Canine articular cartilage:			
before chromatography	13	87	
after chromatography	12.5	70	7

Concentration of link protein was measured by solid-phase radioimmunoassay test (Fig.3), and the relative proportion of subfractions are expressed in per cent, calculated by SDS-electrophoresis in densitometer (Fig.1).

electrophoresis, however, revealed several protein bands even in the rechromatographed material (Fig. 1). The band with the mobility of the link protein b represented 22 % of the total Con A-binding proteins of A_5+A_6 fraction in a densitometer, but 56 % of that proved to be link protein with radioimmunoassay.

DISCUSSION

Earlier studies have revealed that the link protein, even in the absence of hyaluronic acid, can bind to the protein core of proteoglycan monomers, specifically to the hyaluronic acid binding region of core protein (6,7). In spite of that studies, our knowledge on the interaction between link proteins and PG monomers is limited, even it is not yet known which link protein plays a role in the stabilization of the aggregated structure. It is also interesting to note that there is as yet no evidence of immunologically detectable dissimilarities between the link proteins of a given species (9), hence correct information can be expected only by the employment of monoclonal antibodies.

The present study clearly demonstrates the specific binding of a subfraction of link protein b to immobilized Con A via the sugar binding sites of lectin. The active sites of Con A can react with sugars or glycoproteins containing branched terminal non-reducing α-D-mannopyranosyl, α-D-glycopyranosyl or β-D-fructofuranosyl residues, in which list the last one being the least effective (16). Elution profile suggests that the terminal residues are not uniform in the Con A-binding protein. The relative proportion of Con A-binding link protein was found to be highest in the proteoglycan aggregates derived from rib cartilage of old cadavers. Differences in the link protein composition revealed in this work, and the heterogeneity and polydispersity of proteoglycan monomers (14,17,18) suggest that the aggregate composition may depend either on species, age or the cartilage types examined.

The specific interaction of link protein with Con A raises the questions whether or not link proteins associate with proteoglycans via the oligosaccharide residues, and whether or not any link protein bind similarly to the hyaluronic acid binding region of the protein core of proteoglycan monomer which

may also contain Con A-binding residues (19), and whether or not any "link" proteins are recovered in the proteoglycan aggregates formed after extraction and reassociation, which aggregates obtained in vitro may be considered artificial. The presence of a sugar-binding oligopeptide as an undetected component in the aggregate structure could not be excluded, in any case.

ACKNOWLEDGEMENTS

I am grateful to Drs M. Szabolcs and J. Csongor, Institute of Central Laboratory, University of Medicine, Debrecen, for their valuable cooperation in this study. I also wish to thank Mrs G. Nagy and Dr. J. Erdei for their invaluable technical assistance. This work was supported by the Scientific Research Council, Ministry of Health, Hungary (No 2-01-0300-01-1/H).

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