Interaction of Link Protein with Collagen*

Srinivasan Chandrasekhar, Hynda K. Kleinman, and John R. Hassell
From the Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205

Link protein (M, = 42,000) is an integral component of cartilage as well as of some noncartilaginous tissues. In cartilage, it forms a macromolecular complex with cartilage proteoglycan and hyaluronic acid, but its function in other tissues is unknown. We provide evidence here that the link protein of cartilage binds well to native collagen types I and III. The binding occurs only if both link protein and collagen are native. The binding of link protein to collagen type fibrils is higher than to monomeric collagen. Link protein binding to collagen fibrils is saturable and occurs at molar ratio of collagen to link protein of 7:13:1. These data suggest that the link protein binds to collagen and that the binding requires the collagen to be in its native triple helical structure. This interaction may play a role in collagen fibril formation.

Link protein (M, = 42,000) was first discovered in the extracellular matrix of cartilage (1–5). It was originally found in reassociated dissociative extracts of cartilage and was shown to be present as a macromolecular complex along with the cartilage proteoglycan and hyaluronic acid (1–8). Subsequent studies indicated that link protein stabilizes the binding of the cartilage proteoglycan monomer to hyaluronic acid (7–12). These aggregates, along with type II collagen fibers, form the principal components of the cartilage matrix.

The link protein is also present in noncartilaginous tissues including scrotum and sclera (13, 14). However, sclera lacks the cartilage type of proteoglycan. Further, the proteoglycans of sclera appear to associate through their dermatan sulfate side chains, and do not require the link protein to form aggregates (14, 15). Such observations suggest that link protein may serve other as yet unrecognized functions in cartilage and in other tissues.

The interactions of matrix glycoproteins, as well as of proteoglycans, with various collagens regulate the formation of various extracellular matrices. Such interactions are important in establishing the architecture of the matrix and its interaction with the cells in the tissues (reviewed in Refs. 16 and 17). In this paper, we have examined the binding of cartilage link protein to collagen. We find that the link protein has an affinity for the native collagens of the types found in the sclera and in the aorta (types I and III) and that the binding requires the native triple helical conformation of the collagen molecule.

---

MATERIALS AND METHODS

Preparation of Collagen—Type I collagen was prepared from an acid extract of rat tail tendon (18); type II collagen was prepared from the Swarm chondrosarcoma grown in athymic rats (19); type III from a peptic digest of fetal calf skin (20); type IV from a murine tumor, producing a basement membrane (21); and type V from calf skin (22). Ascaris collagen was a gift from C. Sullivan of the National Institute of Dental Research (27). The isolated collagens were examined for purity by SDS-PAGE (24) and amino acid analyses.

Isolation of the Link Protein—Link protein was isolated from the Swarm chondrosarcoma grown in rats by a modification of a previously published procedure (25). The tumor tissue was extracted with an equal volume (weight/volume) of 1 M guanidine hydrochloride, pH 7.0, containing 0.01 M diiodosuccinylbenzylaminepentetraacetic acid, 0.05 M sodium acetate, 0.005 M benzamidine hydrochloride, and 0.1 M 6-amino-hexanoic acid for 18 h at 4 °C. Insoluble material was removed by centrifugation (25,000 × g for 20 min). Cesium chloride was added to the supernatant fraction to a final concentration of 1 g/mL of the extract. The extract was then centrifuged in a 50.2 TI rotor (Sorvall) at 33,000 rpm for 68 h at 10 °C. The bottom one-fifth of the gradient (A1) was collected, diluted with an equal volume of 8 M guanidine hydrochloride, and recentrifuged as described above. The top one-fourth of the gradient (A2) was collected, dialyzed against 8 M urea, and applied to a DEAE-cellulose column equilibrated with 8 M urea (0.05 M Tris, pH 7.4) by centrifugation (25,000 × g for 20 min).

Preparation of Antibody against Link Protein—Antiserum against link protein was raised in young rabbits (27, 28). Link protein (0.2 mg) was injected subcutaneously with complete Freund's adjuvant. Four weeks after the initial immunization, two booster injections (0.1 mg) in incomplete Freund's adjuvant were given in 4-week intervals. Three weeks after the last injection, blood was drawn from the rabbits and the levels of antibody to link protein in the serum were established by an ELISA. The antiserum gave a single line of identity against link protein in the Ouchterlony test.

Specificity of the antiserum was established by an ELISA in which we compared its reaction with link protein and with cartilage proteoglycan (29–31). Using a competitive binding assay, varying amounts (0.5–2 μg/mL) of either link protein or cartilage proteoglycan were mixed with a fixed amount of antibody to link protein in a round bottom microtitro plate (Linbro) at 4 °C for 1 h. The dilutions of antigens and antibodies were then done into PBS plus 0.05% Tween 20 (v/v). Then the solutions were transferred to flat bottom microtitro plates that had been precoated with 0.1 μg of link protein and were incubated for 1 h at room temperature. The solution was then removed, and the wells were rinsed and incubated for an additional 1 h at 37 °C.

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Visiting Postdoctoral Fellow supported by the Fogarty International Center for Health Sciences.

Received for publication, July 16, 1982, and in revised form, January 5, 1983.

Printed in U.S.A.
hour with peroxidase-conjugated goat antibody to rabbit immunoglobulin (1:1000 dilution in PBS-Tween). Subsequently, the plates were rinsed to remove the unbound material and then incubated with substrate (10 mg of o-phenylenediamine in 1.0 ml of methanol, 10 µl of 30% H₂O₂, and 99 ml of distilled H₂O). The amount of reaction product was estimated by measuring absorbance at 492 nm using a Titertek Multiskan (Flow).

The results presented in Fig. 1 demonstrate that the antisera is specific for link protein. Cartilage proteoglycan, a possible contaminant of the link protein preparations, did not compete with link protein, at proteoglycan concentration of 200 µg/well and at an antibody dilution of 1:20. In similar competition ELISA experiments not reported here, we have also observed that the link protein antibody does not recognize type II collagen, fibronectin, or chondronectin (up to 100 µg of the proteins and 1:20 dilution of the link protein antibody).

### RESULTS

#### Binding of Link Protein to Native and Denatured Collagen

The binding of link protein to microtiter wells coated with various collagens was measured by a modification of the ELISA assay (29-31). In brief, flat bottom microtiter plates (Dynatech Laboratories) were coated with collagens (20 µg/well) overnight at 4 °C, and then the coated wells were incubated with various amounts of the link protein dissolved in PBS plus 0.05% Tween 20 (v/v) at room temperature. After 1 h, the solution was removed, and the wells were rinsed (with PBS-Tween) and incubated for an additional hour with antibody to link protein. Subsequently, the ELISA was completed by adding peroxidase-conjugated goat antibodies against rabbit immunoglobulin and the appropriate substrate (see previous section). To test for competition with soluble collagen, link protein was mixed with native or denatured collagen in PBS-Tween in round bottom wells (Linbro) at 4 °C for 16 h, and then transferred to flat bottom microtiter wells coated with native collagen type I. The amount of link protein bound under these conditions was measured using antibody to link protein and the peroxidase-conjugated goat anti-rabbit antibody. As controls, the collagen-coated wells were incubated with either 1) link protein alone, 2) link protein plus antibody to link protein, 3) link protein plus peroxidase-conjugated second antibody, or 4) antibody to link protein plus peroxidase-conjugated second antibody, or 4) antibody to link protein and the second antibody. In each case, the absorbance did not exceed 0.24.

The binding of link protein to collagen was also determined by an immunoblotting procedure (32). Protein solutions (25 µl of native collagen, denatured collagen, and BSA) at four different concentrations (5, 2.5, 1.25, and 0.625 mg/ml) were applied on nitrocellulose paper and were incubated at 4 °C for 8-16 h. The paper strips containing the collagens or BSA were separately incubated with TBS-BSA at 4 °C for 12-16 h with gentle rocking. Each paper strip was treated with 10 µl of link protein in TBS (5 µg/ml in TBS-BSA) and was kept rocking gently at room temperature for 90 min. Control strips coated with either native or denatured collagen or BSA were also incubated with TBS-BSA but without link protein under identical conditions. The paper was then washed five times with PBS and incubated for 90 min with antibody to link protein (1:250 in TBS-BSA). Subsequently the paper strips were treated for 90 min with peroxidase-conjugated goat anti-rabbit immunoglobulin and finally with substrate (5.0 mg of diaminobenzidine, 10 µl of H₂O₂ to a final volume of 40 ml) with distilled water. After 1 min, the paper strips were rinsed thoroughly in distilled water to stop the reaction.

In the ELISA procedures and in the nitrocellulose paper assays, it is possible that denatured collagen may not have bound to the solid substrate. In order to rule out this possibility, we have carried out studies in which we tested the binding of fibronectin to native and denatured collagen applied to microwell plates as well as to nitrocellulose paper. These studies showed a greater binding of fibronectin to denatured collagen than to native collagen (data not presented), in confirmation of previous studies (33, 34), and indicate that denatured collagen binds adequately to microwell wells and nitrocellulose paper. Further ELISA studies using anti-type I collagen antibody established that equal amounts of native and denatured type I collagen bound to microwell plates.

In Vitro Fibril Assays—Fibrils were prepared by incubating collagen in PBS, pH 7.2, at 37 °C for 30 min, either with or without link protein (35, 36). These fibrils were centrifuged at 25,000 × g and washed twice with PBS. The samples were solubilized in sample buffer containing Cleland's reagent and electrophoresed on SDS-PAGE (24). Similar studies were carried out with denatured collagen and link protein or with link protein alone. The amounts of proteins in the precipitates were estimated by scanning the negatives of the photographs (Quick Scan, Helena Laboratories) using gels with known quantities of link protein and collagen to standardize the assay. Scatchard analysis was done by standard procedures.

SDE-PAGE—The discontinuous system of Laemmli was used (24). Briefly, the gel consisted of a 3% polyacrylamide stacking gel and a 7.5% polyacrylamide separating gel. Samples were reduced with 7.5 mg/ml of dithiothreitol. Protein bands were made visible by staining with Coomassie blue R-250.

**RESULTS**

#### Binding of Link Protein to Collagen Films

The affinity of the link protein for various isotypes of collagen was tested by measuring the binding of link protein to collagen films in microtiter wells. In these experiments, an excess of collagen (200 µg) was absorbed onto the plastic surface and various amounts of link protein were added to the wells. A non-ionic detergent, Tween 20 (0.5%), was included in the solution of the link protein, to reduce nonspecific adsorption to the surface of the well. Under these conditions, link protein was found to bind best to type III collagen and somewhat less to type I collagen (Fig. 2). Link protein bound poorly to films of collagen types II, IV, and V.

#### Comparison of Link Protein Binding to Native and Denatured Type I Collagen

More detailed studies were carried out on the binding of link protein with type I collagen. The importance of the triple helical structure of collagen in the interaction with link protein was evaluated by the film assay. In these studies, the plastic wells were coated with type I collagen, preheated to various temperatures (0, 25, 40, 56, and 100°C). Much less binding of link protein was observed to the denatured collagen film (Fig. 3). Ascaris collagen, which resembles other collagens in composition but lacks a fibronectin binding site (37), and BSA did not bind link protein (data not shown). These data indicate that the link protein binds better...
Related studies were carried out by measuring whether the binding of link protein to the collagen-coated surface could be inhibited by prior mixing of soluble collagen with the link protein (Fig. 4). These studies showed that native collagen in solution inhibited the binding of link protein to the native collagen surface. Under identical conditions, denatured collagen in solution did not compete for the binding of link protein with a native collagen surface.

The specific binding of link protein to native, but not to denatured, collagen was further established by an immunoblotting procedure (32). In this assay, different concentrations of native and denatured collagens or BSA dissolved in 0.5 M acetic acid were applied as spots onto nitrocellulose papers and allowed to bind to the paper at 4 °C for 8 h. The paper strips were then treated with 3% BSA solution to block the remaining sites. The proteins on the paper were then treated with link protein in the presence of 3% BSA to prevent nonspecific binding. The amount of link protein bound was determined using antibody against link protein. This study again demonstrates that link protein binds only to native collagens (Fig. 5) and is dependent on the amount of collagen. Little or no binding of link protein to denatured collagen (Fig. 5) or BSA to (Fig. 5) was observed.

**Binding of Link Protein to Collagen Fibrils**—The binding of link protein to collagen was also measured under more physiological conditions. Here, solutions of collagen and a purified preparation of link protein were mixed and then incubated at 37 °C under conditions which allow fibrils to form. The fibrils that formed were isolated by centrifugation, rinsed, dissolved in electrophoresis buffer, and electrophoresed. The results
Link Protein Binding to Collagen

Relative Binding of Link Protein to Monomeric and Fibrillar Collagen—Collagen fibrils were produced by incubating an aliquot of a solution of collagen in PBS at pH 7.2 at 37 °C for 30 min. The suspension of fibrils was transferred to a water bath at 26 °C and incubated for 30 min either with or without link protein. In addition, another aliquot of the original collagen solution was also incubated at 26 °C for 30 min. This temperature (26 °C) was chosen because no fibrils formed from the collagen solution in 30 min and none of the fibrils which had formed at 37 °C dissolved at 26 °C (data not shown). After 30 min, solid NaCl (20% w/v) was added to both samples. The precipitates that formed were collected by centrifugation and then examined by electrophoresis. Some link protein was precipitated by the 20% NaCl when incubated alone (Table I). However, all the collagen added to the samples was precipitated by this procedure. More link protein was present in the fibrillar rather than in the nonfibrillar collagen precipitate, indicating higher binding of link protein to fibrillar collagen than to monomeric collagen.

Molar Ratio and Strength of Link Protein-Collagen Binding—These studies indicate that less than 1 molecule of link protein is bound to each collagen molecule. A more precise estimate was obtained (Fig. 8) by measuring the amount of link protein that precipitated in the presence of increasing amounts of collagen. The amount of link protein that precipitated in the reaction (Fig. 8, lane 6). Some link protein was still present in the precipitate when collagen was added to the reaction at 37 °C to form fibrils (Fig. 8, lane 5). The quantity of link protein in the native fibrils was determined by densitometric scanning of the precipitated link protein and by comparing with the densitometric scans known quantities of purified link protein. The values represent the average ± mean deviation, obtained by two independent experiments.

### Table I

| Material precipitated by NaCl | Amount of link precipitated (µg) |
|------------------------------|---------------------------------|
| Link protein                 | 2.9 ± 0.2                       |
| Native collagen + link protein | 7.1 ± 0.4                       |
| Collagen fibril + link protein | 10.9 ± 0.5                      |

![Fig. 6. Binding of native and denatured link protein to native collagen.](image)

![Fig. 7. Binding of link protein to collagen fibrils.](image)

![Fig. 8. Link protein binding to collagen—effect of collagen concentration.](image)
Link Protein Binding to Collagen

**FIG. 9.** Link protein binding to collagen—effect of link protein concentration. A fixed amount of collagen (250 μg) was mixed with the indicated amounts of link protein and the amount of link bound to collagen fibrils was determined as described above (Fig. 7). The binding was directly proportional to the amount of collagen added. About 5 μg of link protein were precipitated by 450 μg of collagen. Given the ratio of their molecular weights as 6.8, this would indicate that 1 molecule of link protein was binding to 13 molecules of collagen.

In the second experiment (Fig. 9), varying amounts of link protein were added to 250 μg of collagen and allowed to precipitate. A maximum of 5 μg of link protein bound to the 250 μg of collagen fibrils under these conditions, indicating that there are a fixed number of binding sites in the collagen fibrils. This corresponds to a ratio of 1 molecule of link protein to 7 molecules of collagen. Determination of the affinity of link protein-collagen interactions by Scatchard analysis (inset, Fig. 9) indicates that the equilibrium constant of the reaction is $3.8 \times 10^{-7}$ M and the number of equal and independent binding sites on the collagen molecule is 0.26.

**DISCUSSION**

While the link protein of cartilage is known to be an important component of the aggregate structure of cartilage proteoglycan, recent studies suggest that it may have additional functions. For example, link protein is present in non-cartilagenous tissues such as sclera which lack the cartilage type of proteoglycan (13, 14, 15). Further, even in cartilage, a significant proportion of the link protein is extractable without the denaturing solvents required to dissociate the proteoglycan aggregate.  

In addition, recent studies indicate that in cartilage link protein may be associated with collagen fibrils (38). Our studies reported here show that the link protein purified from a chondrosarcoma binds to collagen films, particularly to those formed of types I and III collagen. We have also shown that the binding of link protein requires that both the collagen and the link protein be in their native form. Less than 1 molecule of link protein binds per molecule of collagen. These data also indicate that link protein binds better to fibrillar collagen than to "monomeric" collagen. Conditions were chosen under which collagen molecules and collagen fibrils were stable as such. We find that almost twice as much link protein binds to the fibrillar than to the monomeric collagen preparation. In other systems, fibrillar collagen is more active than monomeric collagen in inducing platelet aggregation (39, 40). Similar structural factors could be involved in the interaction of link protein with fibrillar collagen. Further, studies were carried out with the fibrillar collagen to determine the maximum binding of link protein to collagen. These studies suggest that about 1 molecule of link protein binds to 7-13 molecules of collagen. The binding to fibrils is saturable and is inhibited by native collagen, but not by denatured collagen. The $K_{eq}$ of link protein-collagen interaction as determined by Scatchard analyses (Fig. 9) was $3.8 \times 10^{-7}$ M. In a similar study, the $K_{eq}$ of fibronectin-collagen was found to be $10^{-7}$ M (41). Our data suggest that although the link protein-collagen interactions are not as strong as that of fibronectin-collagen, they are nevertheless significant.

Link protein binds to collagen fibrils which consist of aggregates of monomeric collagens. The ratio of link protein binding to collagen is 1:7-13. This value is probably an underestimate, because a molecular weight of 300,000 for collagen fibrils is too low. The fibrils are known to be formed of collagen monomers in multiples of 5 (42). Alternatively, it is possible that a contaminant in the collagen preparation is actually binding the link protein. We feel that this is unlikely. The collagen used for study here, as well as the link protein, has been chromatographed on a column of DEAE-cellulose to remove azionic materials, such as hyaluronic acid, proteoglycans, or glycosaminoglycans. Analyses of link protein and collagen indicate that they contain less than 1 μg of uronic acid/0.25 mg of material (not shown). Further, no cross-reaction is observed between link protein and cartilage proteoglycan using antibody to either link protein or cartilage proteoglycan (unpublished results). Such results would indicate that the type of material (proteoglycan and hyaluronic acid) known to bind link protein is not involved in the reactions studied here. Also, neither the collagen (Fig. 7) nor the link protein (data not presented) when overloaded onto SDS gels, show any trace of contaminating proteins. Coomassie blue stain is known to detect even 1 μg of protein band (43). In addition, the binding occurs even in the presence of non-ionic detergents such as Tween 20 (ELISA) or in the presence of other proteins such as 3% BSA (immunoblotting). Finally, link protein does not bind to an antigen-antibody precipitate, which was used to measure "trapping" of link by the precipitation. These experiments demonstrate that the binding is specific and is not due to any detectable contamination.

It is not clear why the cartilage link protein binds poorly to type II (cartilage) collagen film. Since link protein is present in both cartilage and noncartilage tissues, it is possible that link protein may have tissue-specific interactions. Alternatively, in tissues, the binding interactions may be modulated by additional factors such as proteoglycans and hyaluronic acid.

The functional significance of link protein-collagen interaction is not known. It is generally agreed that link protein binds to proteoglycans and hyaluronic acid and is responsible for the stabilization of the interaction between those macromolecules. The higher binding of link protein to fibrillar collagen than to native collagen indicates that, in vivo, an opportunity exists for link protein to bind to collagen. Whether that binding occurs either directly or through other components, such as proteoglycans, remains to be studied. Through such binding, link protein may be involved in regulating the formation and the morphology of collagen fibrils.

Acknowledgments—We thank Drs. Vincent C. Hasclall, A. Tyl Hewitt, George R. Martin, and Jaro Sodek for critical evaluation of the manuscript, and Denise Haller for typing the manuscript.

**REFERENCES**

1. Hasclall, V. C., and Sajdera, S. W. (1969) J. Biol. Chem. 244, 2384-2396
2. Keiser, H., Shulman, H. J., and Sandson, J. T. (1972) Biochem. J. 128, 163-169
3. Gregory, J. D. (1973) Biochem. J. 133, 383-386
4. Baker, J. R., and Caterson, B. (1977) Biochem. Biophys. Res. Commun. 77, 1-10

*S. Chandrasekhar, unpublished observation.*
5. Bonnet, F., Péris, J.-P., and Jollès, P. (1978) Biochim. Biophys. Acta 532, 242–248
6. Sapdera, S. W., and Hascall, V. C. (1969) J. Biol. Chem. 244, 77–87
7. Hascall, V. C., and Heinegård, D. (1974) J. Biol. Chem. 249, 4242–4249
8. Heinegård, D., and Hascall, V. C. (1974) J. Biol. Chem. 249, 4250–4256
9. Faltz, L. L., Reddi, A. H., Hascall, G. K., Martin, D., Pita, J. C., and Hascall, V. C. (1979) J. Biol. Chem. 254, 1375–1380
10. Hardingham, T. E. (1979) Biochem. J. 177, 237–247
11. Hascall, V. C., and Heinegård, D. (1974) J. Biol. Chem. 249, 77–87
12. Poole, A. R., Pidoux, I., Reiner, A., Coster, L., and Hassell, J. R. (1982) J. Cell Biol. 93, 910–920
13. Kleinman, H. K., Klebe, R. J., and Martin, G. R. (1981) J. Cell Biol. 88, 473–485
14. Hay E. D. (1981) J. Cell Biol. 91, 205a–223s
15. Linsenmayer, F. F., Gibney, E., Toole, B. P., and Gross, J. (1978) Exp. Cell Res. 116, 470–474
16. Smith, B. D., Martin, G. R., Miller, E. J., Dorfman, A., and Swarm, R. (1975) Arch. Biochem. Biophys. 166, 181–186
17. Epstein, E. (1974) J. Biol. Chem. 249, 3525–3531
18. Orkin, R. W., Gehron, P., McGoodwin, E. B., Martin, G. R., Valentine, T., and Swarm, R. (1977) J. Exp. Med. 145, 204–219
19. Chung, R., Rhodes, R. K., and Miller, E. J. (1976) Biochem. Biophys. Res. Commun. 71, 1167–1175
20. Evans, H. J., Sullivan, C. E., and Piez, K. A. (1976) Biochemistry 15, 1435–1439
21. Mosher, D. F. (1980) Prog. Hemostasis Thromb. 5, 111–151
22. Poole, A. R., Pidoux, I., Reiner, A., and Rosenberg, L. (1982) J. Cell Biol. 93, 921–937
23. Baumgartner, H. R. (1977) Thromb. Haemostasis 37, 1–12
24. Jaffe, R., and Deykin, D. (1974) J. Clin. Invest. 53, 875–881
25. Mosher, D. F. (1980) Prog. Hemostasis Thromb. 5, 111–151
26. Piez, K. A. (1978) in Biochemistry of Collagen (Ramachandran, G. N., and Reddi, A. H., eds) pp. 1–44, Plenum, New York
27. McCormick, P. J., Chandrasekhar, S., and Mills, A. J. T. (1979) Anal. Biochem. 97, 359–367
Interaction of link protein with collagen.
S Chandrasekhar, H K Kleinman and J R Hassell

J. Biol. Chem. 1983, 258:6226-6231.

Access the most updated version of this article at http://www.jbc.org/content/258/10/6226

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/10/6226.full.html#ref-list-1