The Heterogeneity of Link Proteins Isolated from Human Articular Cartilage Proteoglycan Aggregates*

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Link proteins, isolated from proteoglycan aggregates prepared from human articular cartilage, were studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing. When subjected to the former technique the native link proteins resolve into three components of molecular weights 48,000, 44,000, and 41,000. Link protein, isolated following clostripain digestion of proteoglycan aggregate, is present as a single component of molecular weight 41,000. Under all conditions of isoelectric focusing tested, the native link proteins resolve into at least nine subcomponents having pI values between 6.0 and 7.0. The clostripain-treated link protein resolves into at least five subcomponents which have pI values similar to those of the more basic subcomponents observed in the native molecules. One source of heterogeneity contributing to both isoelectric focusing profiles is variation in sialic acid content, since neuraminidase treatment of the link protein preparations produces a shift to subcomponents with more basic pI. The electrophoretic data are consistent with the two larger link protein components representing the same protein core, but being substituted to different degrees with oligosaccharide chains that may have variable sialic acid contents. The smallest link protein component, which may be derived from either of the larger moieties by limited proteolytic cleavage, is also substituted with sialic acid-containing oligosaccharides.

Most of the proteoglycan from cartilaginous tissues are able to form aggregates in the presence of other tissue components, namely hyaluronic acid and a glycoprotein of low buoyant density, termed link protein (1-5). While both molecules can bind independently to proteoglycan subunits (3, 6, 7), only hyaluronic acid promotes the formation of a multi-molecular complex (3, 4). In addition, link proteins can also bind independently to hyaluronic acid (6-9).

The structure of the proteoglycan aggregate has been reviewed by Hascall (10). Briefly, proteoglycan subunits possess a terminal hyaluronic acid-binding region on their core protein which is not substituted with glycosaminoglycan chains. Many subunits interact with a single hyaluronic acid molecule through this region, the interaction requiring a continuous sequence of five disaccharide repeating units of the hyaluronic acid molecule. The separation of the proteoglycan subunits along the hyaluronic acid chain is believed to be determined mainly by the interaction between chondroitin sulfate chains on adjacent proteoglycan molecules. The link proteins stabilize the interaction between proteoglycan subunits and hyaluronic acid, probably through binding to both components in the aggregates. It has been postulated that a single link protein is involved in the stabilization of each proteoglycan subunit in the aggregate (6, 8, 11, 12).

In the absence of link proteins the hyaluronic acid/proteoglycan subunit complex is not stable at low pH, elevated temperature, high ionic strength, or high centrifugal force (13-15). The presence of link proteins serves to stabilize the aggregate under these conditions. In addition, the proteoglycan subunits in the link-free complex can be dissociated in the presence of low molecular weight hyaluronic acid oligomers, but this is not possible in the link-stabilized aggregate (13, 16).

The presence of link proteins also serves to partially protect the hyaluronic acid-binding region of the proteoglycan from proteolytic attack, so enabling this region to be isolated (9).

To date, link proteins have been described in a variety of cartilaginous tissues from different species and are characterized as having molecular weights between 40,000 and 50,000 as determined by SDS-polyacrylamide gel electrophoresis. The link proteins from bovine nasal cartilage have been most widely studied (15, 17-19) and are generally reported as two components with molecular weights of about 44,000 and 48,000. A smaller component has also been observed (19, 20). The link proteins isolated from bovine articular cartilage appear to be identical with those from the nasal cartilage (21). Link proteins also exist in avian cartilages and during the development of the fetal chick limb it appears that only a large link protein is synthesized at early stages in development, whereas later two link proteins are present (22). In contrast, link protein in the Swaro rat chondrosarcoma is present as a single component with a molecular size comparable to the smaller of the two major link proteins in bovine nasal cartilage (8, 12, 23, 24).

The amino acid composition of isolated bovine link proteins indicates that the major components separated by electrophoresis possess similar amino acid compositions (17, 19) and, therefore, may be structurally related. Furthermore, evidence for a structural relationship is provided by studies on the fragmentation of the link proteins by either trypsin (25) or cyanogen bromide (26). Fragmentation with cyanogen bromide also suggests that the link proteins are not structurally related to the proteoglycan subunits (20), and this is confirmed by the absence of immunological cross-reactivity (6, 27, 28). It is likely that the carbohydrate content of the link protein may play a major role in determining the electrophoretic heterogeneity, though the structure of any integral oligosaccharide

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.
has yet to be described. However, it has been reported (19) that the large link protein has a higher carbohydrate content and that both link proteins contain fucose, mannose, galactose, N-acetylgalactosamine, N-acetylglucosamine, and sialic acid, though in varying amounts.

Little is known about the link proteins from human cartilage, though it has been reported that those from human nasal cartilage are similar in composition and molecular size to their bovine counterparts (29). Furthermore, two components have been reported in SDS-polyacrylamide gel electrophoresis (30-32). The purpose of the present study was to investigate further the structure of link proteins from human articular cartilage with particular emphasis on the electrophoretic heterogeneity of the molecules and on the role played by carbohydrate in its origin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Guandinium chloride, clostripain (EC 3.4.22.8), bovine serum albumin, sialyllactose, Tween 20, and Trion X-100 were from Sigma. Cesium chloride and Nonidet P-40 were from BDH Chemicals. Proteinase-free neuraminidase (EC 3.2.1.18) was from Behring Laboratories. Acrylamide, methylenebisacrylamide, SDS, ampholytes (BioLyte), and nitrocellulose sheets were from Bio-Rad. Agarose A45 was from Pharmacia-LKB.

**Sources of Cartilage**—Human articular cartilage was collected at autopsy from the distal femoral epiphyses of newborns in whom there was no macroscopic evidence of cartilage pathology. The tissue was stored at -20 °C prior to extraction.

**Preparation of Link Protein**—The cartilage was finely sliced and extracted with 10 volumes of 4 M guandinium chloride, 0.1 M potassium acetate, pH 5.8, at 4 °C for 70 h. The extraction fluid also contained 1 mM each of EDTA, phenylmethylene-sulfonyl fluoride, and iodoacetamide, and 1/2 mg/ml of pepstatin to inhibit possible endogenous proteolytic activity (33, 34). The extract was then filtered through glass wool, and the filtrate was dialyzed against 400 volumes of 0.1 M potassium acetate, pH 5.8, at 4 °C for 20 h. The diazoy filtrate was adjusted to a density of 1.67 g/ml by the addition of solid CsCl (1.2 g of CsCl/1 ml of filtrate). This solution was centrifuged at 100,000 × g at 10 °C for 48 h, and the resulting density gradients were fractionated. The fractions were monitored for uronic acid (35), absorbance at 280 nm, and density. The proteoglycan aggregate was recovered from fractions with a density greater than 1.68 g/ml (A1) after exhaustive dialysis against water and subsequent freeze drying.

The preparation was dissolved at 1 mg/ml in 4 M guanidinium chloride, 0.1 M potassium acetate, pH 5.8, which also contained 3 M CaCl2 to give a density of 1.50 g/ml. This solution was centrifuged and fractionated as described above. The link protein-rich fractions with a density less than 1.40 g/ml (A1D3) were pooled. This solution was dialyzed against 20 volumes of 4 M guanidinium chloride, 50 mM Tris/ HCl, pH 7.35, at 4 °C for 20 h, and was then concentrated to 5 ml by ultrafiltration using an Amicon PM-10 membrane at 30 p.s.i. The concentrate was applied onto a Sepharose CL-6B column (2.2 × 57.5 cm) equilibrated with 4 M guanidinium chloride, 50 mM Tris/HCl, pH 7.35, and eluted with the same buffer at a flow rate of 17 ml/h. Fractions of 3 ml were collected and monitored for absorbance at 280 nm (Fig. 1c). The elution position of the modified link proteins was established by SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis.

**Neuraminidase Treatment of Link Protein**—Fractions from the dissociative Sepharose CL-6B columns were dialyzed against 1000 volumes of 0.2 M Tris/HCl, pH 7.1, at 4 °C for 20 h, under which conditions the link protein should remain in solution.2 Neuraminidase (35 units/ml of fraction, and degradation was allowed to proceed at 37 °C for 20 h. Controls were performed using the enzyme buffer alone. Under the conditions used, the neuraminidase gave complete degradation of 0.6 nm sialylactose.

**Reduction and Alkylation of Link Protein**—Fractions from the dissociative Sepharose CL-6B columns were made 5 mM in dithiothreitol and incubated at 46 °C for 5 h. The solution was then adjusted to 20 mM in iodoacetamide and incubated at 40 °C for 1 h and then at 4 °C for 20 h. Control incubations were performed in the absence of dithiothreitol and iodoacetamide.

**SDS-Polyacrylamide Gel Electrophoresis**—Samples were dialyzed against 500 volumes of 0.125 M Tris/HCl, pH 6.5, containing 0.1% SDS for 20 h prior to electrophoresis by the method of King and Laemmli (37) using 10% (total concentration) acrylamide slab gels which were stained with Coomassie brilliant blue R250 by the method of Fairbanks et al. (38).

**Isoelectric Focusing in 6 M Urea Polyacrylamide Slab Gels**—Samples were routinely dialyzed against 500 volumes of 6 M urea containing 1% glycerol (pH 6.0) at 4 °C for 20 h prior to focusing. Alternatively, the samples were adjusted to contain 1% Triton X-100, and dialysis was then performed in the presence of 0.1% Triton X-100. Some samples were also treated with 1% SDS, 2.5% mercaptoethanol for 5 min at 100 °C prior to focusing (39). Electrophoresis was conducted using an LKB Multiphor unit and associated gel casting equipment. Polyacrylamide slab gels (6%, 2 mm thick) routinely consisted of 2.92 g of acrylamide, 9.92 g of N,N,N',N'-tetramethylethylenediamine, 21.6 g of urea, (6% final concentration), 3.9 ml of amphotolysis, pH 3-10, and 40 ml of N,N,N',N'-tetramethylethylenediamine in a total volume of 59.8 ml. Polymerization was initiated by the addition of 150 ml of 10% ammonium persulfate. For samples containing Triton, the focusing gel also contained 0.1% Triton X-100, and for samples treated with SDS the focusing gel contained 9.5 M urea and 2% Nonidet P-40 (39).

Samples were applied as streaks on the gel surface. Anode and cathode wicks contained 1M phosphoric acid and 1 M sodium hydroxide, respectively. Electrophoresis across 11 cm of gel (25 cm in length) was for 2-3 h at 25 watts constant power using cold tap water cooling. Before staining, the gel was first washed for 30 min in 3.5% selsalicylic acid, 11.5% trichloroacetic acid to remove urea and ampholytes and then for 30 min in 10% acetic acid, 25% isopropanol. The gel was stained by the method of Fairbanks et al. (38). The pH gradient was established by cutting a 1-cm wide strip of unstained gel into 0.5-cm sections. These were homogenized with 1 ml aliquots of degassed deionized water, and the pH of the supernatant was measured.

**Antiserum**—Rabbit antiserum to bovine nasal cartilage link protein and to clostripain-derived Swarm rat chondrosarcoma link protein were prepared as described previously for bovine (6) and rat species (40). Antibodies and immunoglobulin G were concentrated from immunemun and nonimmune sera by precipitation with 50% ammonium sulfate (15). A pig IgG antibody conjugated with horseradish peroxidase and directed against rabbit F(ab')2 was prepared as described elsewhere (41).

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2. L. Rosenberg, personal communication.
Electrophoretic Transfer for Immune Localization—Polyacrylamide gels were washed three times for 10 min in 24 mM Tris, 192 mM glycine, pH 8.3, containing 20% (v/v) methanol. Electrophoretic transfer to a nitrocellulose sheet was performed in this buffer using an E-C Apparatus Corp. Electroblot system. Transfer was at 0.8 A for 16 h. The sheet was then soaked in PBS (10 mM sodium phosphate, pH 7.2, containing 145 mM sodium chloride and 0.05% sodium azide) containing 3% bovine serum albumin for 30 min using gentle agitation. The sheet was then immersed in rabbit antibody to bovine or rat link protein (1.7 mg/ml) in PBS containing 3% bovine serum albumin and left as above for 30 min at room temperature. It was then washed three times for 5 min each in PBS containing 0.1% Tween 20, and then washed once in PBS alone. Incubation with peroxidase-labeled pig IgG anti-rabbit F(ab)'2 (0.19 mg/ml) in PBS containing 3% bovine serum albumin was for 30 min, followed by four washes of 10 min each in PBS containing 0.1% Tween 20, and then two washes in PBS which did not contain sodium azide. The sheet was incubated for the peroxidase reaction in PBS (no azide) containing 0.25 mg/ml of 3,3',4',4'-tetraminobiphenyl hydrochloride and 0.1 µl/ml of 50% hydrogen peroxide. When the sheet was adequately developed, sodium azide was added to a final concentration of 1% to arrest the reaction, and the sheet was washed in water and dried.

Two-dimensional Electrophoresis—First dimension isoelectric focusing gels were run as described above. Strips (3 mm wide) were then cut and equilibrated in three changes of 63 mM Tris/HCl, pH 6.8, containing 5% mercaptoethanol, 2.3% SDS, and 10% glycerol. Electrophoresis in the second dimension was then performed by the method of O'Farrell using a 10% acrylamide separating gel (42), and staining was by immune localization, following electrophoretic transfer to nitrocellulose, for greatest sensitivity.

Laurell Immunoelectrophoresis—Immunoelectrophoresis was performed in 0.6% agarose gels as described previously (6, 15). Gels contained 25 µl/ml and 50 µl/ml of concentrated antibody to bovine link proteins (24.7 mg/ml) for native and clostripain-treated link preparations, respectively. Solutions of native (4 µl) and clostripain-treated (2 µl) link protein from the Sepharose CL-6B column fractions were electrophoresed at 3 V/cm for 5.5 h into the antibody-containing gel. Gels were washed, dried, and stained as described previously (15).

RESULTS

Purification and Characterization—Native and clostripain-treated link proteins were prepared from human articular cartilage proteoglycan aggregate by methods based on those used previously to prepare analogous material from bovine nasal cartilage (13–15, 17, 18) and Swarm rat chondrosarcoma (36). The native link proteins eluted as the major protein peak (Kw = 0.47) on Sepharose CL-6B chromatography in the presence of 4 M guanidinium chloride (Fig. 1a), and their identity was confirmed by immunoelectrophoretic analysis of the column fractions (Fig. 2) using a cross-reacting specific antibody purified bovine nasal cartilage link protein. The clostripain-treated link proteins were purified by the same chromatographic procedure (Fig. 1c), and their presence in the major protein peak was also confirmed by immunoelectrophoretic analysis.

SDS-Polyacrylamide Gel Electrophoresis—Fractions from the dissociative Sepharose CL-6B columns were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Native link proteins were resolved into three components, having molecular weights of 48,000, 44,000, and 41,000, whose abundance decreased with decreasing molecular weight (Fig. 3). There was no clear resolution of these components by the column, though the smaller components were slightly more retarded. The clostripain-treated link protein was present as a single component of molecular weight 41,000, thus co-migrating with the smallest of the native link components (Fig. 4). The hyaluronic acid-binding region of the proteoglycan subunit was resolved adjacent to the link protein as a broad species having an average molecular weight of about 75,000 (Fig. 4).

Isoelectric Focusing—Fractions from the Sepharose CL-6B columns were also examined by isoelectric focusing in 6 M urea. The only bands visible after staining were in the fractions that had been shown immunologically to contain link protein. For the native link protein at least nine components were resolved all with pH values in the range of 6.0 to 7.0, with those components of intermediate pH being most abundant (Fig. 5). With increasing retention of the fractions and, therefore, decreasing molecular size of the link protein, the more basic components became more prominent. This was more apparent when equal protein concentrations were loaded onto the gel (data not shown) rather than equal fraction volumes as illustrated in Fig. 5.

When the peak fraction from the dissociative Sepharose CL-6B column of the clostripain-treated link protein was examined five components were clearly resolved (Fig. 6, track C), which co-migrated with the components of most basic PI present in the peak fraction of the native link protein (Fig. 6, track A). Components of intermediate PI value were again most prominent. Fractions containing the hyaluronic acid-binding region of the proteoglycan showed no additional bands, as such protein migrated to the anode (data not shown).

Focusing profiles were independent of whether the sample was applied at the cathode or the anode or whether the gel was prefocused prior to sample application, and the presence of a nonionic detergent (Triton X-100) did not simplify the patterns. In addition, there was no simplification of the focus-
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FIG. 3. SDS-polyacrylamide gel electrophoretic analysis of the native link protein preparation. The fractions from the Sepharose CL-6B chromatography of A1D3 (see Fig. 1a) were analyzed on a 10% polyacrylamide gel. Molecular weight standards (A) and unfractionated A1D3 (B) were also analyzed.

FIG. 4. SDS-polyacrylamide gel electrophoretic analysis of the clostripain-treated link protein preparation. The fractions from the Sepharose CL-6B chromatography of A1-clost.-A3-V, (see Fig. 1c) were analyzed on a 10% polyacrylamide gel. Molecular weight standards (A), unfractionated A1-clost.-A3-V, (B), and peak fractions from the chromatography of native link protein (C) and clostripain-treated link protein (D) were also analyzed.

FIG. 5. Isoelectric focusing of the native link protein preparation. The fractions from the Sepharose CL-6B chromatography of A1D3 (see Fig. 1a) were analyzed. The point of sample application is indicated by the arrow.

FIG. 6. Isoelectric focusing of link proteins before and after neuraminidase treatment. Native link protein (fraction 50, see Fig. 1a) (A), native link protein after neuraminidase treatment (B), clostripain-treated link protein (fraction 51, see Fig. 1c) (C), and clostripain-treated link protein after neuraminidase treatment (D) were analyzed in the same gel. The point of sample application is indicated by the arrow. At the concentration used neuraminidase was not detectable after staining.

Neuraminidase Treatment—The peak fractions from the Sepharose CL-6B columns were examined by SDS-polyacrylamide gel electrophoresis and isoelectric focusing after treatment with neuraminidase. The neuraminidase treatment did not substantially alter the pattern observed by SDS-polyacrylamide gel electrophoresis of the native link protein (Fig. 7) under either reducing or nonreducing conditions. There was, however, a small increase in the mobility of all components under both conditions.

In contrast, the isoelectric focusing pattern was changed for both native (Fig. 6, track B) and clostripain-treated (Fig. 6, track D) link proteins. In both cases, the pI values of the neuraminidase-treated samples were more basic than those of the intact molecules, as expected after the removal of sialic acid residues. With the native link protein the pattern was somewhat less complex with respect to the number of bands, though all the bands observed appeared to have counterparts of analogous mobility in the untreated sample. This analogy between neuraminidase-treated and untreated samples was also observed for the clostripain-treated link protein. While one cannot be certain that all sialic acid has been removed from the link protein, maximal possible removal under these conditions was indicated by the observation that twice the neuraminidase concentration did not effect the electrophoretic profiles. However, any remaining sialic acid residues may contribute to the observed profiles. Sialic acid residues showing increased resistance to neuraminidase have been reported in glycoproteins (43).

Nitrocellulose Transfer—The peak fraction from the Sepharose CL-6B column of the native link protein was subjected to SDS-polyacrylamide gel electrophoresis. Protein was then transferred onto a nitrocellulose sheet, and link proteins were identified by peroxidase staining after treatment first with a specific rabbit antibody to the link protein and subsequent treatment with a peroxidase-labeled pig antibody to the rabbit antibody. The first step employed antiserum raised either against the single link protein isolated from the Swarm rat...
chondrosarcoma after clostripain treatment or against the two link components isolated from bovine nasal cartilage.

The pattern visualized using antiserum raised against rat chondrosarcoma link protein displays three components (Fig. 8, track A) and is identical with that observed by staining of the electrophoresis gel with Coomassie brilliant blue. An identical result was obtained with the antiserum raised against bovine nasal cartilage link protein (Fig. 8, track B). The use of an antiserum against the chondrosarcoma-derived link protein which gives a single component on SDS-polyacrylamide electrophoresis indicates that the three components present in the human preparation represent immunologically related forms of link protein of varying molecular weight, which are antigenically and hence structurally related to bovine and rat link proteins.

Two-dimensional Electrophoresis—The link proteins were subjected to isoelectric focusing followed by further separation by SDS-polyacrylamide gel electrophoresis (Fig. 8C). The components of most acidic pl after focusing corresponded to those of largest molecular size, and those of most basic pl were of smaller size. There was notable heterogeneity among the components which corresponded to the largest link protein in the second dimension, and this may explain the diffuse trailing edge observed on single-dimension SDS-polyacrylamide gel electrophoresis. This heterogeneity may in part reflect variation in sialic acid substitution. In addition, some bands which appeared as single entities after focusing resolved into two or three components in the second dimension, suggesting a fortuitous similarity in pl between molecules having a different molecular size or an association of components that is only dissociable under the conditions unique to SDS-polyacrylamide gel electrophoresis but is unaffected by all the focusing conditions described previously.

Fig. 8. Immunological visualization of link protein following one- and two-dimensional electrophoresis. Native link protein (fraction 49, see Fig. 1a) visualized using an antibody to link protein from the Swarm rat chondrosarcoma (A) or to link protein from bovine nasal cartilage (B) following analysis by SDS-polyacrylamide gel electrophoresis. Native link protein (fraction 48, see Fig. 1a) was also subjected to two-dimensional electrophoresis prior to visualization with the antibody to the link protein from bovine nasal cartilage (C).

**DISCUSSION**

The link proteins which are an integral part of the proteoglycan aggregates prepared from cartilage often show multiple components on SDS-polyacrylamide gel electrophoresis (15, 19, 22, 29). This heterogeneity can be theoretically attributed to genetically distinct link proteins, though there is no evidence in the literature to support such a concept. Alternatively, post-translational modification of a single gene product, particularly with respect to proteolytic modification within the extracellular matrix of the tissue, and variation in the carbohydrate substitution of a single link protein would give rise to multiple components. Data currently in the literature suggests that both proteolytic modification and carbohydrate variation may account for link protein heterogeneity.

Bonnet et al. (17) and Baker and Caterson (19) have shown that the two major link proteins from bovine nasal cartilage possess very similar amino acid compositions and may, therefore, arise from the same gene product. They inferred that the major difference was, therefore, due to variation in glycosylation. Analysis of fragments after treatment with V8 protease (19), trypsin (25), and cyanogen bromide (26) also suggests analogy in protein sequence between the two components. Treatment of the two major bovine nasal link proteins with trypsin results initially in a common degradation product without any evidence of prior conversion of the large link component to the smaller (25). Link protein degradation products of a similar size are also produced by proteolytic treatment of proteoglycan aggregates with either trypsin (12), papain (44), or clostripain (36). A moiety of similar size has also been noted as a minor component in native link protein preparations (19). This component may, therefore, represent a derivative of the major link proteins resulting from limited proteolysis.

The human articular cartilage used in the present work was found to contain three link protein components on analysis by SDS-polyacrylamide gel electrophoresis, similar in size to those isolated from bovine cartilage (15, 19). The two larger link proteins were also detected in adult human nasal cartilage.
(29) and articular cartilage (31). Treatment of proteoglycan aggregates with clostripain produced a single link protein having an identical mobility with the smallest native link component, consistent with the hypothesis that this component is derived from the larger link proteins by limited proteolysis. The cross-reaction of all three link components with an antiseraum raised against the single link component from the Swarn rat chondrosarcoma also suggests that all the human link protein components are structurally related.

The electrophoretic heterogeneity of the human link proteins was studied by isoelectric focusing. The single link protein component derived by clostripain treatment was resolved into at least five subcomponents, while the native link protein was resolved into at least nine subcomponents. Many of the subcomponents present on focusing of the native link protein were resolved into two or three bands when subjected to further SDS/polyacrylamide gel electrophoresis. This may indicate that self-association of the link proteins contributes to the focusing profile, though for a fortuitous similarity in pl for the different components is also possible. Self-association of bovine articular cartilage link protein has been suggested (15). However, as the complex focusing pattern could not be simplified by a variety of highly dissociative conditions, it would appear that self-association contributes to the production of the subcomponents it is highly stable. Alternatively, subcomponents may represent isoproteins and/or be due to variation in constituent oligosaccharides. If charge heterogeneity is due to oligosaccharides then one possible cause is variation in sialation. This proved to be partly true as neuraminidase treatment of the native link protein produced a simplification in the focusing pattern with a diminution in the abundance of subcomponents with acidic pl and an intensification in those with basic pl. Clostripain-treated link proteins also showed a shift toward more basic pl values after neuraminidase treatment. Furthermore, the clostripain-treated subcomponents appeared to be analogous in pl to the more basic subcomponents in the native link protein preparation, suggesting that proteolytic treatment may cleave a peptide rich in sialated oligosaccharides. The neuraminidase treatment did not, however, simplify the pattern obtained on polyacrylamide gel electrophoresis and, therefore, sialation alone is not the difference between the two major link components. Evidence in support of further structural heterogeneity of link protein molecules with the same molecular size has also been derived from immunochemical studies of isoelectric focused rat and bovine link proteins.5

In conclusion it has been shown that the link proteins from human articular cartilage occur as three electrophoretically distinct components on SDS-polyacrylamide gels. Our data combined with those from Péron et al. (29) suggests that the two larger components observed on SDS-polyacrylamide gels may have identical protein structure but differ in oligosaccharide substitution and that the smallest component is derived from them by proteolytic modification. All the link proteins possess sialic acid-containing oligosaccharides, and a sialated peptide is cleaved by proteolytic treatment. The link components are all heterogeneous in the sialic acid substitution of their oligosaccharides, and other variations in oligosaccharide structure may also be present. However, it would appear that the functional capacity of the link protein is unaffected by the structural variations outlined above as all the link protein was prepared from intact proteoglycan aggregates.

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