Link Protein Interactions with Hyaluronate and Proteoglycans

CHARACTERIZATION OF TWO DISTINCT DOMAINS IN BOVINE CARTILAGE LINK PROTEINS*

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Hyaluronic acid-binding region and trypsin-link protein were prepared from bovine nasal cartilage proteoglycan complex after trypsin digestion. Binary complexes were reformed between trypsin-link protein and hyaluronic acid-binding region or hyaluronate. Upon trypsin treatment of these complexes, two fragments deriving from trypsin-link protein were characterized. One of them, of 20 kDa, corresponds in fact to a 140-amino acid long fragment and bears the glycosylated site of trypsin-link protein; it appears to be involved in proteoglycan/link protein interaction. The other, of 22 kDa, corresponds to the 200 C-terminal amino acids of trypsin-link protein; it appears to be involved in the binding of link protein with hyaluronic acid. A structural model of bovine trypsin-link protein depicting two distinct domains involved in hyaluronate and proteoglycan subunit interactions is proposed.

In the cartilage, the major part of the proteoglycans is involved in an aggregated structure (1) which plays an important role in the maintenance of the plasticity of the tissue (2) as the free proteoglycan subunits may be able to diffuse out of the matrix (3). This aggregated structure, the proteoglycan complex, is built of noncovalently bound hyaluronic acid (HA), proteoglycan subunits (PGS) and link proteins (LP) (3). LP are glycoproteins of 44.5 and 48.5 kDa (4, 5); they stabilize the binding between HA and PGS (6, 7) and influence the spacing of the monomers along the hyaluronate filament (8). The ability of LP to bind to either HA or PGS independently (9, 10) suggests the existence of two functionally distinct binding sites; specific chemical modifications performed on bovine articular cartilage LP corroborate this statement (11). Two major protein fractions have been isolated from a trypptic digest of the proteoglycan complex (12): one of them, the hyaluronic acid-binding region (HABR), derives from the PGS and is located at its N terminus (13), whereas the other (TLP) is a common trypsin fragment from LP. TLP differs from native LP by the removal of a short N-terminal peptide from the latter (14). HABR and TLP still possess the HA binding capacity (10, 12) and also interact with each other (15), indicating that the two binding sites are maintained in TLP. A structural model of the bovine TLP, based on its cyanogen bromide splitting, has been proposed (14): two fragments could thus be characterized, in accordance with the presence of a single methionine residue in the molecule (16). One of them, the 18-kDa fragment, constitutes the N-terminal moiety of TLP and bears a glyclosidic side chain; the other, the 27.5-kDa fragment, is not glycosylated and consists of the C-terminal region (14, 16). Recently, the complete primary structures of rat and chicken link proteins have been reported revealing a tandemly repeated sequence in the C-terminal part of both (17, 18); this redundant structure has been proposed to participate in their interaction with HA. However, definite arguments permitting the precise localization of the PGS- and HA-binding sites in LP are still lacking. The aim of this work is to characterize such substructures in bovine nasal cartilage TLP.

EXPERIMENTAL PROCEDURES

Materials

The tryptic fragments TLP and HABR arising from bovine nasal cartilage proteoglycan complex were obtained by trypptic treatment of the complex and purified according to Ref. 19; their homogeneity was assessed by SDS-PAGE. HA from human umbilical cord and diphenylcarbamyl chloride-treated trypsin (EC 3.4.21.4) were from Sigma. Fractogel TSK HW 55 (F) was from Merck, Sephacryl S-300 and Superose 6 (HR 10/30 column) were from Pharmacia Biotechnology, Inc. The Novablot electrophoretic transfer system was from LKB and the Biotrap apparatus from Schleicher & Schüll. All other reagents (analytical grade) were from Prolabo (Paris, France), Sigma, Merck, and Carlo Erba (Milan, Italy).

Methods

Separation Methods—Fractogel TSK HW 55 (F) gel filtrations were performed with 25 mM ammonium bicarbonate, 1 mM EDTA, pH 7.8, buffer as eluent (buffer A) on an analytical scale on a 30 × 0.7-cm column, flow rate 0.5 ml/min or at a preparative scale on a 60 × 2-cm column, flow rate 1.5 ml/min. Sephacryl S-300 gel filtration was performed in a 50 mM sodium acetate, 4 M GdnHCl, pH 5.8 buffer (buffer B) on a 180 × 1-cm column, flow rate 0.1 ml/min. Superose 6 gel filtration was performed with the aid of a FPLC chromatograph (Pharmacia) on an HR 10/30 column using buffer B as eluent, flow rate 0.5 ml/min. Recordings of the elution profiles were obtained with the aid of a Pharmacia UV-1 monitor at 280 nm. Samples recovered from Sephacryl S-300 and Superose 6 gel filtrations in buffer B were dialyzed against distilled water before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on 12 or 15% polyacrylamide slab gels (6.7 mm) following the method of Laemmli (20). Samples were applied in 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, pH 6.8 buffer with or without 5% 2-mercaptoethanol (reducing and nonreducing conditions) with bromophenol blue as tracer dye. Proteins were detected with 0.05% Coomassie Brilliant Blue G in methanol/acetic acid/water (6:1.5, v/v/v) and glycoproteins or glycopeptides were stained with the periodic acid-Schiff reagent following Zacharius et al. (21).
Analytical Methods—SA4 monoclonal antiserum, directed against rat chondrosarcoma LP, was a gift of B. Caterson (University of West Virginia) (22); it recognized epitopes localized on the C-terminal moiety of LP (17). Western blot experiments were carried out from SDS-PAGE; in brief, electrotransfer on 0.22-μm nitrocellulose sheets was performed in 50 mM sodium borate, pH 8.0 buffer containing 20% methanol for 1 h at constant current (0.8 mA/cm²) using a Novablot electrophoretic transfer system. The nitrocellulose sheets were revealed following the method of Campbell (23) by horseradish peroxidase-labeled anti-mouse IgG (Amersham Corp.) using 2,2'-azino-di-(3-ethylbenzthioazoline sulfonate) as substrate. Electrophotoblotting on polyethylene-coated glass fiber sheets was performed from SDS-PAGE following Vandekerckhove et al. (24); in brief, two polyethylene-coated sheets of glass microfiber paper GF/C (Whatman) were used for every transfer. The electrotransfer was carried out as described for the immunoblot experiments and only the most anodal sheet was revealed with fluorescamine. The sheet in contact with the polyacrylamide gel was used for protein sequencing experiments. Electroelution from SDS-PAGE using the Biotrap apparatus was performed according to Ref. 25 in a 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3 buffer at constant voltage (200 V) for 8 h; the recovered material was then dialyzed against distilled water before N-terminal amino acid sequence analysis. About 70–80% of the material applied to SDS-PAGE could be recovered by this method. N-terminal amino acid sequences were determined using a Bio-Applied 470 A sequencer equipped with a 120 PTH analyzer. Cyanogen bromide (CNBr) treatment was performed as previously described (16). Uronates were determined by the carbazole method (26).

Tryptic Treatment of the Complexes—TLP-HA reassociations were performed by mixing various amounts of HA with TLP in buffer B (20 mM TLP in 1 ml) at room temperature for 2 h, and the GdnHCl concentration was brought to 1 M by stepwise addition, every 15 min, of 1 volume of buffer A; the samples were then exhaustively dialyzed against buffer A. Aliquots (1/10) of these mixtures were submitted to gel filtration on an analytical TSK HW 55 (F) column and the area under the excluded peak corresponding to the TLP-HA complex was estimated. Due to its insolubility, TLP was lost on the column if not washed with buffer B in order to entirely remove insoluble TLP and was re-equilibrated with buffer A. The binding remained constant for samples containing less than 10 μg of HA, 1 nM TLP; the column was washed with buffer B in order to entirely remove insoluble TLP and was re-equilibrated with buffer A. The binding remained constant for samples containing 15–25 μg of HA for 1 nM TLP. The smallest hyaluronate oligomers required for binding to TLP is a decasaccharide (9); thus, under our conditions, it appears that the maximum recovery is reached with an excess of 7–8 potential binding sites. Therefore, in subsequent preparative experiments, TLP-HA mixtures were done with a TLP/HCA ratio of 1 nM/15 μg, corresponding to an 8-fold excess of HA potential binding sites per TLP molecule, and were directly used for tryptic digestion experiments.

TLP-HABR reassociation was performed by pooling the two protein-protein complexes (molar ratio 1:1) and stepwise dilution of the mixture followed by extensive dialysis in buffer A (10); this mixture was directly used for tryptic digestion experiments; free TLP or HABR, upon tryptic digestion, did not give rise to any of the protected fragments characterized in this study (see "Results").

Tryptic treatments were, in all instances, carried out in buffer A at 37 °C for TLP-HA and TLP-HABR complexes with enzyme/substrate ratios ranging from 1/50 to 1/10 by weight. After 18 h (TLP-HA) or 6 h (TLP-HABR) aliquots of the digests were then subjected to analytical SDS-PAGE as described above.

RESULTS

Tryptic Treatment of TLP-HA—TLP in TLP-HA complex was highly protected from tryptic digestion when compared to TLP alone. Long time intervals (18 h) were necessary to produce any significant cleavage of TLP in the presence of HA. Under these conditions TLP-HA gave rise to split products observed in the nonreduced digests even at a trypsin/TLP ratio of 1/50. One of them (band X in Fig. 1) increased in intensity with increasing amounts of trypsin; the others did not accumulate (Fig. 1, lanes 3). At an enzyme substrate ratio of 1/10, almost the totality of TLP was digested (Fig. 1c, lane 3). The excluded peak areas after TSK HW 55 (F) gel filtrations of the digests became lower with increasing amounts of trypsin, reflecting the TLP digestion. The split products of TLP-HA complex characterized by SDS-PAGE were recovered in these peaks (Fig. 1, lanes 4), demonstrating their interaction with HA as the latter eluted in the void volume of the column. The more retarded peaks contained fragments not detectable by SDS-PAGE. Thus large amounts of trypsin (trypsin/TLP = 1/10) and long digestion periods are required for an almost complete digestion of TLP in TLP-HA complex.

Molecular Characterization of the HA-protected Tryptic Fragment (X) from TLP-HA Digest—The HA-associated degradation products from an 18-h tryptic digest of TLP-HA complex (trypsin/TLP = 1/10) were freed of small non-HA-associated peptides by preparative TSK HW 55 (F) gel filtration (not shown). The elution profile was similar to that shown in Fig. 1c. HA was separated from the peptidic materials by Sephacryl S-300 gel filtration under dissociative conditions. HA eluted in the void volume and 280 nm absorbing materials were recovered into two included peaks (Fig. 2). The first of them contained trace amounts of apparently non-degraded TLP and the second, the major one, the HA-protected fragments. After reduction and alkylation of the material contained in the latter, the component with fast electrophoretic mobility (band X in Fig. 1) was isolated in a pure state after electroelution from SDS-PAGE as described under "Experimental Procedures"; its relative molecular mass was estimated at 22 kDa. Its N-terminal amino acid sequence was determined as: Asp-Leu-Gln-Gly-Val-Val-Phe-Pro-Tyr-Phe-

FIG. 1. Characterization of the tryptic digests (18 h) by TSK HW 55 (F) gel filtration and SDS-PAGE (unreduced samples) of TLP-HA complex at various trypsin/TLP ratios. Enzyme substrate = (a) 1/50, (b) 1/25, (c) 1/10. Lane 1, untreated TLP; lane 2, trypsin-digested TLP; lane 3, trypsin-digested TLP-HA complex; lane 4, trypsin-digested TLP-HA complex, material recovered in the corresponding excluded peak (—) from TSK HW 55 (F) gel filtration. X, the TLP-HA-protected fragment. Standard markers were α-lactalbumin, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin.

FIG. 2. Sephacryl S-300 gel filtration of a TLP-HA 6-h tryptic digest (trypsin/TLP: 1/10). —— represents the fraction containing the TLP protected fragments.
Pro-Arg; its electrophoretic mobility was not modified upon CNBr treatment. Such a sequence is absent in the 101 first amino acids in bovine TLP (27) but was already determined in a tryptic peptide isolated from its 27.5-kDa CNBr-fragment (data not shown). These data demonstrate that the 22-kDa fragment isolated from the tryptic digest of TLP·HA complex arose from the C-terminal moiety of TLP.

Trypsin Treatment of TLP·HABR—The reformed TLP·HABR complex was digested for 6 h by various amounts of trypsin; as controls, TLP and HABR alone were submitted to the same treatment under identical conditions. Analytical SDS-PAGE of the nonreduced digests is presented in Fig. 3. TLP·HABR digests gave rise to a major compound characterized as band Y (Fig. 3, lanes 12-14). This compound was not detected in the free HABR nor in the free TLP digests. TLP was extensively digested when free as well as when involved in TLP·HABR, whereas large amounts of HABR remained apparently intact. However, reduced digests revealed that HABR splitting occurred to the same extent when free or when involved in TLP·HABR (not shown); band Y mobility was not modified upon reduction. For every trypsin/TLP·HABR ratio, band Y was present in an equal amount. These results clearly demonstrate that band Y is a final degradation product protected against tryptic digestion. Thus relatively milder digestion conditions were required for a complete splitting of TLP in the TLP·HABR complex when compared to the TLP·HA complex.

Molecular Characterization of the Tryptic Fragment (Y) from the TLP·HABR Digest—The split products from a 6-h nonreduced tryptic digest of TLP·HABR (trypsin/TLP·HABR = 1/25) were separated by FPLC using a Superose 6 column under dissociative conditions. The two first eluted peaks contained compounds whose electrophoretic mobilities were identical to those of apparently intact HABR and TLP, respectively, when submitted to analytical SDS-PAGE under nonreducing conditions. The third eluted peak (Fig. 4) contained the material characterized as band Y in Fig. 3; no protein band could be detected on SDS-PAGE in the more retarded peaks. Based on its relative electrophoretic mobility, band Y molecular mass was estimated at 20 kDa (Fig. 5, lane 2) and was stained by the periodic acid-Schiff reagent (Fig. 5, lane 5). By Western blot analysis the 8A4 monoclonal antibody revealed that band Y did not derive from the C-terminal end of TLP and did not rule out its HABR origin. However, the following structural data will establish its TLP origin.

Upon CNBr treatment, the 20-kDa fragment mobility was unaffected on SDS-PAGE under nonreducing conditions; however, its cleavage into two fragments of 18 and 9 kDa relative molecular mass was observed after reduction (Fig. 5, lane 3). Only the 18-kDa CNBr fragment was periodic acid-Schiff reagent-positive (Fig. 5, lane 4). The N-terminal amino acid sequences, determined after electroblotting on polybrene-coated glass filter sheets, of the 20-kDa fragment (band Y) and of its 18-kDa CNBr fragment were identical to that of the native TLP (Table I); the N-terminal sequence of the 9-kDa fragment was identical to that of the 27.5-kDa fragment corresponding to the TLP C-terminal CNBr peptide (16).

**DISCUSSION**

TLP in TLP·HA complex is more highly protected from tryptic digestion than when involved in TLP·HABR complex. Table I summarizes the molecular properties of the two TLP fragments described in this study.

The TLP-protected region in TLP·HA is localized in the C terminal moiety of TLP, whereas in TLP·HABR its N-terminal region is protected.

Recently the primary structures of the link proteins from rat chondrosarcoma and chick limb bud chondrocytes have been elucidated, revealing a tandemly repeated structure in both, which has been proposed to participate in their HA binding ability (17, 18). Redundant peptides have been iso-
TABLE I
Molecular characterization of the two fragments of TLP origin obtained by tryptic treatment of TLP-HA and TLP-HABR complexes

<table>
<thead>
<tr>
<th>Complex</th>
<th>Molecular mass after CNBr treatment and reduction (kDa)</th>
<th>N-terminal sequence</th>
</tr>
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<tbody>
<tr>
<td>TLP-HA</td>
<td>22</td>
<td>DLQMVPGYFR</td>
</tr>
<tr>
<td></td>
<td>140°</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td>1°</td>
<td>LLVEAEQAKVFSRGG</td>
</tr>
<tr>
<td></td>
<td>25°</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>LLVEAEQAKV</td>
</tr>
<tr>
<td></td>
<td>25°</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>GYHKKT</td>
</tr>
<tr>
<td></td>
<td>84°</td>
<td></td>
</tr>
</tbody>
</table>

TLP-HABR

The one-letter amino acid abbreviation system is used. * Amino acid position in bovine TLP (27).  
Amino acid position in rat chondrosarcoma LP (17).

Fig. 6. Tentative model based on rat chondrosarcoma LP (17) depicting the two subsites in bovine TLP protected from tryptic digestion by HA or HABR. The one-letter amino acid abbreviation system is used. * Amino acid position in bovine TLP (27).  
Amino acid position in rat chondrosarcoma LP (17).  

Concerning bovine LP (14, 16) it is clear that its 18-kDa CNBr fragment corresponds to a 59-amino acid-long peptide, the apparent M, of this fragment being largely overestimated due to its glycosylation. Therefore, the 20-kDa protected fragment is in fact a ~140-amino acid-long N-terminal peptide from TLP. Fig. 6 presents a tentative model based on rat chondrosarcoma LP structure (17) depicting the regions protected by HABR and HA from tryptic hydrolysis. From these results it can be postulated that the binding of LP with PGS and HA involves two different substructures in LP: an N-terminal domain, which has been demonstrated to present structural homologies with the immunoglobulin superfamily (27), and a redundant C-terminal domain possessing homologies with a part of the proteoglycan core (28, 29), respectively.

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REFERENCES