Purification and characterization of a hyaluronan-binding protein from rat chondrosarcoma

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Swarm rat chondrosarcoma contains a hyaluronan-binding protein of molecular mass 102 kDa (HABP102). The protein is present in 4 M-guanidinium chloride extracts of the chondrosarcoma and can be incorporated into reconstituted proteoglycan aggregates, but it is not present in native proteoglycan aggregates or in 0.5 M-guanidinium chloride extracts. HABP102 is unlikely to be an integral membrane protein, as it does not require detergent for extraction, is not enriched in hydrophobic amino acids and does not bind avidly to octyl-Sepharose. The protein stains poorly with Coomassie Blue and is only visible on PAGE gels after staining with silver. Disulphide bonds are essential for the binding of HABP102 to hyaluronan, and bivalent cations are not required for this interaction. HABP102 can be purified from dissociative chondrosarcoma extracts by sequential density-gradient centrifugation, hyaluronan-Sepharose affinity chromatography and hydrophobic-interaction chromatography. The amino acid composition is similar to that of domains 1–4 of the chondrosarcoma proteoglycan core protein, but peptide analysis after digestion with *Staphylococcus aureus* V8 proteinase and chymotrypsin and different immunoreactivity suggest that HABP102 is not closely related to proteoglycan hyaluronan-binding region. HABP102 is a glycoprotein containing *N*-acetyl-galactosamine, *N*-acetylglucosamine, mannose and galactose.

INTRODUCTION

Hyaluronan (HA) plays an important structural role in cartilage extracellular matrices as the central backbone in proteoglycan (PG) aggregates (Hascall, 1977). PG subunits bind non-covalently via a glycosaminoglycan-free binding region (HABR) at the *N*-terminus of the molecule to an HA chain (Heinegård & Hascall, 1974). This interaction is stabilized by link protein, a molecule that has considerable sequence similarity to HABR and that binds to both HA and PG monomer (Hardingham, 1979; Franzén *et al.*, 1981). It is the presence of these large PG aggregates in the collagen network that gives articular cartilage its ability to withstand high compressive forces (Kempson *et al.*, 1976). It is likely that the role of HA in cartilage is not just that of an inert binding unit upon which PGs are replaced during the course of PG catabolism, but that HA is metabolically active and may have regulatory as well as structural influences. Morales & Hascall (1988) have suggested that the catabolism of HA and PG is co-ordinately regulated by the chondrocyte, with uptake perhaps being achieved via specific receptors similar to those involved in specific endocytosis of HA before catabolism in liver endothelial cells (Laurent *et al.*, 1986). As well as being present in the extracellular matrix of cartilage, HA is also present in the chondrocyte pericellular matrix, where it is responsible for retaining PG at the cell surface (Goldberg & Toole, 1986) and where it may have some control on PG synthesis, perhaps acting via a specific receptor (Wiebkin & Muir, 1973; Solursh *et al.*, 1974; Handley & Lowther, 1976; Bansal *et al.*, 1986). HA evidently has an important role in cartilage development, as it inhibits chondrogenesis *in vitro* and its concentration decreases markedly

during chondrogenesis *in vivo* (Singley & Solursh, 1981; Knudson & Toole, 1985). It has been suggested that these effects are mediated by HA-binding sites that have been shown to appear in limb buds at the time of condensation but that are not present before this (Knudson & Toole, 1987).

HA receptors have not been isolated from chondrocytes, but various HA-binding proteins have been detected in a number of other tissue types. Brain contains a soluble 68 kDa HA-binding glycoprotein, hyaluronectin (Delpech & Halavet, 1981). In the liver, endothelial cell-surface receptors have been characterized that are responsible for receptor-mediated endocytosis of HA for catabolism (Laurent *et al.*, 1986; Raja *et al.*, 1988). Chick-embryo heart fibroblasts synthesize a 66 kDa soluble HA-binding protein (Turley, 1982) and a large (1000–2000 kDa) cell-associated protein complex with an HA-binding site and protein kinase activity (Turley, 1989). An 85 kDa HA-binding protein associated with the cytoskeleton and first described in 3T3 cells (Underhill *et al.*, 1983) has now been located in numerous other tissues (Underhill, 1989).

We have recently described a hyaluronan-binding protein, HABP102, isolated from the Swarm rat chondrosarcoma (Mason *et al.*, 1989). In the present paper we describe the further characterization and purification of HABP102 and discuss its possible location and any relationship to previously described HA-binding species.

MATERIALS AND METHODS

Materials

Guanidinium chloride was from Aldrich Chemical Co.

Abbreviations used: PG, proteoglycan; HA, hyaluronan; HABP, hyaluronan-binding protein; HABR, hyaluronan-binding region.

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(Gillingham, Dorset, U.K.). Hyaluronan (from human umbilical cord), chondroitin ABC lyase (EC 4.2.2.4), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide hydrochloride, molecular-mass markers for SDS/PAGE (MW-SDS-70L and MW-SDS-200), 6-aminohexanoic acid, benzamidine hydrochloride, phenylmethane-sulphonyl fluoride, *N*-ethylmaleimide, BSA (radio-immunoassay grade), chymotrypsin and *Staphylococcus aureus* V8 proteinase were from Sigma Chemical Co. (Poole, Dorset, U.K.). AH-Sepharose 4B, Sepharose CL-6B and octyl-Sepharose 4B were from Pharmacia LKB (Milton Keynes, Bucks., U.K.). Acrylamide (grade I), *NN'*-methylenebisacrylamide, CsCl and glycine were from BDH Chemicals (Poole, Dorset, U.K.). CHAPS was from Boehringer Corp. (Lewes, East Sussex, U.K.). The 1C6 and 8A4 monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A., and the Department of Biology, University of Iowa, Iowa City, IA, U.S.A., under Contract NOI-HD-6-2915 from the National Institute of Child Health and Human Development. Goat anti-(mouse IgG) antibody-horseradish peroxidase conjugate and 4-chloro-1-naphthol were from Bio-Rad Laboratories (Watford, Herts., U.K.). Nitrocellulose membranes were from Schleicher und Schüll (Dassel, Germany). Immobilon [poly(vinylidene difluoride)] transfer membranes were from Millipore Corp. (Bedford, MA, U.S.A.). Minicon-B15 sample concentrators were from Amicon (Stonehouse, Glos., U.K.). Aurodye colloidal gold stain was from Janssen Life Science Products (Olen, Belgium).

Methods

Tumour maintenance and harvesting. The Swarm rat chondrosarcoma was maintained in male Sprague-Dawley rats by subcutaneous injection of tumour mince in Hanks balanced salt solution (Mason & Bansal, 1987). Tissue was harvested at 5–6 weeks. The tumour outer capsule was removed and the nodules were dissected with a scalpel blade and any necrotic areas discarded. The tumour pieces were forced through a 1 mm²-mesh stainless-steel sieve by use of the bottom of a small beaker and the resultant mince was used in extractions.

Extraction. Tumour mince was extracted for three periods of 3 h at 4 °C with agitation in 5 ml of 0.5 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing proteinase inhibitors (50 mM-disodium EDTA, 0.1 M-6-aminohexanoic acid, 5 mM-benzamidine hydrochloride and 0.5 mM-phenylmethane sulphonyl fluoride)/g wet wt. After each time period the extract was centrifuged at 1000 *g* for 10 min, the supernatant removed (associative extract) and fresh extracting solution added to the mince. After extraction under associative conditions the unextracted residue was extracted in 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing proteinase inhibitors and with or without 0.5% CHAPS for 16 h at 4 °C with agitation (dissociative extract). In some cases this extract was dialysed against 0.5 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing proteinase inhibitors overnight at 4 °C to allow reassociation of PG aggregates.

Density-gradient centrifugation. The chondrosarcoma extracts were subjected to CsCl-density-gradient centrifugation under associative or dissociative conditions essentially as described by Faltz *et al.* (1979). For preparation of PG aggregates, associative extracts or dissociative extracts dialysed to associative conditions were adjusted to a starting density of 1.58 g/ml by the addition of solid CsCl. The solutions were centrifuged at 100 000 *g* (r_{av} , 8.1 cm) for 48 h at 10 °C in a Sorvall TFF 50.38 rotor. The resultant gradients were fractionated and assayed for density, uronic acid content and protein by absorbance at 280 nm. Material in the bottom quarter (density > 1.6 g/ml) was retained and is referred to as a-A1 for associative extracts processed in associative gradients (containing native aggregates) and A1 for dissociative extracts processed in associative gradients (containing reconstituted aggregates). For preparation of chondrosarcoma proteins with an affinity for hyaluronic acid, a-A1 fractions (native aggregates) or A1 fractions (reconstituted aggregates) were made 4 M with respect to guanidinium chloride and adjusted to a starting density of 1.48 g/ml with solid CsCl. Centrifugation and fractionation were as described above for associative gradients. Material in the top quarter of the gradient (density < 1.4 g/ml) was retained and is referred to as a-A1.D4 for protein components of native aggregates and A1.D4 for protein components from reconstituted aggregates. For the preparation of chondrosarcoma protein, associative extracts (made 4 M with respect to guanidinium chloride) or dissociative extracts were adjusted to a starting density of 1.48 g/ml with solid CsCl. Centrifugation and fractionation were as described above. Material in the top quarter of the gradient (density < 1.4 g/ml) was retained and is referred to as a-D4 for protein from associative extracts and D4 for protein from dissociative extracts.

SDS/PAGE and blotting. Some samples were digested with chondroitin ABC lyase before electrophoresis. A 5 ml portion of an A1.D4 fraction, containing 80 µg of protein, was dialysed to 10 mM-NaF/60 mM-sodium acetate/50 mM-Tris/HCl buffer, pH 8.0, containing proteinase inhibitors. One unit of chondroitin ABC lyase was added and digestion was allowed to proceed overnight at 37 °C. Samples for immunolocalization with antibody 1C6 were reduced and alkylated before electrophoresis (Heinegård, 1977).

All samples were concentrated and exchanged into 2% (w/v) SDS/0.001% Bromophenol Blue/62.5 mM-Tris/HCl buffer, pH 6.8, with or without 5% (v/v) 2-mercaptoethanol with the use of Minicon B-15 sample concentrators, and then heated at 100 °C for 3 min. Electrophoresis was performed in 5–16%-linear-gradient polyacrylamide slab gels (14 cm-wide 11 cm-long resolving gel with a 2.5 cm stacking gel), by the discontinuous method of Laemmli (1970). After electrophoresis, proteins were either first stained with Coomassie Brilliant Blue R250 (Fairbanks *et al.*, 1971) and then with silver (Morrissey, 1981), or transferred on to nitrocellulose or Immobilon membranes (Towbin *et al.*, 1979). Stained protein bands on gels were quantified by scanning densitometry (Joyce-Loebl Chromoscan 3). To determine efficiency of transfer of proteins on to nitrocellulose, duplicate lanes were cut out of the nitrocellulose and stained with Aurodye colloidal gold stain according to

the manufacturer's instructions (Janssen Life Science Products).

Peptide mapping. Peptide mapping was carried out essentially as described by Cleveland *et al.* (1977). Bands containing polypeptides for mapping were cut out of SDS/PAGE gels and equilibrated for 30 min in 10 ml of 0.1 % SDS/125 mM-Tris/HCl buffer, pH 6.8. An 8–20 % linear-gradient SDS/PAGE gel was prepared with a tall stacking gel (4.5 cm). Polypeptide bands from the first PAGE separation were placed in the sample wells and overlaid with 25 ng of *S. aureus* V8 proteinase or 400 ng of chymotrypsin in 0.1 % SDS/0.001 % Bromophenol Blue/125 mM-Tris/HCl buffer, pH 6.8. Electrophoresis was carried out until the tracking dye had traversed two-thirds of the stacking gel. The current was switched off for 40 min, during which time digestion occurred. Electrophoresis, staining and densitometry were as described above for SDS/PAGE.

Immunodetection. Following transfer, the nitrocellulose was blocked by incubation with 3 % (w/v) BSA in Tris-buffered saline (0.5 M-NaCl/20 mM-Tris/HCl buffer, pH 7.5) overnight at room temperature. The blot was incubated with primary antibody (8A4 at 1:800 or 1C6 at 1:100) in BSA/Tris-buffered saline for 16 h at room temperature, washed in several changes of BSA/Tris-buffered saline and then incubated in horseradish-peroxidase-conjugated anti-(mouse IgG) antibody (1:2000 in BSA/Tris-buffered saline) for 4 h. After further washes colour development was achieved by incubation in Tris-buffered saline containing 0.6 mg of 4-chloro-1-naphthol/ml, 0.02 % H_2O_2 and 20 % (v/v) methanol.

HA-Sepharose affinity chromatography. HA was depolymerized with ascorbic acid (Swann, 1967). After depolymerization, HA was precipitated by the addition of 4 vol. of ethanol. The HA was pelleted by centrifugation, washed in 80 % (v/v) ethanol and then redissolved in water and freeze-dried. Depolymerized HA had a mean molecular mass of 40 kDa as determined by Sepharose 6B chromatography (Wasteson, 1971). Depolymerized HA was coupled to AH-Sepharose 4B by using a carbodi-imide coupling procedure (Tengblad, 1979). The amount of HA bound to the Sepharose was 1 mg/ml of swollen gel as determined by uronic acid assay (Bitter & Muir, 1962) after releasing it by digestion with chondroitin ABC lyase (Yamagata *et al.*, 1968). Fractions for the affinity chromatography were mixed with an equal volume of HA-Sepharose and then dialysed in small steps to 1 M-NaCl/50 mM-sodium acetate buffer, pH 6.8, containing proteinase inhibitors in order to maximize any interaction (Bansal *et al.*, 1986). In some experiments the buffer also contained 10 mM-dithiothreitol or 10 mg of HA (40 kDa)/ml prepared by ascorbate depolymerization as described above. The affinity gel was washed sequentially with 1 M-, 2 M- and 3 M-NaCl and then eluted with 4 M-guanidinium chloride/0.5 % CHAPS (all in 50 mM-sodium acetate buffer, pH 6.8, containing proteinase inhibitors).

Hydrophobic-interaction chromatography. Hydrophobic-interaction chromatography was carried out essentially as described by Woods *et al.* (1984). Octyl-Sepharose 4B was equilibrated in HIC buffer (4 M-

guanidinium chloride/20 mM-Tris/HCl buffer, pH 7.0, containing proteinase inhibitors). A1.D4 fraction was exchanged into the same buffer and incubated with the gel overnight at 4 °C. The octyl-Sepharose was washed with several volumes of HIC buffer and then eluted stepwise with increasing concentrations of Triton X-100 in HIC buffer.

Amino acid analysis. HA-binding fractions were separated on SDS/PAGE and blotted on to Immobilon membranes as described above. The HABP102 bands were cut out, washed extensively in 2 M-NaCl to remove any residual glycine from the PAGE running buffer and then dried. The protein was hydrolysed in 6 M-HCl for amino acid determination, or 4 M-methanesulphonic acid for amino sugar determination, under N_2 for 24 h at 110 °C. For cysteine determination, samples were first oxidized with performic acid (Hirs, 1967). Amino acids and amino sugars were analysed on an LKB Alpha-plus analyser fitted with a 25 cm column (in the Na^+ form) with ninhydrin detection system. Norleucine was used as an internal standard.

Carbohydrate analysis. A g.l.c. method was used for the analysis of neutral sugars and the amino acid analyser was used for the amino sugars (see above).

The trimethylsilyl derivative method was carried out according to the procedure of Chambers & Clamp (1971). The HA-binding fraction was separated by SDS/PAGE and blotted on to an Immobilon membrane, and the bands of interest were cut out. After extensive washes in 2 M-NaCl and then water, the protein on the Immobilon membrane was methanolysed in 1 M-HCl in methanol under N_2 at 85 °C for 4 h, with mannitol as internal standard. The methanolysate was dried under a stream of N_2 and the methyl glycosides were converted into the trimethylsilyl derivatives by the addition of trimethylchlorosilane/hexamethyldisilazane/pyridine (1:3:9, by vol.). After incubation for 10 min at 25 °C the trimethylsilyl derivatives were separated with N_2 by a capillary g.l.c. system on a WCOT fused-silica column with a coating of CP-SIL8 (Chrompack, Middeburg, The Netherlands). The gas chromatograph was a Varian model 3300 fitted with flame ionization detection and the peaks were integrated by a Varian 4290 integrator.

RESULTS

In our initial experiments we used HA-Sepharose affinity chromatography of a protein fraction (D4), derived from dissociative extracts of rat chondrosarcoma, to isolate a HA-binding fraction. This contained link protein, small amounts of a 66 kDa protein and a protein that we have named HABP102 (Mason *et al.*, 1989). SDS/PAGE analysis of the protein components of reconstituted aggregates (an A1.D4 fraction), also derived from dissociative extracts of rat chondrosarcoma, revealed that this fraction contained the same protein species as the fraction that bound to the HA-Sepharose, but in using this second method for purification a better yield was achieved in a shorter time period. Consequently, for all subsequent characterizations of HABP102, this was the method of preparation of the HA-binding fraction. Link protein was the only protein visible in the A1.D4 fraction on Coomassie Blue-stained SDS/PAGE gels (Fig. 1a). After silver staining of the same gel (Fig.

Fig. 1. Staining characteristics of HABP102 in PAGE gels

An A1.D4 fraction from the CsCl-density-gradient centrifugation of dissociative extracts of rat chondrosarcoma was analysed by SDS/PAGE under reducing conditions. Protein was detected (a) by staining with Coomassie Blue and scanning densitometry of the gel and then (b) by silver staining the same gel and scanning densitometry of the gel. Percentages shown on the densitometer traces are absorbance at 530 nm expressed as percentages of the total absorbance at 530 nm of the whole lane.

1b) HABP102 was the most heavily stained protein in the fraction (63 % of total absorbance at 530 nm). HABP102 could therefore be incorporated into reconstituted aggregates. Experiments were then carried out to determine whether it was also present in the 0.5 M-guanidinium chloride extract, which contained approx. 70 % of the total chondrosarcoma uronic acid. Firstly, to determine whether HABP102 was a constituent of native aggregates, an a-A1.D4 fraction was prepared from an associative extract of the chondrosarcoma and the proteins in this fraction were compared on SDS/PAGE separations with those in the A1.D4 fraction. No HABP102 was detectable in the a-A1.D4 fraction even when the gels were intentionally overloaded to reveal any minor components. In addition to link protein, a major component of molecular mass 66 kDa was present in this fraction (result not shown). Although HABP102 was not detectable as a component of native aggregates, it may be present in associative extracts. In order to determine whether this was the case, a total protein fraction (a-D4) was prepared from an associative extract of the chondrosarcoma and subjected to HA-Sepharose affinity chromatography under conditions identical with those used previously to prepare HABP102 from a sequential dissociative extract (Mason *et al.*, 1989). No HABP102 was detectable by silver stain after SDS/PAGE of the HA-binding fraction (Fig. 2, lane b). A 66 kDa protein was

Fig. 2. HABPs in associative extracts of rat chondrosarcoma

The total protein fraction (a-D4) from the CsCl-density-gradient centrifugation of rat chondrosarcoma associative extracts was chromatographed on an HA-Sepharose affinity column. The HA-binding fraction eluted from the affinity column with 4 M-guanidinium chloride/0.5 % CHAPS was analysed by SDS/PAGE under reducing conditions and either subjected to Coomassie Blue staining (lane a) or silver staining (lane b) or blotted on to nitrocellulose and probed with monoclonal antibodies directed against PG HABR (lane c) or against link protein (lane d).

the most abundant protein in this fraction on both Coomassie Blue-stained (Fig. 2, lane a) and silver-stained (Fig. 2, lane b) gels. On Western blots the 66 kDa protein cross-reacted with monoclonal antibody 1C6 directed against PG HA-binding region (HABR) (Caterston *et al.*, 1986). Link protein was also identified in this fraction by using an anti-(link protein) monoclonal antibody, 8A4 (Fig. 2, lane d). A number of other proteins were also present but were not identified.

We were previously unable to elute HABP102 or link protein from HA-Sepharose by using HA in solution (Mason *et al.*, 1989). However, inclusion of 10 mg of HA (40 kDa)/ml in the dialysis solutions during stepwise dialysis of a D4 fraction and HA-Sepharose to conditions that result in binding in controls prevented the binding of HABP and link protein to the affinity gel (Fig. 3).

HABP102 failed to focus into a discrete band on SDS/PAGE, and this could indicate a degree of glycosylation, perhaps due to the presence of glycosaminoglycan chains. Small dermatan sulphate PGs have been identified in cartilage that have molecular masses of the same order as that of HABP102 and that have core proteins of molecular mass 45 kDa as determined by SDS/PAGE after chondroitin ABC lyase digestion. Although these dermatan sulphate PGs have no known HA-binding activity, such molecules might co-purify in an aggregate preparation. We investigated whether HABP102 contained any dermatan sulphate or chondroitin sulphate chains by investigating its mobility on SDS/PAGE after chondroitin ABC lyase digestion. There was no change in the electrophoretic mobility of

Fig. 3. HA oligosaccharides prevent HABP from binding to HA-Sepharose

A protein fraction from a dissociative extract of chondrosarcoma (D4 fraction) was mixed with HA-Sepharose in the presence or in the absence of 10 mg of HA (40 kDa)/ml and then dialysed to binding conditions. The proteins bound to the affinity gel in the absence (lane b) or in the presence (lane c) of HA (40 kDa) were analysed by SDS/PAGE and detected by silver staining. Lane a, molecular-mass markers.

HABP102 following the enzyme treatment (Fig. 4). After the digestion with chondroitin ABC lyase a 150 kDa protein was detectable (Fig. 4, lane b), indicating that the A1.D4 fraction contained small amounts of a PG that was normally excluded from the resolving gel.

Link protein and the HBR of the chondrosarcoma aggregating PG contain a number of disulphide bonds, and so experiments were carried out to determine whether HABP was a disulphide-bonded multimer and to assess the extent of any intrachain disulphide bonding. The electrophoretic mobility of the A1.D4 fraction was measured before and after reduction with 5% 2-mercaptoethanol. The results (not shown) demonstrated that, although the mobility of link protein decreased from an apparent molecular mass of 35 kDa to 45 kDa after reduction, the mobility of HABP102 was unchanged. However, in a separate experiment it was shown that disulphide bonding is required for the interaction of HABP102 with HA, as HABP failed to bind to HA-Sepharose in the presence of 10 mM-dithiothreitol (results not shown).

HABP102 is extractable from the chondrosarcoma with 4 M-guanidinium chloride in the absence of de-

Fig. 4. Chondroitin ABC lyase digestion of A1.D4 fraction

An A1.D4 fraction from the CsCl-density-gradient centrifugation of dissociative extract of rat chondrosarcoma was subjected to chondroitin ABC lyase digestion and then analysed by SDS/PAGE under reducing conditions. Protein was detected by silver staining. Lane a, chondroitin ABC lyase alone; lane b, A1.D4 fraction after digestion; lane c, A1.D4 fraction undigested; lanes d and e, molecular-mass markers.

tergent, although the yield is increased in the presence of 0.5% CHAPS (results not shown). This evidence suggested that HABP102 is not an intercalated membrane protein, and in order to obtain more information about its properties and possible tissue location the relative hydrophobicity of the protein was assessed by hydrophobic-interaction chromatography of an A1.D4 fraction on octyl-Sepharose 4B (Fig. 5). HABP102 did not undergo a strong interaction with the column and was completely eluted with 0.06% Triton X-100 (Fig. 5, lane 6). Link protein was slightly more hydrophobic, with most of it being eluted at 0.1% Triton X-100 (Fig. 5, lane 10). A complete separation of HABP102 and link protein was achieved by using octyl-Sepharose chromatography (Fig. 5), but only partial separation could be achieved by gel-filtration chromatography under dissociative conditions on Sepharose 6B or Sephadex G-100 columns (results not shown).

Peptide analysis was carried out to investigate the degree of similarity between HABP102 and HBR66. HABP102 was purified electrophoretically from an A1.D4 fraction, and HBR66 was purified electrophoretically from the HA-Sepharose-affinity-column-bound fraction of a-D4. The peptides resulting from their digestion with chymotrypsin and *S. aureus* V8 proteinase were separated by SDS/PAGE. The peptide maps show that HABP102 and HBR66 have few bands in common (Fig. 6), and therefore are unlikely to be closely related. The amino acid composition of HABP102 is shown in Table 1, with the compositions of rat chondrosarcoma link protein (Neame *et al.*, 1986) and proteoglycan core protein domains 1-4 (Doege *et al.*, 1987) for comparison. HABP102 was found to be

Fig. 5. Hydrophobic-interaction chromatography of A1.D4 fraction

An A1.D4 fraction from the CsCl-density-gradient centrifugation of dissociative extract of rat chondrosarcoma was chromatographed on octyl-Sepharose 4B. Protein was eluted with increasing concentrations of Triton X-100 as shown, and each fraction was analysed by SDS/PAGE under reducing conditions. Protein was detected by silver staining.

Fig. 6. Peptide maps of HABP102 and HABR66

Electrophoretically purified HABP102 or HABR66 was digested with chymotrypsin or *S. aureus* V8 proteinase and the resultant peptides were analysed by SDS/PAGE. The electrophoretograms were silver-stained and peptide profiles were compared by measuring the absorbance at 530 nm of each lane in a scanning densitometer. (a) and (d) Peptide maps of HABP102, and (b) and (e) peptide maps of HABR66, after digestion with 25 ng of *S. aureus* V8 proteinase (a and b) or 400 ng of chymotrypsin (d and e). (c) and (f) show profiles of 25 ng of *S. aureus* V8 proteinase and 400 ng of chymotrypsin alone respectively. The positions of the undigested proteins, HABP and HABR, are indicated with arrows.

Table 1. Amino acid composition of HABP102

The amino acid compositions of rat chondrosarcoma link protein (RC LP2) and rat chondrosarcoma (RC) PG HABR are shown for comparison. The composition of link protein is taken from Neame *et al.* (1986). The composition of PG HABR is that of domains 1–4 of the proteoglycan core protein (amino acid residues 20–798; molecular mass approx. 85 kDa without oligosaccharide residues) calculated from the complete core protein sequence determined by Doege *et al.* (1987). Abbreviations: N.D., not detected; N.M., not measured.

	Amino acid composition (residues/100 residues)		
	HABP102	RC LP2	RC PG HABR
Aspartic acid	10.4	12.1	7.1
Threonine	5.9	4.4	9.0
Serine	6.5	5.0	6.4
Glutamic acid	17.4	8.6	13.0
Proline	5.4	5.0	9.5
Glycine	13.7	10.0	7.5
Alanine	7.0	7.9	8.6
Cysteine*	1.7	2.8	2.4
Valine	7.9	6.9	7.6
Methionine	N.D.	3.9	0.9
Isoleucine	3.8	3.7	4.1
Leucine	5.3	8.5	6.0
Tyrosine	2.5	6.1	4.5
Phenylalanine	3.8	5.8	3.3
Histidine	1.3	2.7	1.4
Lysine	3.2	5.4	2.2
Arginine	4.3	7.2	5.0
Tryptophan	N.M.	N.M.	1.5

* Determined as cysteic acid after oxidation in performic acid.

enriched in aspartic acid/asparagine, glutamic acid/glutamine and glycine. Methionine was not detected; hydroxyproline was also not detected, indicating that the protein is non-collagenous. HABP102 is not enriched in hydrophobic amino acid residues and therefore probably does not contain a large hydrophobic domain. Sugar analyses revealed that HABP102 also contains *N*-acetylglucosamine, *N*-acetylgalactosamine, mannose and galactose (results not shown).

DISCUSSION

We have described a glycoprotein of molecular mass 102 kDa present in the Swarm rat chondrosarcoma that binds to HA. The fact that this protein has not been previously identified in chondrosarcoma extracts may in part be due to its low binding to Coomassie Blue on PAGE gels. Degree of Coomassie Blue or silver staining is not proportional to concentration for all proteins; acidic proteins such as pepsin and sialic acid-containing proteins for example do not stain well with Coomassie Blue (Fairbanks *et al.*, 1971). Faltz *et al.* (1979) observed a faint protein band at approx. 100 kDa on heavily loaded Coomassie Blue-stained SDS/PAGE separations of the protein components of chondrosarcoma PG aggregates that may correspond to HABP102. The apparently small amounts found in that study might have been due

to poor Coomassie Blue binding, but could also have been due to the use of high loading densities (1.63 g/ml) in the preparation of an A1 fraction. We have found that this results in low yields of high-buoyant-density PG aggregates, possibly as the result of a high protein content decreasing their density. This is consistent with previous observations that large amounts of protein interfered with aggregate preparation at high starting densities from adult but not neonatal cartilage (Roughley *et al.*, 1984, 1985; Bayliss & Roughley, 1985). This possibly reflects an accumulation of HABR-containing PG fragments with age. The chondrosarcoma contains large amounts of HABR in the form of the 66 kDa species that was found in associative extracts in this study. It is unlikely that they arise from post-isolation cleavage, since a variety of proteinase inhibitors and low temperatures were consistently used in all procedures. Similar fragments have been reported by others (Roughley *et al.*, 1985).

The fact that HABP is not present in 0.5 M-guanidinium chloride extracts but can be incorporated into reconstituted aggregates from 4 M-guanidinium chloride extracts is interesting in terms of its possible tissue location. These results suggest that HABP102 is not associated with aggregates *in vivo* but is released from its site by 4 M-guanidinium chloride and then binds to the HA backbone of aggregates when conditions are altered to favour association. Alternatively the results are consistent with HABP102 being a component of a population of naturally occurring aggregates that are not extractable in 0.5 M-guanidinium chloride. HABP102 is extractable in the absence of detergent and is not very hydrophobic in nature, as assessed by hydrophobic-interaction chromatography and amino acid composition. This indicates that it is not a transmembrane protein but does not preclude the possibility of extrinsic location at the chondrocyte cell membrane, perhaps by a small hydrophobic anchor. Multiple freeze-thaw cycles during chondrosarcoma extraction again resulted in HABP102 being isolated in dissociative and not associative extracts (results not shown), results that argue against an intracellular location.

There is no evidence to suggest that HABP102 is related to any known cartilage matrix proteins or HA-binding proteins that have been characterized in other tissues. The protein does not react on Western blots with antibodies directed against link protein, PG HABR, hyaluronectin or type II collagen (Mason *et al.*, 1989) or with antibody K3 (M. V. Crossman & R. M. Mason, unpublished work), which is directed against the 85 kDa HA-binding glycoprotein associated with the cell cytoskeleton (Underhill *et al.*, 1987). Of the HA receptors characterized to date, HABP102 is closest in apparent molecular mass to the 99.5 kDa HA-binding protein in alveolar macrophages (Green *et al.*, 1988), which reacts with the K3 monoclonal antibody. The previously characterized HA-binding proteins appear to fall into two classes: those that bind a minimum of an HA decasaccharide, including link protein (Tengblad, 1981), PG HABR (Christner *et al.*, 1978) and hyaluronectin (Bertrand & Delpech, 1985), and those that require a minimum of six sugar residues of HA for recognition, these including the 3T3 receptor (Underhill *et al.*, 1983) and the liver receptor (Laurent *et al.*, 1986). Further experiments are required to determine to which class HABP102 belongs.

The amino acid composition of HABP102 is similar to that of domains 1–4 of rat chondrosarcoma PG, but the lack of immunoreactivity with antibody 1C6 and the difference in its peptide profiles compared with the 66 kDa PG HABR isolated from native aggregates suggest that it is not closely related to PG HABR.

In common with link protein and the PG HABR, HABP102 requires intact disulphide bonds to interact with HA. Bivalent cations are not required for the interaction of HABP with HA, since binding occurs in the presence of EDTA.

The function of HABP102 is not known. Receptors for HA in cartilaginous tissues could be involved in extracellular matrix structure, retention of the pericellular matrix at the chondrocyte cell surface, regulation of chondrogenesis, mediation of the effects of HA on PG synthesis and uptake of HA for turnover. We have been unable to detect HABP102 in cultures of a permanent chondrocyte cell line derived from the Swarm chondrosarcoma (Mason *et al.*, 1989). Further work is required to establish the role of HABP102 in the chondrosarcoma and whether it is present in other cartilaginous tissues.

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