Proteoglycans from the Swarm Rat Chondrosarcoma

STRUCTURE OF THE AGGREGATES EXTRACTED WITH ASSOCIATIVE AND DISSOCIATIVE SOLVENTS AS REVEALED BY ELECTRON MICROSCOPY*

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Matthias Mörgelin‡, Jürgen Engel†, Dick Heinegård¶, and Mats Paulsson**

From the ‡M. E. Müller Institute for High Resolution Electron Microscopy and ¶Department of Biophysical Chemistry, Biocenter of the University of Basel, Küngelberstrasse 70, CH-4056 Basel, Switzerland, the †Department of Medical and Physiological Chemistry, University of Lund, P. O. Box 94, S-22100 Lund, Sweden, and the **M. E. Müller Institute for Biomechanics, University of Bern, Postfach 76, CH-3010 Bern, Switzerland

Proteoglycan aggregates were extracted from Swarm rat chondrosarcoma tissue in the native state and compared with proteoglycan aggregates isolated dissociatively with 4 M guanidine HCl. Purified aggregates were examined with a variety of electron microscopic techniques. In some cases they showed a structure of the central filament identical to that of the link-stabilized central filament observed in earlier experiments where the separated constituents were allowed to reconstitute (Mörgelin, M., Paulsson, M., Hardingham, T. E., Heinegård, D., and Engel, J. (1988) Biochem. J. 253, 175–185). The tight packing of proteoglycan monomers along the hyaluronate with a minimum distance of 12 nm between adjacent E1 strands also could thus be confirmed for never dissociated aggregates. The results therefore show that the organization of proteoglycan aggregates assembled in vitro from the participating molecules is representative for conditions in situ.

An additional structural type of central filament was observed in the preparations. This contained long stretches of free hyaluronate interspaced by short stretches of central filament with condensed arrays of link protein-proteoglycan. Chemical cross-linking in combination with low shear electron microscopical techniques showed that this discontinuous central filament structure is not an artifact of specimen preparation. The addition of suprastoichiometric amounts of exogenous link protein did not affect the central filament structure with the low packing density.

Densely and loosely packed types of central filament were isolated in varying relative amounts with different associative and dissociative solvents.

Proteoglycans constitute the major non-collagenous extracellular matrix component of normal hyaline cartilage. They occur predominantly as a very large aggregating species with molecular weights ranging from $1 \times 10^6$ to $4 \times 10^6$ (for review see Heinegård and Paulsson, 1984; Hardingham, 1986; Heinegård and Sommarin, 1987). The average molecule consists of a protein core (M, approximately 220,000, Doerge et al., 1987) which is heavily substituted with about 100 chondroitin sulfate chains, 30 keratan sulfate chains, and 60 N- and O-linked oligosaccharides (Hascall and Saajdera, 1970; Hassel et al., 1972; Heinegård and Axelsson, 1977; Löhmander et al., 1980; Heinegård et al., 1985).

The aggregating proteoglycans (aggrecan) bind specifically to a decasaccharide or larger segment of hyaluronate (Hascall and Heinegård, 1974). Because this non-covalent interaction and the ensuing formation of large aggregates is fundamental for retaining these molecules in cartilage, much attention has been focused on the mechanism of aggregation. It involves a specialized portion of the core protein, the hyaluronate binding region (Heinegård and Hascall, 1974), which has been identified as the globular domain G1 at the NH₂ terminus of the molecule (Doerge et al., 1987; Paulsson et al., 1987; Mörgelin et al., 1988). The interaction is further stabilized by the binding of link protein (Heinegård and Hascall, 1974; Hardingham, 1979) with affinity for both the hyaluronate and hyaluronate binding region (Tengblad, 1981; Franzén et al., 1981), leading to the formation of a very stable ternary complex (Bonnet et al., 1985). A large number of proteoglycans may bind to hyaluronate, and the resulting aggregated structure with its very high negative charge density is responsible for cartilage elasticity and resilience.

Chastropic solvents such as 4 M guanidine HCl effectively dissociate the components in the aggregates by abolishing non-covalent interactions. The dissociated components are readily extracted in high yields (Sajdera and Hascall, 1969). Dialysis into an associative solvent such as 0.5 M guanidine HCl allows a large portion of the molecules to reaggregate.

Taking advantage of this behavior, we have previously studied the assembly of intact monomers and fragments thereof with hyaluronate and link protein by electron microscopy (Mörgelin et al., 1988) using glycerol spraying/rotary shadowing (Shotton et al., 1979; Tyler and Branton, 1980). Aggregates reconstituted in the absence of link protein exhibited a rather loose structure of the central filament consisting of G1 and hyaluronate, where individual G1 domains were distinguished and apparently had a statistical distribution along the hyaluronate. Although gaps of variable size were present, closest center-to-center distances of 12 nm between adjacent G1 domains were measured. A more condensed continuous central filament structure, with a tight packing of monomers along hyaluronate, was observed in the presence of link protein. Individual domains of either G1 or link protein
were not resolved. Long stretches of densely packed central filament regions alternating with short regions of free hyaluronate indicated a high degree of cooperativity in protein binding. Again the closest distances between neighboring monomers were 12 nm. The same continuous central filament structure was previously demonstrated when A1 preparations of bovine nasal cartilage were visualized by this technique (Wiedemann et al., 1984).

The protein components employed in these reconstitution experiments had presumably been denatured during isolation and purification. The significance of the results depends on the proper refolding of the molecules into a native conformation and their self-assembly into supramolecular structures representative of in situ organization. Therefore, in the present study, we compared reassociated to native aggregates. The latter can be extracted in large amounts from the Swam rat chondrosarcoma (Choi et al., 1971).

Proteoglycan monomers from rat chondrosarcoma tissue have a molecular structure similar to those obtained from cartilage of other species, except that they do not contain any keratan sulfate chains (Choi et al., 1971), and the proline-rich keratan sulfate attachment polypeptide sequence is lacking (Doege et al., 1987; Antonsson et al., 1989). Whereas proteoglycans of hyaline cartilage resist quantitative extraction with associative solvents, those from the tumor tissue can be easily isolated in non-denaturing buffers, i.e. with 0.5 M guanidine HCl, 0.05 M sodium acetate, pH 5.8. Under these conditions the non-covalent interactions of the central filament components are not dissociated (Oegema et al., 1975; Faltz et al., 1979a). In the present study the detailed structure of native aggregates was studied using different electron microscopy replica techniques and turned out to be indistinguishable to that of dissociatively prepared (A1) or reconstituted link-stabilized aggregates. We could also demonstrate that two forms of central filament structure in native aggregates can be differentially extracted from the chondrosarcoma by use of a variety of associative solvents. A preliminary report on some aspects of this work has been presented elsewhere (Mörgelin et al., 1990).

MATERIALS AND METHODS

Maintenance of Swarm Rat Chondrosarcoma—Frozen tumor cells were kindly provided by A. Blättler, Ciba-Geigy AG, Basel, Switzerland. The tumor was maintained in male Sprague-Dawley rats, weighing 250–350 g, by subcutaneous injection of 0.5–1 ml of tumor tissue minced in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4 (TBS, about 200 mg/ml suspension). At 4–5-week intervals, the rats were killed and dissected free of surrounding fascia. The cleaned tumor tissue was rapidly frozen on dry ice and stored at –20 °C.

Extraction Procedures—Extraction were carried out at 4 °C and, unless indicated otherwise, in the presence of the following protease inhibitors: 0.05 M EDTA, 0.1 M 6-aminohexanoic acid, 5 mM N-ethylmaleimide, 5 mM benzamide hydrochloride, and 0.5 mM phenylmethylsulfonyl fluoride. The latter two inhibitors were added to the solvents just prior to the extraction. Tumor tissue was sliced with a razor blade and briefly (10 s) homogenized in 5 ml/g wet weight of prechilled 0.05 M guanidine HCl (GdnHCl).2 0.05 M sodium acetate, pH 5.8, using a Polytron homogenizer (Kinematica, Littau, Switzerland) at half-maximal speed, and subsequently extracted for 4 h with stirring. Alternatively, briefly homogenized tumor tissue was extracted dissociatively for 4 h with 5 ml/g wet weight of prechilled 4 M GdnHCl, 0.05 M sodium acetate, pH 5.8, in the presence of protease inhibitors.

In subsequent extraction experiments, dissociated tumor nodules were homogenized in 5 volumes of prechilled TBS, containing 5 mM benzamide hydrochloride, 5 mM N-ethylmaleimide, and 0.5 mM phenylmethylsulfonyl fluoride. The resulting tissue dispersion was extracted for 1 h with stirring. Residues were maintained at 4 °C for 20 min at 8,000 rpm (4 °C, Sorvall, GSA rotor, 10,000 × g). This extraction cycle was repeated twice, and the three supernatants were immediately frozen and stored at –20 °C. The tissue residues were then extracted three times as described above in the same buffer containing in addition 10 mM EDTA (TBS-EDTA). Pellets were extracted from these with 0.5 M GdnHCl, 0.05 M sodium acetate, pH 5.8, and finally with 4 M GdnHCl, 0.05 M sodium acetate, pH 5.8. These extracts were dialyzed into associative conditions, i.e. against 10 volumes 0.05 M sodium acetate, pH 5.8. Aliquots (1 ml) of the first extract obtained with each solvent were chromatographed on Sepharose CL-2B as described below.

Preparation of a-A1 Fractions—0.5 M GdnHCl extracts were cleared by centrifugation in capped polycarbonate tubes (13,000 rpm, 27,000 × g, 45 min, Sorvall GSA rotor). The density was adjusted to give 1.63 g/ml by the addition of 0.5 g of solid CsCl/g of extract. The solutions were then centrifuged in Beckman Quick Seal polycarbonate tubes (12,000 rpm, 20 min) at 10,000 × g (Ultracentrifuge TPT 50.38 rotor). The gradients were separated into four fractions after freezing the centrifuge tubes in liquid nitrogen. Fractions were analyzed for protein and proteoglycan contents by measuring the optical density at 280 nm, dot blots on nitrocellulose developed with toluidine blue, SDS-polyacrylamide gel electrophoresis, and electron microscopy. Material in the bottom one-fourth of the gradient was collected and dialyzed twice against 40 volumes of 0.5 M sodium acetate, pH 7.0. This fraction is subsequently referred to as a-A1 (associative extract, A1 fraction of associative gradient).

Preparation of Link Protein—A 4 M GdnHCl extract was clarified by centrifugation (8,000 × g, 10,000 × g, 100 min, Sorvall SS-34 rotor) and dialyzed overnight at 4 °C into associative conditions. The dialyzed extract was then centrifuged in cesium chloride density gradients as described above. The A1 fraction was recovered and mixed with an equal volume of 8 M GdnHCl, 0.1 M sodium acetate, pH 5.8, and centrifuged under dissociative conditions (starting density 1.53 g/ml). The resulting A1D4 fraction was purified further by chromatography on Sepharose CL-6B, followed by chromatography and rechromatography on Sephacryl S-200. Columns were eluted with 4 M GdnHCl, 0.05 M sodium acetate, pH 5.8.

Digestion with Chondroitinase ABC—Proteoglycan aggregate samples from an a-A1 preparation were either dialyzed extensively against 0.1 M sodium acetate, 0.1 M Tris, pH 7.3, or mixed with the appropriate volume of concentrated buffer. They were usually digested with 1unit of chondroitinase ABC/100 mg proteoglycan for 4–6 h at 37 °C in the presence of ovoalbumin (10 μg/ml) as a protease inhibitor. Proteoglycan concentrations were 5–10 mg/ml. Digested samples were subsequently purified by gel filtration on Sepharose CL-2B as described below.

Column Chromatography—Gels for column chromatography were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. For large scale preparative purposes a Sepharose CL-2B column (67 cm × 2.5 cm) or Sephacryl S-200 (67 cm × 2.5 cm) was used. For the determination of hexuronic acid, 0.5 M sodium acetate, pH 7.0, and 0.05 M sodium acetate, pH 5.8, were used as elution buffer at 0.5 ml/min. The resulting A1D4 fraction was purified further by chromatography on Sepharose CL-6B, followed by chromatography and rechromatography on Sephacryl S-200. Columns were eluted with 4 M GdnHCl, 0.05 M sodium acetate, pH 5.8.

1 The nomenclature of Heinegård (1977) is used throughout. Additionally, the term a-A1 is used to indicate associatively extracted A1 fractions.

2 The abbreviations used are: GdnHCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DSP, dithiobaia(succinimidyl)propionate.
Protein was determined with the bicinchoninic acid Pierce protein assay (Pierce Chemical Co.). Column effluents from Sepharose CL-2B gel filtration of proteoglycan preparations were assayed for protein contents by a simplified microtiter plate bicinchoninic acid assay.

SDS-polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (1970). Usually aliquots of extracts of proteoglycan samples were concentrated by ethanol precipitation. Approximately 25–50 μg of protein were applied to 3–15% polyacrylamide gradient gels. When desired, samples were reduced with 2% β-mercaptoethanol in the sample buffer. Proteins were detected by staining with Coomassie Brilliant Blue R.

Addition of Link Protein to Proteoglycan Aggregates—Link protein from chondrosarcoma, dissolved at a concentration of 1 mg/ml in 4 M GdnHCl, 0.05 M sodium acetate, pH 5.8, was added in increasing amounts (1.5, 3, 6, and 15% of proteoglycan mass, respectively) to aliquots of a purified α-A1 preparation in 0.5 M sodium acetate, 0.5% CHAPS (Fluka, Buchs, Switzerland), pH 7.0. The samples were allowed to react overnight at 4 °C during dialysis against 0.2 M ammonium hydrogen carbonate, pH 7.9. In parallel control experiments link-free aggregates were reconstituted by mixing proteoglycan monomers from bovine nasal cartilage or rat chondrosarcoma with 0.1% (w/v) high molecular weight hyaluronate (Healon, Pharmacia, Upsala, Sweden) in 0.5 M sodium acetate, pH 7.0. The samples were allowed to react overnight at 4 °C, and then equimolar amounts of link protein were added. The CHAPS concentration was adjusted to 0.5% by adding appropriate amounts of a concentrated CHAPS solution. The samples were then dialyzed for 48 h at 4 °C against 0.2 M ammonium hydrogen carbonate, pH 7.9. Reconstituted and link protein-treated aggregates were dialyzed to final concentrations of 50–100 μg/ml with the same buffer and visualized by rotary shadowing electron microscopy.

Chemical Protein Cross-linking—Elastin or chondroitinase-digested proteoglycan α-A1 preparations were purified by Sepharose CL-2B chromatography and dialyzed overnight at 4 °C against 0.15 M NaCl, 10 mM HEPES, pH 7.0. Samples (0.5–1 mg/ml) were incubated after addition of 2% (v/v) dithiobis(succinimidyl)propionate (DSP, Pierce) in dimethyl sulfoxide (10 mg/ml) for 1 h at room temperature. Alternative samples (0.5–1 mg/ml) were fixed in solution by the addition of 1% (v/v) glutaraldehyde and incubation for 1 h at room temperature. The reagents were subsequently removed by dialysis overnight at 4 °C against 0.2 M ammonium hydrogen carbonate, pH 7.9, and the samples were either subjected to SDS-polyacrylamide gel electrophoresis or prepared for electron microscopy as described below.

Electron Microscopy—Extracted or reconstituted aggregates were dialyzed overnight at 4 °C against 0.2 M ammonium hydrogen carbonate, pH 7.9, in a dialysis apparatus designed for small volumes (5–50 μl, Biowest, Basel, Switzerland). Proteoglycan samples were then diluted with the same solvent to obtain a final concentration of 50–100 μg/ml. They were used for electron microscopy after either glycerol spraying (Shotton et al., 1979; Tyler and Branton, 1980), mica sandwich squeezing (Mould et al., 1985), or mica centrifugation (Nave et al., 1989). Specimens were subsequently dried at high vacuum for 1–2 h and rotary shadowed at a 9° angle with platinum/carbon, followed by carbon coating at 90°. Replicas were floated onto distilled water and picked up with 400 mesh copper grids. Electron micrographs were taken on a Zeiss 109 transmission electron microscope operated at 50 or 80 kV accelerating voltage. Magnifications were calibrated by photography of a calibration grid (Balzers, Liechtenstein) under the same electron-optical conditions. Measurements were performed on a screen after ×10 enlargement of the negatives.

RESULTS

Isolation of Proteoglycan Aggregates from the Swarm Rat Chondrosarcoma—Proteoglycan aggregates were isolated from the tumor tissue under mildest possible conditions, i.e., by extraction with 0.5 M GdnHCl containing protease inhibitors according to previously published protocols (Oegema et al., 1975; Falztz et al., 1979a). Extracts were purified by size exclusion chromatography on Sepharose CL-2B either directly or after associative CaCl2 density gradient centrifugation. Proteoglycan aggregates were recovered in high yields from void volume peaks of column eluents (results not shown).

In some cases the tumor tissue was sequentially extracted with different associative solvents, and finally with 4 M GdnHCl, in the presence of protease inhibitors. For comparison, in some experiments the tumor was directly extracted with 4 M GdnHCl containing protease inhibitors, followed by dialysis into associative conditions. A schematic presentation of the different extraction procedures is given in Fig. 1. Aliquots of the clarified extracts were directly chromatographed on a Sepharose CL-2B column. All extracts gave essentially the same profile, with a sharp aggregate peak in the void volume, minimal amounts of material in the included portion, and a large peak due to small proteins and protease inhibitors in the total volume (results not shown).

Material from the different extracts, excluded from the column, was identified by electron microscopy as proteoglycan aggregates (see below). Interestingly, large amounts of aggregates were solubilized from the tissue already in physiological buffer, i.e., TBS, without use of chaotropic or chelating additives (Table I).

Extraction of the chondrosarcoma either for 4 h with 0.5 M GdnHCl, or in sequence with TBS, TBS-EDTA, and 0.5 M GdnHCl, solubilized about 70% of the total hexuronic acid. Further extraction of the residues with 4 M GdnHCl yielded an additional 20% of the total hexuronic acid. Upon extraction with 4 M GdnHCl, yields of about 96% of the total hexuronic acid were obtained (Table I). These results are in general agreement with published data (Falztz et al., 1979a).

Electron Microscopy of Native Aggregates—To find a specimen preparation technique that exposed aggregates to a

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**TABLE I**

| Extract          | Hexuronic acid | Continuous central filament structure | G3 present |
|------------------|----------------|----------------------------------------|------------|
| Sequential extraction with | % of total weight conc. | % total length of identified structures | % of particles |
| TBS              | 53             | 5/95                                   | 20         |
| TBS-EDTA         | 9              | 60/40                                  | 85         |
| 0.5 M GdnHCl     | 8              | 20/80                                  | 30         |
| 4 M GdnHCl*      | 20             | 0                                      | 1–2        |
| Direct extraction with 4 M GdnHCl* | 86             | 0                                      | 55         |

* This extract is designated d-4 M GdnHCl in the text.
minimum of shear forces and thereby gave optimal structure preservation, samples of purified a-A1 fractions from 4 h 0.5 M GdnHCl extracts were visualized in the electron microscope by different preparation techniques. These were glycerol spraying/rotary shadowing (Shotton et al., 1979; Tyler and Branton, 1980), mica sandwich squeezing (Mould et al., 1985), and mica centrifugation (Nave et al., 1989).

Long aggregates with varying amounts of monomers bound to hyaluronate strands at different packing densities were observed with each method. The central filament of a more densely packed aggregate appeared identical to that of aggregates isolated under dissociative conditions from a number of tissues and then reconstituted (Figs. 2 and 3). When aggregate samples were prepared for electron microscopy by glycerol spraying, only about 5% of the monomers were in aggregates, the major fraction of the molecules remaining unbound (Fig. 3a). There was large variation in the ratio of bound to unbound particles between individual droplets, and especially the smaller ones were often completely devoid of aggregate structures. Sometimes hyaluronate strands with only some proteoglycan monomers bound in a very oriented fashion were visible (Fig. 3a, inset), suggesting partial removal of proteoglycan monomers due to shear during spraying or upon impact on the mica surface.

In contrast, aggregate preparations after mica sandwich squeezing or mica centrifugation exhibited large fields densely covered with aggregates (Fig. 3, b and c). About 90% of the monomers were bound, and strands of unsubstituted hyalu-
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A higher degree of preservation was also demonstrated by the much larger aggregate length observed by the two latter methods (results not shown). It seems as if the mica squeezing or centrifugation techniques exert less shearing stress on the samples, yielding less destruction of long and thin structures compared to the glycerol spraying technique. This has previously been shown for other filamentous systems (Mould et al., 1985; Nave et al., 1989).

On the other hand glycerol spraying/rotary shadowing consistently gave the lowest background staining of 2–3 nm platinum crystallites, whereas clusters of varying sizes were often present in specimens prepared with the other two techniques. These artifactual structures are probably due to traces of salt and do sometimes cover large areas of the grids. Furthermore, glycerol spraying/rotary shadowing usually gave the highest resolution of the central filament and the glycosaminoglycan chains. In contrast, mica squeezing and centrifugation frequently showed the protein cores of individual monomers along their whole lengths, sometimes with particularly high contrast due to collapsed side chains. Hence, the three techniques were applied in parallel for optimal information.

Examination of the associatively prepared aggregates with a high packing density of monomers along the hyaluronate showed a structure of the central filament identical to that of link-stabilized aggregates seen in our previous reconstitution experiments (Mörgelin et al., 1988, 1989). Continuous stretches of central filament were seen as compact protein-covered strands extending over long distances, occasionally interspaced by free hyaluronate strands (Fig. 2). Individual G1 domains could not be resolved, but tight packing of proteoglycan monomers along the hyaluronate, with closest center-to-center distances of 12 nm, was demonstrated by measuring the distances between adjacent E1 strands.

The length of the extended domain E2 was 282 ± 21 nm and 210 ± 24 nm for those intact and chondroitinase digested monomers that contained G3, respectively. This is somewhat shorter than the corresponding lengths of the E2 domain of the bovine nasal cartilage proteoglycan (405 ± 37 nm, intact and 263 ± 27 nm, chondroitinase digested, Mörgelin et al., 1989). The observed difference correlates with the finding that a proline-rich peptide (about 100 amino acids long), corresponding to the keratan sulfate attachment region, is present in the sequence of mature bovine cartilage core protein, whereas it is not found in the chondrosarcoma proteoglycan sequence (Doege et al., 1987; Antonsson et al., 1989).

Interestingly, a different aggregate type with a more loosely packed central filament structure was found in the same extracts (Fig. 4b). The prominent feature thereof is a similarly tight packing of monomers in densely staining clusters, but these are rather short and do usually not exceed 10 adjacent proteoglycan monomers. In addition to the clusters, single monomers with a binding region globe enlarged in diameter, presumably representing one G1 domain complexed with one or more link proteins, were often seen to be bound to the hyaluronate. Both types of central filament structure were observed with all electron microscopic preparation techniques, and it is therefore unlikely that the loosely packed central filaments are artifacts caused by the shearing forces of the glycerol spraying method. It should be noted, however, that occasionally both central filament types were found to somewhat overlap by transitional forms, or to be present within one single aggregate (Fig. 4c). Additional details on the molecular structure of the aggregate types are shown in Table II. An average a-A1 preparation contained 60% of densely packed central filament structure, with mean distances of 12 ± 5 nm between adjacent monomers along the central filament (Fig. 5b). The loosely packed central filament stretches accounted for 40% of the total central filament length. They showed broad distributions of distances between

Table II: Structural details of proteoglycan aggregates extracted from the Swarm rat chondrosarcoma with 0.5 M GdnHCl

| Total length of identified structures | G3 present (total particles) |
|-------------------------------------|-------------------------------|
| Densely packed central filaments    | 60% (d, n) 12 ± 5%, 50%      |
| Loosely packed central filaments    | 40% (d, n) 21 ± 27%, 50%      |
neighboring monomers (27 ± 21 nm, Fig. 5d). Similar distances between adjacent monomers were measured for whole aggregates with overall predominant dense or loose central filament packing density (Fig. 5, a and c). In both central filament types closest center-to-center distances between monomers were 12 nm. The COOH-terminal globular domain showed that both reagents were able to covalently cross-link neighboring monomers (27 ± 21 nm, Fig. 5d). Similar distances between adjacent monomers were measured for whole aggregates with overall predominant dense or loose central filament packing density (Fig. 5, a and c). In both central filament types closest center-to-center distances between monomers were 12 nm. The COOH-terminal globular domain was present in about 50% of the particles both in the densely and loosely packed types.

In some experiments 0.5 M GdnHCl extracts were directly applied to a preparative Sepharose CL-2B column in order to determine whether exposure to high salt concentrations and prolonged high centrifugal force during cesium chloride density gradient centrifugation might influence the central filament structure. In all cases, however, both densely and loosely packed central filaments were seen in the electron microscope, showing the same structural features as summarized for a-A1 preparations in Table II. When the lengths of central filament patches or interspersing free hyaluronate strands were measured we obtained similar results as for aggregates from a-A1 preparations (results not shown). Therefore, it is unlikely that the pool of molecules with loosely packed central filaments is created during purification.

**Chemical Cross-linking**—Experiments were specifically designed to examine whether the interrupted structure of loosely packed central filaments was due to electron microscopic preparation artifacts. Therefore, proteoglycan aggregates were fixed by chemical cross-linking in solution either with glutaraldehyde or DSP. Subsequent SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions showed that both reagents were able to covalently cross-link proteoglycan monomers and link protein in the central filament (Fig. 6) and thus presumably stabilized its structure. When aliquots of native a-A1 samples were electrophoresed under reducing or non-reducing conditions a link protein band at about M, 40,000 appeared. The link protein band was neither present in preparations after fixation with glutaraldehyde nor in non-reduced DSP-treated samples. It was, however, visible in the lane of reduced DSP-treated samples, included as a control for successful cross-linking by the thiol cleavable DSP spacers. A similar electrophoretic behavior was seen for fixed chondroitinase-digested aggregates from the a-

![Fig. 5. Length distributions of the distances between adjacent proteoglycan monomers in aggregates from an a-A1 preparation. Distances were measured in densely (b) or loosely (d) packed central filament stretches and in whole aggregates with a predominant overall dense (a) or loose (c) central filament structure. Mean values ± S.D. are given in the diagrams.](image)

![Fig. 6. Chemical cross-linking of native proteoglycan aggregates. SDS-polyacrylamide gel electrophoresis of rat chondrosarcoma proteoglycan aggregates extracted under non-denaturing conditions after chemical cross-linking with glutaraldehyde or DSP. Electrophoresis was performed in 3–15% gradient gels with (lanes a–g) and without (lanes h–n) prior reduction with β-mercaptoethanol. Samples were a-A1 (b and h), a-A1 fixed with glutaraldehyde (c and i), or DSP (d and j). Chondroitinase ABC-digested samples were a-A1CB (e and k) and a-A1CB fixed with glutaraldehyde (f and l) or DSP (g and m). After digestion with chondroitinase ABC the core protein is visible as a faint smear with a similar electrophoretic mobility as the laminin A-chain (400 kDa). The positions of standard proteins of known M, are indicated (a and n).](image)

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A1 preparation, where the core protein was seen as a faint smear with a mobility at about 400 kDa in addition to the link protein band (Fig. 6).

When cross-linked aggregates were visualized in the electron microscope, again densely and loosely packed central filament structures were seen at the same ratios as for untreated aggregate preparations (Fig. 7). Furthermore, when aliquots of a-A1 samples were digested with chondroitinase ABC and subsequently purified by Sepharose CL-2B chromatography, free stretches of hyaluronate had been digested and long aggregates were no longer visible, but instead short, continuous aggregates were observed by electron microscopy. The length distributions of their central filaments were the same as the sum of those from the central filament patches of undigested aggregates with densely and loosely packed regions (Fig. 8). These observations suggest that both central
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filament types are present in situ.

Differences between Proteoglycan Aggregates Sequentially Extracted by Different Solvents—Rat chondrosarcoma tissue was consecutively extracted with TBS, TBS-EDTA, 0.5 M GdnHCl, and 4 M GdnHCl. Proteoglycan aggregates from each fraction were recovered by Sepharose CL-2B chromatography and compared by electron microscopy (Fig. 9). From each extract, aggregates with continuous and interrupted central filament structures were identified in the relative amounts given in Table I. The different extracts also varied in their content of G3-containing monomers. Representative aggregate structures are shown in Fig. 9, a–d. Interestingly, the

FIG. 7. Structure of native proteoglycan aggregates after chemical cross-linking. Electron micrographs taken after mica sandwich squeezing of native proteoglycan aggregates which were fixed with glutaraldehyde (a) or DSP (b). The appearance of the aggregates was not influenced by the fixatives. Densely and loosely packed central filament structures in both samples are indicated by arrows and arrowheads, respectively.

FIG. 8. Length distributions of central filament patches in native and chondroitinase-digested proteoglycan aggregates. Aliquots of a-A1 samples were digested with chondroitinase ABC and visualized in the electron microscope after mica sandwich squeezing. The central filament lengths of such aggregates (a) were compared to the sum of the lengths of uninterrupted central filament patches of undigested aggregates (b). Note the highly similar length distributions and mean values in both cases.

FIG. 9. Structure of sequentially and dissociatively extracted proteoglycan aggregates as seen by rotary metal shadowing electron microscopy. Electron micrographs taken after mica sandwich squeezing of proteoglycan aggregates solubilized from Swarm rat chondrosarcoma tissue as described in the text. Aggregates were extracted sequentially with TBS (a), TBS-EDTA (b), 0.5 M GdnHCl (c), 4 M GdnHCl (d), or directly with 4 M GdnHCl (e). All samples were dialyzed and inspected in 0.2 M ammonium hydrogen carbonate. Note the degraded appearance of monomers and the noncontinuous central filament structure in aggregates from TBS and 4 M GdnHCl extracts. The highest fraction of densely packed aggregates with the largest number of G3-containing proteoglycan monomers were seen in TBS-EDTA-solubilized material.
proteoglycans from the TBS-EDTA extract exhibited a much higher proportion of densely packed central filament structure (60%) and an unusually large fraction of G3-bearing particles (85%). In contrast in the 4 M GdnHCl extracts, only loosely packed aggregates with a rather degraded appearance were observed. It should be noted, however, that these are reconstituted and were not observed in the presence of 4 M GdnHCl. Only few intact monomers were bound to the hyaluronic strands, and quite often whole aggregates consisting of only globular structures, presumably the G1 fragment, attached to hyaluronate were found. Aggregates solubilized in TBS had a similar but less degraded appearance. In each case closest center-to-center distances between adjacent monomers of 12 nm were observed within central filament patches. In all densely packed central filament stretches which were examined, average monomer distances of about 12 nm were found. In contrast, the loosely packed central filaments from all extracts exhibited rather broad distributions of intermolecular distances of 27 ± 21 nm.

In control experiments chondrosarcoma tissue was directly extracted dissociatively with 4 M GdnHCl, and the clarified extracts were dialyzed into associative conditions and chromatographed over Sepharose CL-2B. About 70% of the proteoglycan monomers were present in aggregates and appeared in the void volume (results not shown). Electron microscopical inspection of aggregates recovered from void volume fractions showed only loosely packed aggregates (Fig. 9e). Average distances of 40 ± 2 nm between neighboring monomers along the hyaluronate were measured for such aggregates. Similar results were obtained when a-A1 samples were dialyzed into 4 M GdnHCl and back into associative conditions (results not shown). These data indicate an overall molar excess of hyaluronate in the tissue, resulting in low aggregate packing densities after dissociation and reassociation of central filament patches.

Treatment of Aggregates with Link Protein—A possible explanation for the presence of a loosely packed central filament structure would be that link protein is present only in substoichiometric amounts in the tissue, since in the absence of link protein proteoglycans bind to hyaluronate in a statistical way (Morgelin et al. 1988). To test this hypothesis, link protein was purified from an A1D4 preparation and added in varying amounts to purified a-A1 aggregate samples. In parallel control experiments it was added to link-free complexes reconstituted in vitro from hyaluronate and proteoglycan monomers. After dialysis into 0.2 M ammonium hydrogen carbonate such aggregates were visualized in the electron microscope. After addition of equimolar amounts of link protein, the reconstituted, previously link-free aggregates exhibited a link-stabilized central filament structure (Fig. 10, c and d). In aggregates from the a-A1 preparation, however, even a large excess of added link protein did not affect the relative amounts of densely and loosely packed central filament forms (Fig. 10, a and b). The gaps between adjacent short central filament pieces were not closed. These results provide evidence that substoichiometric amounts of link protein are not responsible for the noncontinuous structure of loosely packed central filaments.

**DISCUSSION**

Proteoglycan aggregates can be extracted in high yields from the Swarm rat chondrosarcoma with associative solvents which do not disrupt their native supramolecular structure. It was shown in earlier work that an effective associative solvent is 0.5 M GdnHCl, 0.05 M sodium acetate, pH 5.8, which when used together with protease inhibitors yields the highest proportion of aggregates in an a-A1 preparation with least indication of proteolytic degradation of the proteoglycans (Oegema et al., 1975; Faltz et al., 1979a). In addition, as shown here, intact proteoglycan aggregates can be solubilized in physiological saline, i.e. TBS or TBS containing EDTA. The a-A1 preparation, or comparable preparations obtained in consecutive extraction steps with associative buffers, represented about 70% of the total tissue hexuronic acid, and an additional 20% were solubilized in 4 M GdnHCl. Dissociative extraction of the tumor with 4 M GdnHCl yielded 86% of the total hexuronic acid present. These values are in accordance with previously published results (Faltz et al., 1979a).

Electron microscopical examination of aggregates recovered from different associative extracts did in all cases reveal a structure of the densely packed central filament type identical to that of link-stabilized aggregates seen in earlier reconstitution experiments performed with dissociatively extracted proteoglycans from cartilage (Morgelin et al., 1988) or other tissue sources (Morgelin et al., 1989). These data, however, were obtained by the recombination of molecules which had been exposed to 4 M GdnHCl and high cesium chloride concentrations and centrifugal force during isolation and purification. It cannot be taken for granted that the structure of such reconstituted aggregates is in all respects representative.
of native aggregates. Our present results, however, confirm that this organization is representative for in situ conditions and that associatively extracted aggregates will be a suitable material for further detailed examinations of the central filament structure as well as of the molecular arrangement of hyaluronate binding region and link protein within the central filament.

The present studies led to the detection of apparently different central filament structures in aggregates. The more densely packed aggregates had the same central filament structure as known from reconstitution experiments, with the proteoglycan monomers being tightly packed in a continuous protein shell extending over long distances. The other novel proteoglycan monomers being tightly packed in a continuous to hyaluronate were observed. Faltz et al. (1989) demonstrated that link-free aggregates, in which individual monomers were bound statistically to the hyaluronate, exhibited the well-known continuous densely packed central filament structure after addition of link protein. In previous studies strong evidence was provided that equimolar amounts of hyaluronate binding region and link protein are present in the Swarm rat chondrosarcoma (Oegema et al., 1977; Faltz et al., 1979b). We assume that this is also the case in our preparations, and in future work we will examine in more detail the molar ratios of the different components in the central filament.

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