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The cartilage proteoglycan aggregate: assembly through combined protein–carbohydrate and protein–protein interactions

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Abstract

In vitro reassembled aggregates of cartilage proteoglycan (aggrecan) were studied by glycerol spraying/rotary shadowing electron microscopy and compared to the corresponding native (i.e. never dissociated) structures. In both cases a tightly packed central filament structure was observed consisting of the hyaluronate binding region (HABR) of the proteoglycan, link protein (LP) and hyaluronate (HA). This differs from earlier results where a discontinuous central filament structure was seen after spreading proteoglycan aggregates at a water/air interphase. Binding of isolated HABR to HA is random but upon addition of link protein a clustering of the HA-binding proteins is observed, indicating a cooperativity. In a fully saturated aggregate the HA is covered by a continuous protein shell consisting of HABR and LP. When added in amounts below saturation HABR and LP bind to the HA in clusters which are interrupted by free strands of HA. The proteoglycan aggregate is thus an example for a structure where a polysaccharide forms a template for a supramolecular assembly largely stabilized by protein–protein interactions.

Key words: Aggrecan; Hyaluronate; Link protein; Electron microscopy; Cartilage; Swarm rat chondrosarcoma

1. Introduction

Proteoglycans are a specialized family of glycoproteins typically consisting of a protein core, one or more covalently bound glycosaminoglycan chains and often shorter N- and O-linked oligosaccharides. They form a major class of non-fibrous components of the extracellular and pericellular matrix where they exert a profound influence on the local physical environment. Many proteoglycans contain distinct, often well conserved, protein and carbohydrate domain structures that confer specific functional properties. The protein cores display a great diversity, where many have complex modular structures containing also protein motifs that are of homologous sequence to those found in other extracellular protein families (for a recent review, see ref. [1]).

In the present article we review the structure and interactions of the large aggregating proteoglycan in cartilage, which is now generally referred to as aggrecan. This proteoglycan has been the subject of extensive biochemical investigation

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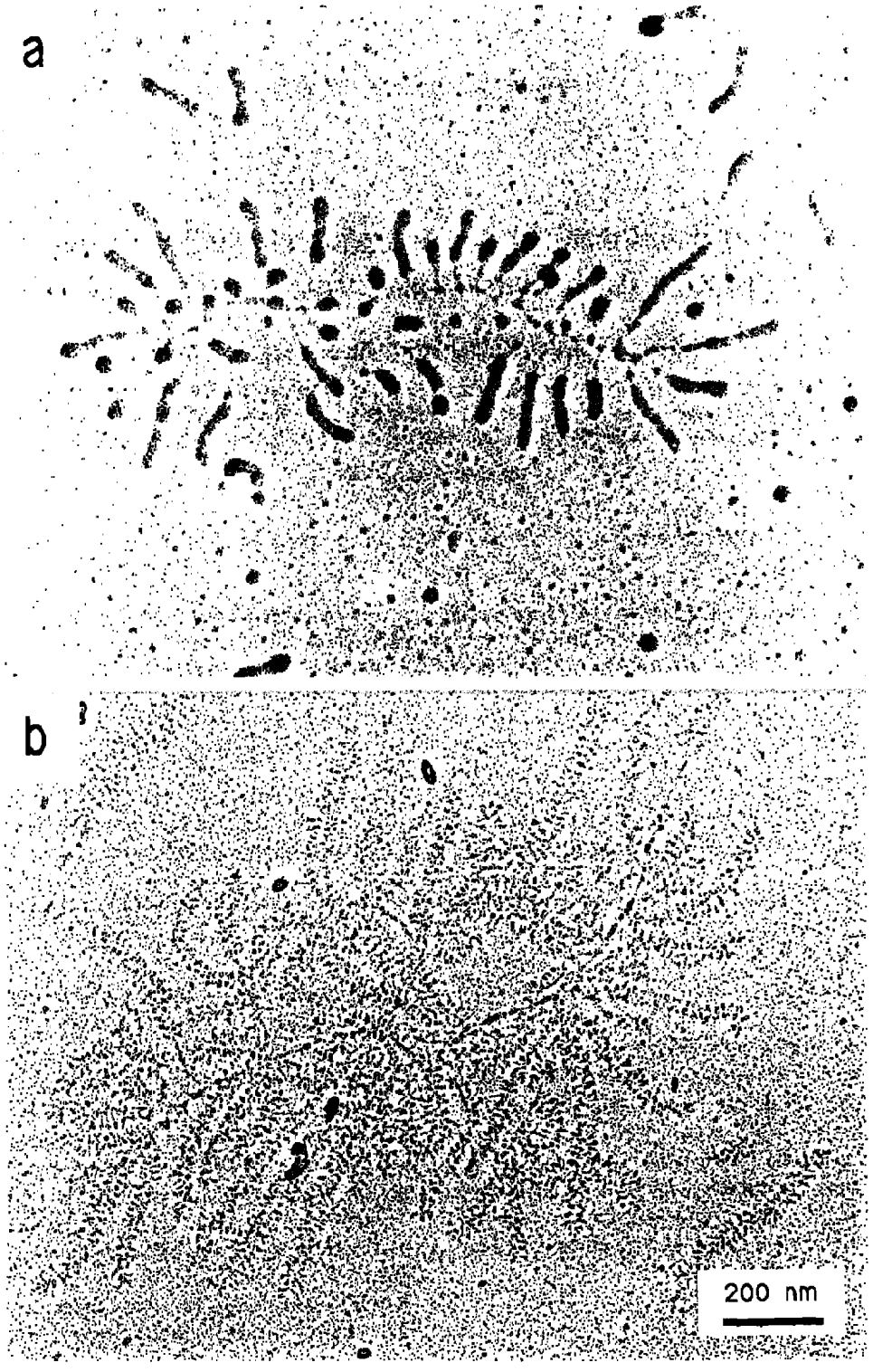
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for decades, and in recent years detailed information on its molecular structure has become available from studies employing the tools of modern molecular and structural biology. It is the prototype for a family of high molar mass proteoglycans which all have the ability to form a strong and specific non-covalent interaction with hyaluronate and link protein via a specialized domain of their protein cores. The resulting multimolecular aggrecan aggregates constitute the bulk of the cartilage extracellular matrix, and are present at high concentrations (50–100 mg/ml). They interact with a network of hybrid fibers of collagens II, IX, XI and XII.

The large number of fixed negative charges which reside in the polyanionic glycosaminoglycan chains contribute to the high solubility of these molecules in water and do also form the basis for the elasticity and resilience of cartilage. At the physiological proteoglycan concentrations the molecules exist in an underhydrated state occupying not more than 20% of their fully expanded volume. They are kept in this compressed state by the inextensible collagen network, which counteracts any swelling of the tissue. As a consequence, the aggrecan molecules will preclude any further decrease in volume due to strong electrostatic repulsions between glycosaminoglycan chains and to osmotic swelling pressure created by the chains. Cartilage can thus be viewed as a composite, where a polyelectrolyte matrix is reinforced by fibers which are embedded in the matrix (for reviews see refs. [2–4]).

2. Cartilage aggrecan structure

Early studies of cartilage proteoglycan structure were hampered by the fact that extraction with low-salt buffers does not yield representative samples of intact molecules. Therefore the discovery that chaotropic solvents such as 4 M guanidine hydrochloride extract tissue aggrecan with 80–90% yield [5,6] was catalytic for the development of this research field. These solvents protect the molecules against proteolytic degradation during the extraction and allow them to be solubilized without the necessity to disrupt the fibrillar collagen network. Once extracted the proteoglycans may be purified by cesium chloride equilibrium density gradient centrifugation [7], taking advantage of the properties of the highly anionic glycosaminoglycan chains. In addition to equilibrium density centrifugation, size exclusion chromatography on porous agarose gels [8,9] as well as ion exchange chromatography and rate zonal centrifugation have been used for the purification and characterization of aggrecan.

The characteristics of proteoglycans from different cartilages have been described by biochemical and biophysical analysis [6,8–18]. The typical aggrecan is a very large macromolecule with a molar mass exceeding 3×10^6 Da. It consists of a central extended protein core of average molar mass of 2×10^5 Da, to which some 100 chondroitin sulfate chains, 30 keratan sulfate chains, up to 8 N-linked and 100 O-linked shorter oligosaccharides are covalently attached [10,19–22]. At one end of the molecule the hyaluronate binding region is located [9,23,24], which is substituted with N-linked oligosaccharides [21,25] but lacks glycosaminoglycan chains. It was found to have a globular structure [26] and a molar mass of 60000–100000 Da [24,26,27]. The interaction with hyaluronate is stabilized further by the binding of an accessory small glycoprotein, link protein. Distal to the hyaluronate binding region the keratan sulfate-rich region is located, which contains about $\frac{2}{3}$ of the keratan sulfate of the proteoglycan in a very dense arrangement [20]. The chondroitin sulfate attachment region, bearing most of the chondroitin sulfate and some keratan sulfate, is found adjacent to the keratan sulfate region. The chondroitin sulfate chains are arranged in

Fig. 1. Electron microscopy after Kleinschmidt spreading of aggrecan aggregates in the presence of cytochrome c (a) or benzyldimethylhexadecylammonium chloride (BAC) (b). Note the interrupted central filament structure along the hyaluronate in both cases. In (a) individual glycosaminoglycan chains in proteoglycan monomers are collapsed to the protein cores, whereas they are resolved in (b).

clusters of in average 4–5 chains [24]. This region is of variable length, probably due to physiological proteolytic degradation in the matrix [28], and usually constitutes about half of the protein of the molecule.

This structural model was confirmed and extended by molecular electron microscopy, which was initiated in the 1970s. In pioneering studies, aggrecan aggregate preparations were visualized by heavy metal shadowing after spreading at a monolayer of cytochrome c or peptides derived from this protein [12,29]. Alternatively, complexes of negatively stained cartilage aggrecan were visualized by dark-field electron microscopy after cytochrome c spreading [30]. The authors essentially used the Kleinschmidt technique as originally designed for electron microscopy of DNA [31]. Images obtained by this technique [12,29,30, 32–34] revealed the general distribution of monomers in aggregates, the organization of side-chain constituents on monomers, and the mode of binding of monomers to the hyaluronate central filaments (Fig. 1a). Up to several hundred aggrecan molecules of 100–400 nm in length, bound to hyaluronate strands with lengths up to several μm, were clearly resolved. After purification under dissociative conditions it was also possible to visualize single proteoglycan monomers,

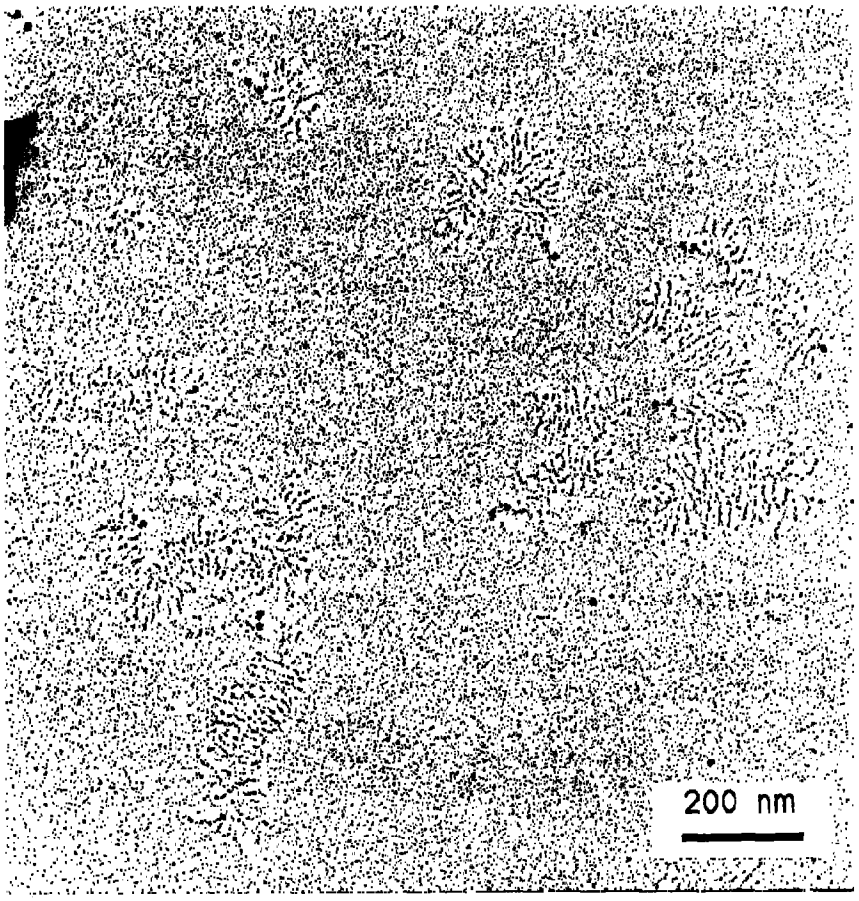


Fig. 2. Electron microscopy after glycerol spraying/rotary shadowing of aggrecan monomers from bovine nasal cartilage. Note the regular appearance of the globular and extended domains of the core protein as schematically indicated in Fig. 3. The glycosaminoglycan side-chains are clearly resolved along their entire length.

where occasionally even individual chondroitin sulfate chains, about 45 nm long, could be resolved. These appeared attached to the protein core at equal intervals of some 11 nm [32]. In most instances, however, the side-chains were collapsed onto the core proteins or formed bundles which rendered a determination of their exact number difficult. Furthermore, it was not possible to derive details of the aggrecan core protein domain organization from these images, as the protein parts were not resolved. Kleinschmidt spreading of proteoglycans can be improved by using benzyldimethylhexadecylammonium chloride (BAC) instead of cytochrome c. This modification results in a better structural preservation of the glycosaminoglycan chains, but no details of the protein core are revealed (Fig. 1b).

Novel structural information was obtained by the glycerol spraying/rotary shadowing technique, applied according to a similar protocol as described for other proteins and macromolecules [35–37]. Proteoglycan samples, dissolved in a volatile buffer (i.e. 0.2 M ammonium hydrogen carbonate, pH 7.9), are mixed with an equal volume of 80% glycerol and sprayed onto freshly cleaved mica discs, followed by decoration with platinum/carbon while rotating [38]. Alternatively specimens are prepared for electron microscopy by mica sandwich squeezing [39] or mica centrifugation [40]. The latter two techniques have the advantage of exerting less shear forces on the molecules than the spraying procedure [41], although they often result in a higher background staining. Extended and globular domains in aggrecan molecules can be very easily recognized after glycerol spraying/rotary shadowing [38,42], as shown in Fig. 2. It is also possible to resolve individual glycosaminoglycan chains, but they are revealed only at optimal thickness and grain size of the metal deposit. Their detection is mainly based on the recognition of ordered single rows of metal crystallites. As opposed to the images obtained after Kleinschmidt spreading, the chondroitin sulfate chains are well spread out and neither collapsed nor bundled. They are usually difficult to trace along their entire length due to overlaps between the closely spaced chains, but in some instances it has been possible to measure the length of individual chains. The protein core is often partially obscured by the brush-like array of side-chains and is clearly visible in intact molecules only under optimal decoration conditions. It can be seen much more clearly and traced along its entire length after removal of the chondroitin sulfate by digestion with chondroitinase ABC [38,42].

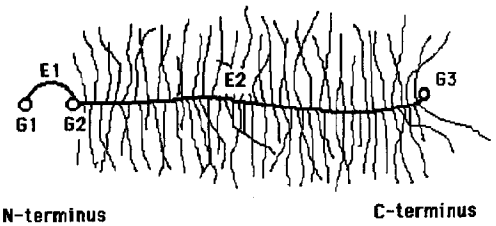
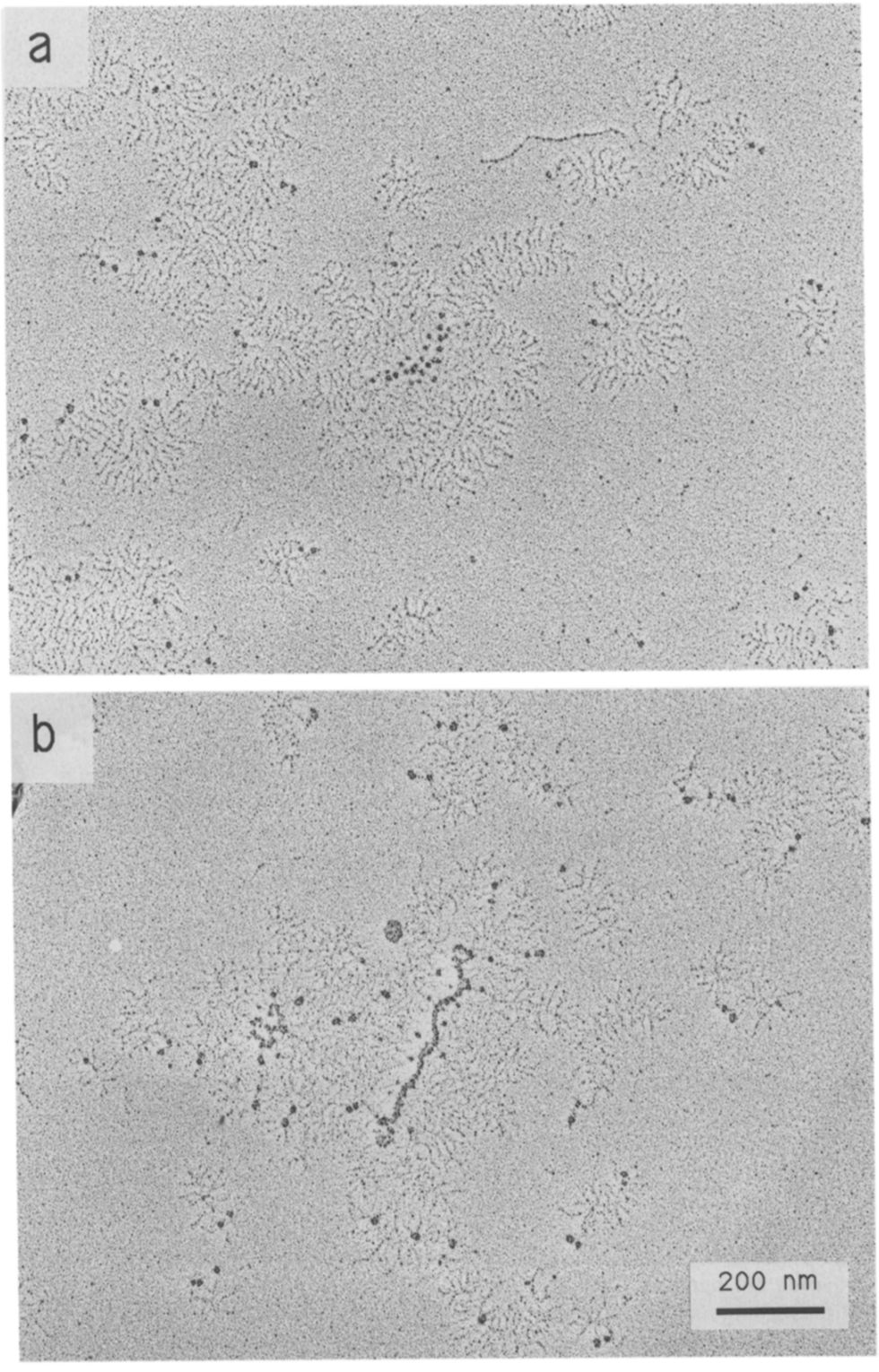


Fig. 3. Schematic representation of an aggrecan monomer as visualized by glycerol spraying/rotary shadowing. The molecule exhibits two globular domains, G1 and G2, at the N-terminus, separated by an extended domain E1, followed by a long extended domain E2, which is terminated by a C-terminal globular domain G3. E2 carries the main bulk of covalently attached glycosaminoglycan side-chains.

Electron microscopy after rotary shadowing, in combination with sequence analysis at the protein [43] and cDNA level [44–49], allowed the proposal of a more detailed model of aggrecan [38,42,50,51]. Several structural and functional domains can be distinguished in the core protein (Fig. 3), which has a molar mass of about 210000 Da [45]. At the N-terminus a globular domain G1 with a diameter of 11 nm corresponds to the hyaluronate binding region. It comprises two homologous loops, called the proteoglycan tandem repeat, and a single loop, similar to those found in immunoglobulins [52]. The specific non-covalent interaction of this structure with hyaluronate requires five disaccharide units [24]. The binding is rather strong with a K_D of $\approx 10^{-8}$ M [27,53–55]. Link protein binds to hyaluronate with similar affinity and does in addition interact with the hyaluronate binding region [24,27,56,57], so that a very stable ternary complex with a K_D decreased about two orders of magnitude is formed [58], as schematically indicated in Fig. 6. The link protein is present in purified aggregates in a one-to-one ratio to aggrecan monomers [24,59]. Consistent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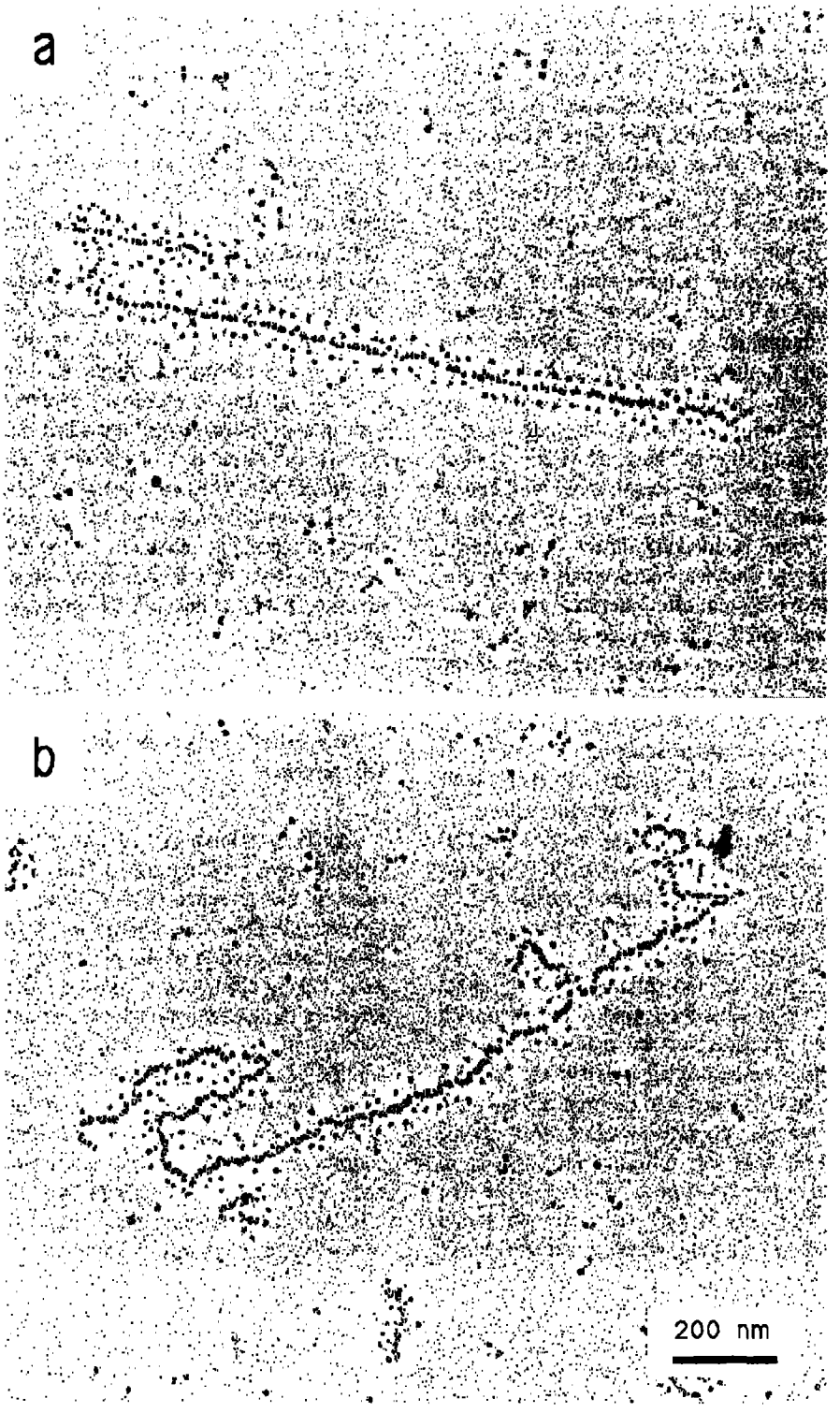
with their similar binding characteristics, the hyaluronate binding region and the link protein contain a closely related loop structure [43,45,60] with the proteoglycan tandem repeat of the link protein [60] and probably also of the hyaluronate binding region being involved in binding to hyaluronate. In the direction of the C-terminus of the aggrecan, G1 is followed by a second globular domain G2 with a diameter of 9 nm, interspaced by a short extended peptide stretch E1 with a mean length of 35 nm. G2 shows a high degree of sequence homology to G1, also containing the proteoglycan tandem repeat, but lacking affinity for hyaluronate [50,61]. Beyond G2 the extended domain E2 follows, carrying the bulk of glycosaminoglycan chains. These are visible as faint strands with an average length of 36 nm, bound to E2 at intervals of average 7 nm. The length of E2 after rotary shadowing differs considerably, depending on whether intact monomers or the core protein fragment after chondroitinase digestion are measured. The average length of intact monomers is 405 nm, whereas the length of the core protein fragment is only 263 nm, implying a stretching effect by glycosaminoglycan repulsion along the protein core in intact monomers [51]. In its N-terminal part E2 contains a stretch of sequence corresponding to the keratan sulfate attachment region and being extremely rich in glutamate and proline [48]. This peptide is glycosylated with $\frac{2}{3}$ of the total keratan sulfate and is present in bovine cartilage aggrecan but not in rat chondrosarcoma aggrecan [45,49], which is not substituted with keratan sulfate. The C-terminal part of E2 represents the chondroitin sulfate-rich region and accounts for the major part of the protein core. Sequence analysis shows that this segment can be divided into different subdomains all containing ser-gly repeats which are putative glycosylation sites, but differing with respect to the length of internal repeats [45]. It appears that almost all of these ser-gly sites are substituted with chondroitin sulfate chains as the number of such dipeptides closely equals the number of side chains determined by biochemical analysis. The C-terminus of the molecule is represented by a third globular domain G3 with a diameter of about 7.5 nm. It is distinct from G1 and G2 in sequence but shares homology with a vertebrate hepatic lectin and related carbohydrate binding proteins [44–48]. A cDNA segment from the rat corresponding to this domain was expressed and could be demonstrated to interact specifically but with low affinity with galactose and fucose [62]. G3 is present in up to 50% of the particles extracted from mature cartilage [42]. It would be of great interest to identify a matrix molecule serving as its physiological ligand. It has recently been reported that lack of G3 due to a pathological mutation in chicken nanomelia leads to failure of transfer of the aggrecan precursor from the endoplasmatic reticulum to the Golgi compartment [63]. This may indicate an alternative function of G3 in intracellular processing of the core protein.

3. Interaction of aggrecan with hyaluronate and link protein

Aggrecan monomers and link protein can be isolated from cartilage under dissociative conditions in the presence of 4 M guanidine hydrochloride. Dialysis into associative conditions allows a large portion of the molecules to reaggregate. Taking advantage of this behaviour the interaction of aggrecan monomers and various fragments with hyaluronate and link protein was studied by electron microscopy after rotary shadowing. The participation of the different domains of the protein core in the binding to the polysaccharide was examined. The structure of aggregates formed, and in particular the influence of link protein on the aggregate structure and mode of proteoglycan binding was studied.

Fig. 4. Electron micrographs after glycerol spraying/rotary shadowing of (a) a complex of aggrecan monomers with hyaluronate and (b) a link-stabilized aggregate formed of hyaluronate, aggrecan monomers and link protein. Note the interrupted central filament structure in (a), the compact, uninterrupted central filament in (b), and the brush-like appearance of the aggregates.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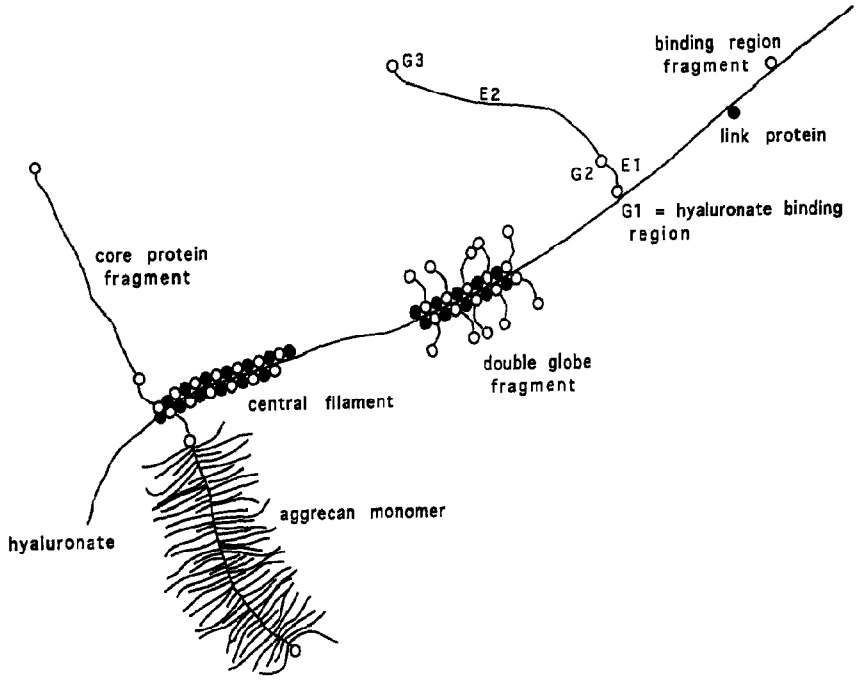


Fig. 6. Schematic model of the aggrecan aggregate structure, based on rotary shadowing electron microscopy. The aggrecan monomers bind to the hyaluronate by their N-terminal hyaluronate binding region (G1). The extended domain E1 acts as a spacer of about 25 nm between G1 and G2. In its C-terminal part G2 is followed by the long extended domain E2 (some 280 nm) which is heavily substituted with glycosaminoglycan side chains. The protein core is terminated by the C-terminal globular domain G3, which is found in up to 60% of the particles, depending on the source. The binding of G1 to the hyaluronate is further stabilized by link protein so that a continuous compact central filament is formed. The spatial arrangement of G1, link protein and hyaluronate is hypothetical.

In a typical reconstitution experiment aggrecan monomers (2 mg/ml) or fragments thereof (100–500 μg/ml) were mixed with appropriate amounts of hyaluronate (2–5 μg/ml) and, if required, link protein (5–150 μg/ml) in 4 M guanidine hydrochloride/0.2 M ammonium hydrogen carbonate, pH 7.9. After removal of the dissociating agent by dialysis, assembly products were visualized in the electron microscope after preparation of replicas. In such images link-stabilized aggregates prepared from whole monomers and hyaluronate reveal an organization with a heavily stained central filament consisting of the hyaluronate, the hyaluronate binding region and link protein [38,50]. In Fig. 4b a typical electron micrograph is shown for bovine nasal cartilage, but similar results were obtained with proteoglycan preparations from other sources [50,51]. The central filament appears as a continuous structure and is surrounded by a brush-like array of thinner strands, corresponding to the chondroitin sulfate side chains attached to the E2 domains of individual monomers. In addition, some globular structures can be observed adjacent to the central filament and in the periphery of the aggregate.

This rather complex structure could be inter-

Fig. 5. Assembly of double globe fragment (G1–E1–G2) with hyaluronate in the absence (a) and presence (b) of link protein. In both cases one globule can be seen to bind to hyaluronate, and the other one points away. Without link protein individual particles bind statistically to the hyaluronate, and with link protein an uninterrupted densely packed central filament structure is seen.

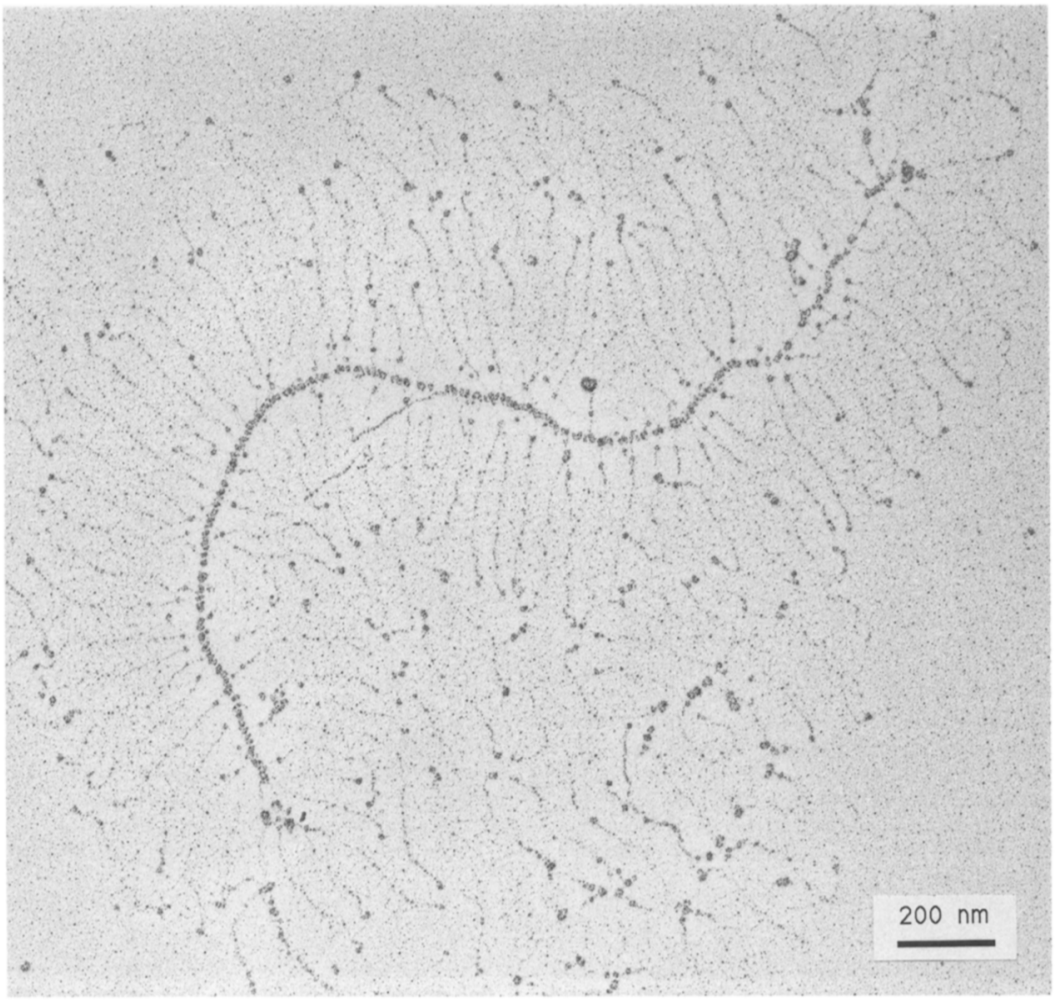


Fig. 7. Structure of a native proteoglycan aggregate from the Swarm rat chondrosarcoma as visualized by mica sandwich squeezing/rotary shadowing. The specimen was isolated from the void volume faction from Sepharose CL-2B chromatography after extraction with 0.5 M guanidine hydrochloride.

preted on the basis of the domain organization of the aggrecan monomer [42] and with the aid of further reconstitution studies in which aggrecan fragments were used instead of intact monomers. In complexes formed both in the absence and presence of link protein the interaction with hyaluronate involves only the N-terminal globular domain G1. The other domains, including G2, can be seen adjacent to the central filament and are not involved in binding [50]. This demonstrates that G1 is the only part of the protein core which interacts directly with hyaluronate, which is interesting in the light of its sequence homology with the apparently nonfunctional G2 [44–48]. The extended domain E1 acts as a spacer between G1 and G2 and determines the distance between the central filament and the heavily glycosylated part of the core protein. Domain E2 with its many chondroitin sulfate side chains gives the aggregates their brush-like appearance (Fig. 4). The arrangement is more easily discerned when the chondroitin sulfate chains or parts of

the protein core are removed by enzyme treatment. Such aggregates resemble those formed with intact monomers (Fig. 5), demonstrating that the side chains or core protein domains other than G1 have little effect on the architecture along the central filament [50]. In aggregates formed with intact monomers or with core protein fragment the C-terminal domains G3 are seen in the periphery of the structures. Their location is well accessible for the putative interaction with other cartilage matrix components, see ref. [62].

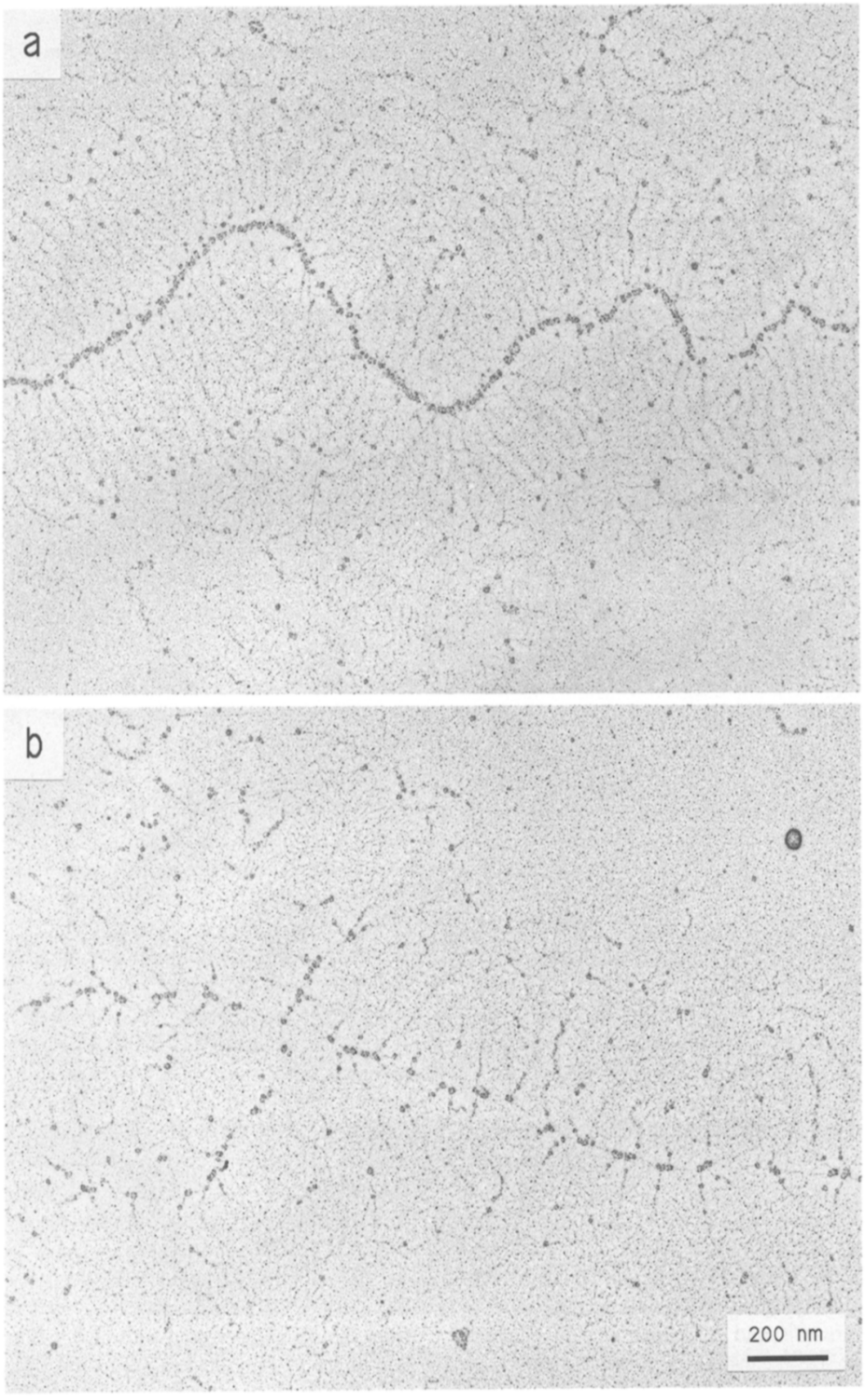
In assembly products formed in the absence of link protein individual G1 globes are found to bind to hyaluronate with a random spacing depending on the degree of saturation. The structure of the central filament is rather loose as shown in Figs. 4a and 5a and the closest centre-to-centre distances between adjacent G1 domains are 12 nm [50]. When link protein is added prior to aggregate formation, the central filament takes on a densely stained appearance (Fig. 4b) in which individual protein domains of either G1 or link protein are not resolved. The link-stabilized central filament extends as an uninterrupted protein shell over long distances, only occasionally interrupted by short stretches of free hyaluronate strands. This compact structure is also seen with negative staining and is partly due to the added mass contributed by the link protein. In addition, link protein appears to bridge adjacent G1 domains and thereby induces a highly cooperative set of protein-protein interactions along the hyaluronate template. A similar effect of link protein on the central filament structure is also visible when aggrecan fragments are used in reconstitution experiments [50] (Fig. 5b).

The details of the spatial arrangement of G1, hyaluronate and link protein in the central filament are not yet known. Tight packing of these components is, however, clearly demonstrated, with average centre-to-centre distances of 12 nm between adjacent G1 domains, as determined from the distances of E1 domains along the central filament (Fig. 6). The same mode of binding to hyaluronate and a similar central filament structure in reconstituted link-free and link-stabilized aggregates was also observed for large proteoglycans of both the aggrecan and versican type from non-cartilage origin [51], which demonstrates a functional conservation.

Earlier studies after Kleinschmidt spreading of proteoglycan aggregates indicated 2–4 times larger spacings and exclusively non-continuous central filament structures (see Fig. 1) with closest packing of monomers along the hyaluronate of about 20 nm [12,30,34], but we believe that this interrupted structure is an artefact of the spreading technique. The observation of an apparently continuous protein shell around the hyaluronate in the central filament implies that this structure is more ordered than has been assumed. The dense arrangement of aggrecan monomers in aggregates with hyaluronate and link protein should lead to severe electrostatic repulsions, unless the central filament has a defined spatial arrangement in solution, where the monomers point out in different azimuthal orientations.

4. Aggrecan from the Swarm rat chondrosarcoma

The protein components employed in the so far described reconstitution experiments had presumably been denatured during extraction and purification by exposure to high concentrations of chaotropic agents. Consequently the significance of the results largely depends on the ability of the protein domains to refold into a native conformation. It cannot be taken for granted that the structure of such reconstituted aggregates is in all respects representative of native aggregates. We adressed this issue by studying aggrecan aggregates from the Swarm rat chondrosarcoma, a tumor tissue in which cartilage-type proteoglycans are abundant [64]. Aggrecan monomers from this tumor have a molecular structure very similar to those present in normal hyaline cartilage, except that they lack keratan sulfate chains [64] and the proline-rich keratan sulfate attachment peptide [45,49]. Native aggregates may be extracted from the chondrosarcoma with high yields under non-denaturing conditions, i.e. with 0.5 M guanidine hydrochloride. In this solvent the non-cova-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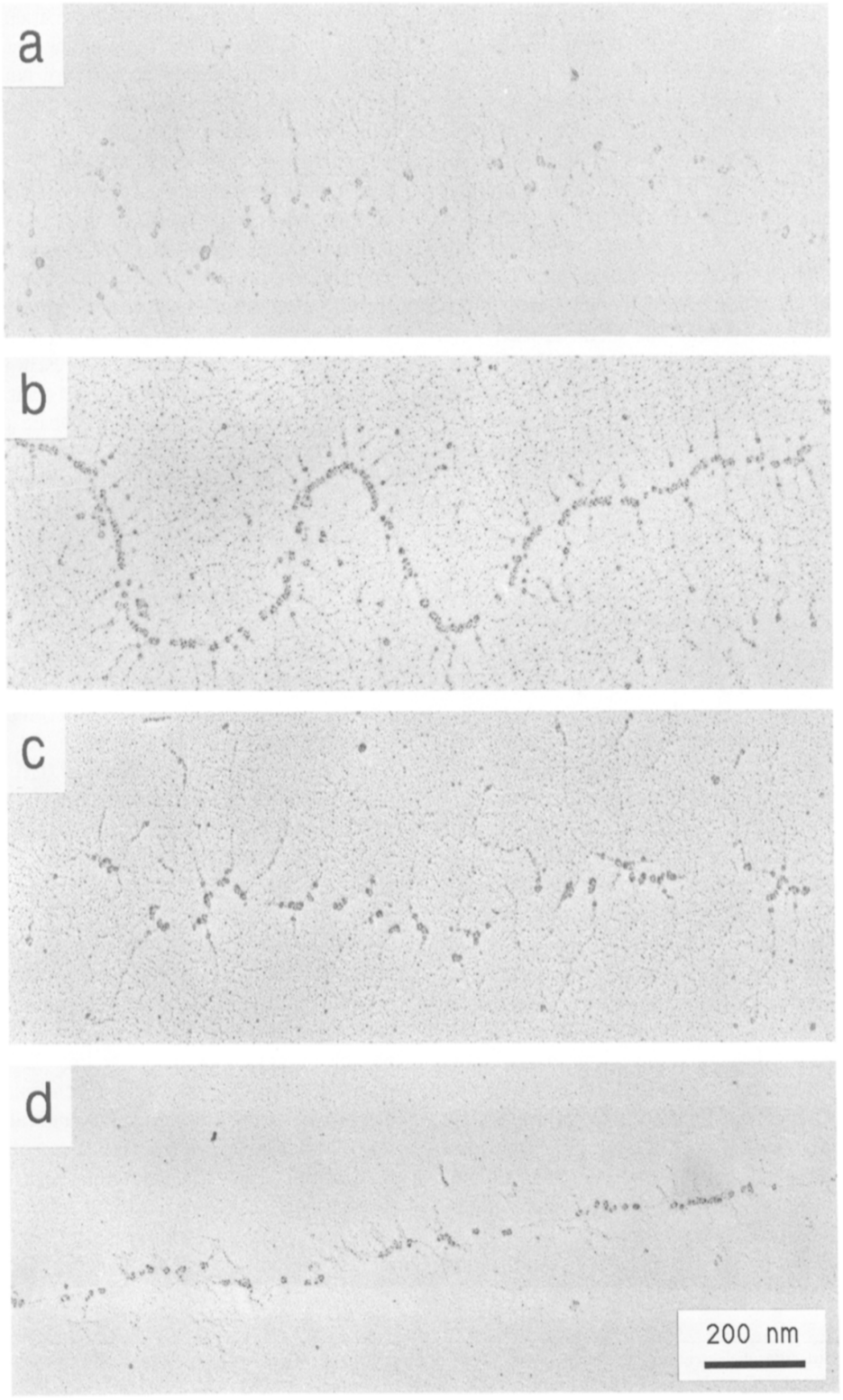
lent interactions within the central filament are not dissociated during extraction and subsequent purification steps [65,66].

Aggrecan aggregates were isolated from the chondrosarcoma tissue under mildest possible conditions with maximum guanidine hydrochloride concentrations of 0.5 M. Such aggregates were examined after different forms of electron microscopical specimen preparation to find conditions which exposed the aggregates to a minimum of shear forces and thereby gave optimal stuctural preservation. The methods used were glycerol spraying/rotary shadowing [35–37], mica sandwich squeezing [39] and mica centrifugation [40]. Under all experimental conditions proteoglycan aggregates with a central filament structure identical to that of link-stabilized reconstituted aggregates from earlier experiments [50, 51] were observed, thereby confirming the physiological relevance of our structural model for *in vivo* conditions [41]. Again, long tightly packed central filament stretches are visible, which are only occasionally interspaced by short pieces of free hyaluronate (Fig. 7). The close spacing of adjacent monomers along the hyaluronate with minimum centre-to-centre distances of 12 nm is also found by measuring the distances between neighbouring E1 strands. The average length of E2 is shorter than the corresponding length of this domain of bovine nasal cartilage aggrecan, which correlates with the absence of the keratan sulfate attachment region in the rat chondrosarcoma aggrecan sequence [45,49].

Interestingly, a second type of aggregate structure is present in the same extracts. This species has a central filament structure with comparably low packing density, where aggrecan monomers are tightly packed only in short clusters, which do usually not exceed 10 adjacent molecules. Fig. 8b shows this loosely packed aggregate population and allows comparison with the other, more densely packed aggregate type with an uninterrupted central filament structure (Fig. 8a). In addition to the short proteoglycan clusters very often single molecules with a somewhat enlarged G1 globule, presumably representing one link-stabilized binding region domain, are bound to the hyaluronate. Densely and loosely packed central filament structures are present in 0.5 M guanidine hydrochloride extracts in a 1.5 : 1 ratio, respectively, regardless of purification scheme and electron microscopic specimen preparation procedure. Addition of purified link protein to such preparations, or fixation by chemical crosslinking prior to electron microscopy, does not influence this ratio. We therefore propose that both densely and loosely packed aggregates are present in situ.

Further support for this hypothesis comes from sequential extraction experiments, where the tumor tissue is treated with a series of associative and dissociative solvents. With this experimental setup densely and loosely packed proteoglycan aggregates can be isolated in different relative amounts and visualized in the electron microscope after rotary shadowing (Fig. 9). Interestingly a pool of predominantly densely packed aggregates with a high content of intact (i.e. G3-containing) monomers is solubilized by EDTA in physiological saline, whereas a large proportion of the aggregates, containing less intact monomers, may be extracted without EDTA. In all instances closest centre-to-centre distances of 12 nm between adjacent aggrecan monomers are found within central filament patches. In contrast, only loosely packed, very degraded aggregates with a high content of single globes, presumed to be G1 fragments bound to hyaluronate, are extracted in a last step under dissociative conditions. Taken together with earlier work demonstrating a sequence homology [45–48] and possibly a functional similarity [62] of the C-terminal domain G3 to a Ca^{2+}-dependent group of hepatic lectins, this result may indicate that the EDTA-soluble pool represents recently synthesized aggregates which have not been subject to physiological proteolytic degradation and are able to interact with a still unknown ligand in the matrix. The proteoglycans extractable only under

Fig. 8. Structure of densely (a) and loosely packed (b) central filaments of native aggrecan aggregates from the Swarm rat chondrosarcoma as seen by glycerol-spraying/rotary metal shadowing electron microscopy.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dissociative conditions may represent the oldest and, during normal tissue turnover, most degraded aggregates, which are strongly bound in the tissue by a yet unknown mechanism.

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Fig. 9. Structure of sequentially and dissociatively extracted aggrecan aggregates as seen by mica sandwich squeezing/rotary shadowing electron microscopy. Aggregates were solubilized associatively with physiological saline in the absence (a) or presence (b) of EDTA, with 0.5 M guanidine hydrochloride (c), and in a last dissociative step with 4 M guanidine hydrochloride (d). Note the degraded appearance of monomers and the loosely packed central filament structure in aggregates in (a) and (d). The highest fraction of densely packed aggregates with the largest number of G3-containing aggrecan monomers are seen in EDTA-solubilized preparations (b).

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