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Domain structure of cartilage proteoglycans revealed by rotary shadowing of intact and fragmented molecules

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The rotary-shadowing technique for molecular electron microscopy was used to study cartilage proteoglycan structure. The high resolution of the method allowed demonstration of two distinct globular domains as well as a more strand-like portion in the core protein of large aggregating proteoglycans. Studies of proteoglycan aggregates and fragments showed that the globular domains represent the part of the proteoglycans that binds to the hyaluronic acid, i.e. the hyaluronic acid-binding region juxtapositioned to the keratan sulphate-attachment region. The strand-like portion represents the chondroitin sulphate-attachment region. Low-M_r proteoglycans from cartilage could be seen as a globule connected to one or two side-chain filaments of chondroitin sulphate.

The major class of proteoglycans in cartilage are large polyanionic glycoconjugates that because of their physical properties and their ability to form aggregates with hyaluronic acid provide resiliency to the tissue. A structural model for these complex multidomain molecules has been suggested, based on chemical data and electron microscopy (for references see Heinegård & Paulsson, 1984). In summary, at one end the core protein contains a hyaluronic acid-binding region that mediates the interaction with hyaluronic acid (Heinegård & Hascall, 1974) and with link protein (Franzén *et al.*, 1981), important for aggregate formation. At the other end, a long segment of the core protein is densely substituted with chondroitin sulphate chains and is accordingly called the chondroitin sulphate-attachment region (Heinegård & Axelsson, 1977). Between these two regions, a segment of the core protein contains a large portion of the keratan sulphate chains in the proteoglycan, i.e. the keratan sulphate-attachment region (Heinegård & Axelsson, 1977).

A minor proportion of the proteoglycans in cartilage have a completely different structure. They are of low M_r (76000) and consist of a monodisperse core protein (M_r 20000) and on average two chondroitin sulphate side chains (Heinegård *et al.*, 1981).

Electron microscopy was first introduced as a tool in the study of cartilage proteoglycans by Rosenberg *et al.* (1970). The technique used, spreading of molecules on films of cytochrome *c*, allowed determination of size parameters of the molecules (Thyberg *et al.*, 1975), as well as giving an insight into their structural organization (Heinegård *et al.*, 1978). The resolution of this technique did not allow observation of substructures in the proteoglycan core protein. In the present paper we describe the use of rotary shadowing of proteoglycans sprayed on to freshly cleaved mica for this purpose.

Materials and methods

Bovine nasal-cartilage proteoglycan aggregates (A1 fraction) and monomers (direct D1 fraction) and fragments thereof were prepared by standard procedures (for references see Heinegård and Paulsson, 1984). Proteoglycan core protein was prepared by chondroitinase ABC treatment of proteoglycan monomers, and keratan sulphate-attachment-region and chondroitin sulphate-attachment-region peptides by consecutive digestion with trypsin and chondroitinase ABC. Hyaluronic acid-binding region was isolated from trypsin digests of proteoglycan aggregates. Low-M_r

proteoglycans were isolated from bovine nasal cartilage as previously described (Heinegård *et al.*, 1981).

Samples for electron microscopy were dissolved in 0.2M-NH_4HCO_3, pH7.9, at 30–50 μg/ml and sprayed and shadowed as described previously (Kühn *et al.*, 1981).

Results

Rotary shadowing of proteoglycan monomers (Plate 1*a*) gave distinct images consisting of two rather densely stained globules attached at one end to a brush-like array of fine filaments. These filaments correspond to the chondroitin sulphate chains, which with this technique remain extended. They have a similar low contrast, as previously seen with heparan sulphate chains obtained from a basement-membrane proteoglycan (Fujiwara *et al.*, 1984). Occasionally a thin strand could be observed, extending from the globules through the array of chondroitin sulphate side chains. This strand probably represents the core protein. The two globules appear to be connected by a somewhat thicker strand and occur in a regular manner about 22nm apart.

In pictures of A1 fractions (Plate 1*b*) proteoglycan aggregates are seen, as well as monomers with the general appearance described above. In the aggregates the globular domains are assembled in the centre, forming a densely staining central filament, thereby outlining the hyaluronic acid. It is difficult to distinguish if both or only one of the globules are in direct contact with the hyaluronic acid, especially as link proteins are present in the central filament, contributing to the contrast. It is clear, however, that the globules are present at the same end of the proteoglycan as the hyaluronic acid-binding region.

More insight into the structure of the protein core is gained by electron microscopy of proteoglycan monomers treated with chondroitinase ABC to remove chondroitin sulphate side chains (Plate 2*a*). Again the two globules can be seen, 22nm apart, at one end of the core protein. From these a thin but well-defined strand extends, representing mainly the chondroitin sulphate-attachment region.

Pictures of the hyaluronic acid-binding region (Plate 2*b*) clearly show that at least one of the two globules is present in this part of the molecule. A proportion of the globules occur in pairs, again with a distance of approx. 22nm, and some of the globules appear as single particles. This could represent a certain heterogeneity in the hyaluronic acid-binding region as prepared by trypsin digestion, which may cleave the connection between the two globular domains. Similar pairs of globules can be seen after negative staining (result not shown).

The peptides representing the keratan sulphate-attachment region (Plate 2*c*) often also contain a globular structure attached to a short strand. These images would indicate that one of the globules is present near this region of the proteoglycan.

Peptides derived from the chondroitin sulphate-attachment region, in contrast, show up only as short rods (Plate 2*d*). Their general appearance agrees well with the assumption that they are derived from the long strand seen in the isolated core protein.

The low-M_r proteoglycan from cartilage gave images consisting of one or two thin filaments extending from a small globule (Plate 2*e*). The filaments look very similar to the side chains in the large proteoglycans and presumably represent the chondroitin sulphate chains, while the small globule corresponds to the core protein.

Discussion

Rotary shadowing appears to be a very useful technique in the study of large proteoglycans, particularly because of its ability to resolve structural domains in the core protein. The results obtained support the established model of cartilage proteoglycan structure and aggregate organization (for review see Heinegård & Paulsson, 1984). The most striking new observation is the occurrence of two globular domains at one end of the core protein. The hyaluronic acid-binding region has been assumed to be globular, and results obtained by neutron scattering are consistent with an ellipsoid shape (Perkins *et al.*, 1981). The second globule has, however, not been detected before, and the observation raises interesting questions about its functional significance. From the image of the various core-protein fragments it can be concluded that the second globule is present in the hyaluronic acid-binding region or between this region and the keratan sulphate-attachment region. Further studies will, however, be required to obtain a more exact correlation between the chemically defined proteoglycan regions and the substructures in the proteoglycan core protein observed by rotary shadowing.

We could also observe a small chondroitin sulphate proteoglycan with a size (M_r 76000) close to the resolution limits of the rotary-shadowing technique. The shapes of the major particles (a globule connected to one or two chondroitin sulphate chains) is in agreement with the chemical data (Heinegård *et al.*, 1981).

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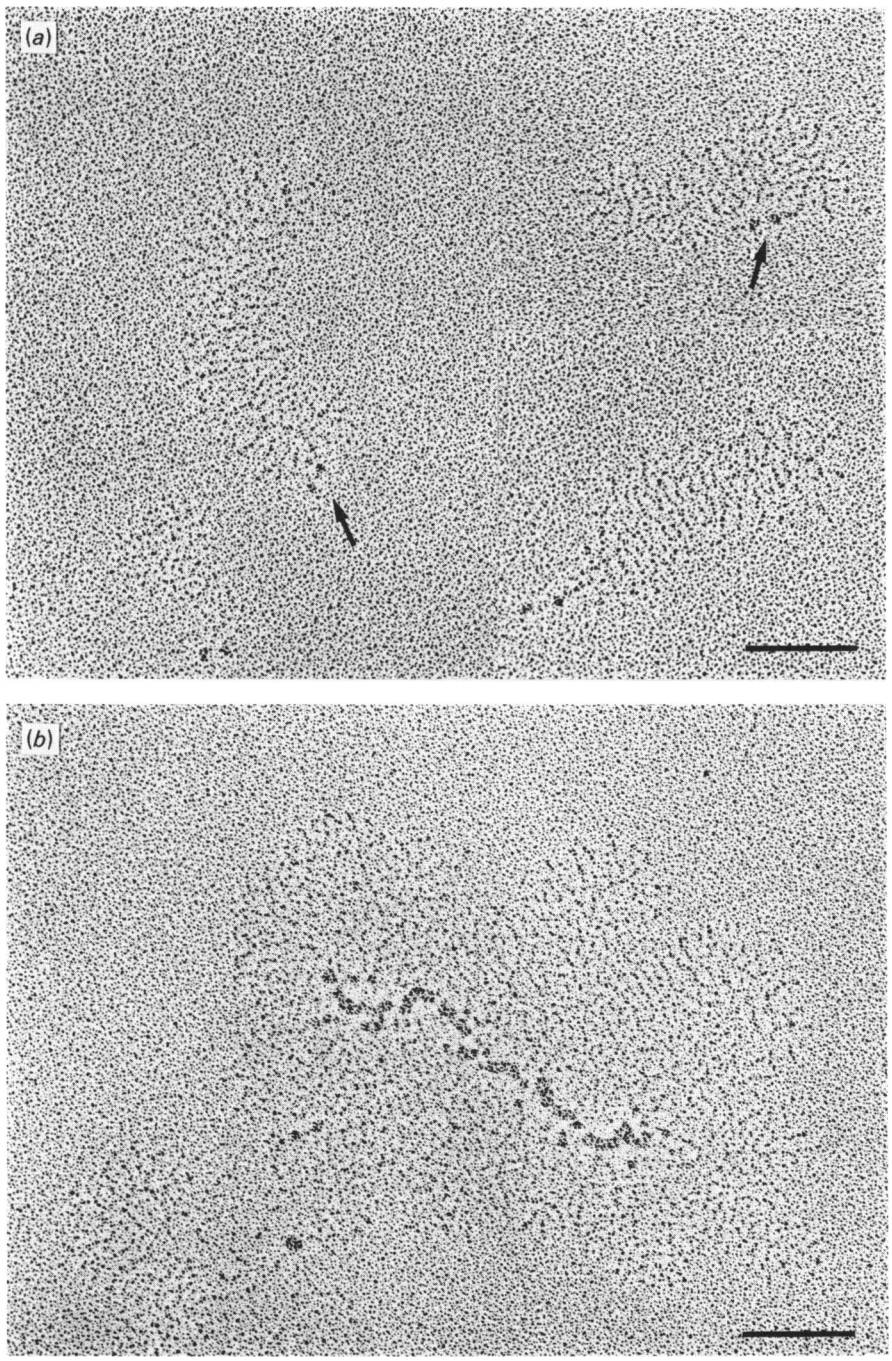
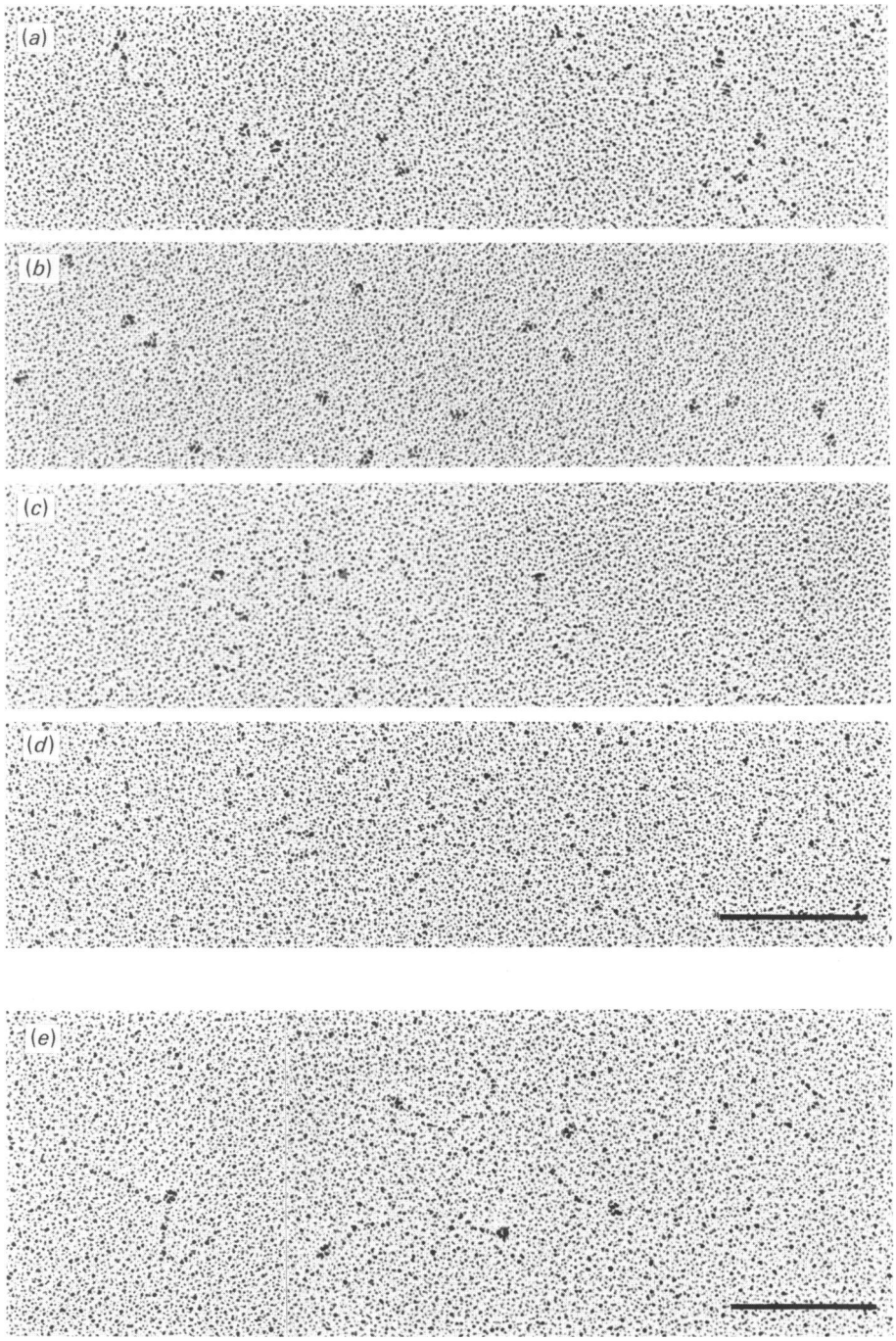


EXPLANATION OF PLATE 1

Electron microscopy after rotary shadowing of (*a*) *proteoglycan monomers and* (*b*) *proteoglycan aggregates*
Note the regular appearance in (*a*) of two globules (arrow) in close proximity to brush-like structures. Bars represent 100nm.



EXPLANATION OF PLATE 2

Rotary-shadowing images of fragments of large cartilage proteoglycans (a–d) and of the intact low-M_r cartilage proteoglycan (e)
The fragments were (*a*) proteoglycan core protein, (*b*) hyaluronic acid-binding region, (*c*) keratan sulphate-attachment-region peptides and (*d*) chondroitin sulphate-attachment-region peptides. Bars represent 100nm.

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