1-C-6 epitope in cartilage proteoglycan G2 domain is masked by keratan sulphate

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The presence of the protein epitope recognized by monoclonal antibody 1-C-6 was investigated on the globular G1 and G2 domains of pig cartilage proteoglycan core protein. After reduction of disulphide bonds and removal of keratan sulphate chains with keratanase, both G1 and G2 domains were shown to contain the epitope. However, without keratanase digestion the epitope on the G2 domain was poorly detected. The results suggest that a keratan sulphate chain substituted close to the epitope sequence in the G2 domain prevents antibody access to the epitope and thus masks its detection. This shows the 1-C-6 epitope to be a conserved protein sequence in the G2 domain of proteoglycans from different species, but its detection may be masked by glycosylation.

INTRODUCTION

The large aggregating proteoglycan from cartilage contains a high-molecular-mass protein core (~230 kDa) with a multi-domain structure (Hardingham *et al.*, 1990). There are two *N*-terminal globular domains, G1 and G2, linked by a short (21 nm) extended segment, and most of the keratan sulphate and chondroitin sulphate chains are attached to long extended segments between G2 and a *C*-terminal globular domain, G3 (Fig. 1). The G1 domain is responsible for proteoglycan aggregation and has sites for binding to hyaluronan and to link protein to which it is structurally related (Bonnet *et al.*, 1985). It consists of two structural motifs, an immunoglobulin fold and a double-loop structure (proteoglycan tandem repeat, PTR) (Bonnet *et al.*, 1986; Neame *et al.*, 1987; Doege *et al.*, 1987; Perkins *et al.*, 1989). The G2 domain contains a single protein motif similar to the PTR sequence found in the G1 domain. No binding properties of the G2 domain have been detected and its function remains unknown (Fosang & Hardingham, 1989).

In studies of the protein core structure of pig laryngeal cartilage proteoglycan, a fragment containing the G1 and G2 domains was isolated and characterized (Mörgelin *et al.*, 1988; Fosang & Hardingham, 1989). It appeared as a 'double-globe' structure by rotary shadowing electron microscopy and interacted via the G1 domain with hyaluronan and link protein (Mörgelin *et al.*, 1988). From the G1–G2 fragment a G2 domain was isolated after mild *V*₈ proteinase digestion, and a polyclonal antiserum specific for the G2 domain was raised in rabbits (Fosang & Hardingham, 1989). A previously characterized (Ratcliffe & Hardingham, 1983) antiserum to the G1 domain was shown not to react with the G2 domain. These antisera thus provided sensitive means for the separate identification of the globular G1 and G2 domains and showed that, in spite of considerable sequence similarity between G2 and part of G1 in the PTR motif (Perkins *et al.*, 1989), these domains each contained separate and quite distinct antigenic determinants. The purified G1 domain contained about 25% (w/w) carbohydrate, including *N*-linked oligosaccharides and keratan sulphate (Bonnet *et al.*, 1985). The G2 domain was of much higher, but variable, keratan sulphate content, and because of this was of slower electrophoretic mobility and larger apparent size than G1 on SDS/PAGE analysis (Fosang &

Hardingham, 1989). The G1–G2 fragment also ran anomalously slowly on SDS/PAGE (apparent molecular mass 150–180 kDa) because of its structure and keratan sulphate content.

The monoclonal antibody 1-C-6 was originally raised against rat chondrosarcoma proteoglycan and was shown to recognize an epitope in the hyaluronan-binding region (G1 domain) (Stevens *et al.*, 1984). The epitope was subsequently identified in tryptic fragments of the G1 domain, but further work established that, among the tryptic fragments of the whole proteoglycan, the epitope was present in not just one but in two peptide sequences

Fig. 1. Location of 1-C-6 epitopes in the amino acid sequence of rat chondrosarcoma proteoglycan

(a) Schematic representation of the location of epitopes in the G1 and G2 domains of the proteoglycan core protein KS, keratan sulphate; CS, chondroitin sulphate; S·S, disulphide bridges; Ig fold, immunoglobulin fold. The shading indicates the 1-C-6 epitope. (b) The aligned amino acid sequences of the G1 and G2 domains of rat chondrosarcoma proteoglycan showing the 1-C-6 epitope. Numbers show the positions of each amino acid residue in the sequence from the protein core *N*-terminus.

Abbreviation used: PTR, proteoglycan tandem repeat.

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(Hejna *et al.*, 1987). The explanation for this was revealed later by the complete sequence of the G1–G2 domains, which showed the PTR motifs of both G1 and G2 to contain the epitope sequence (Doege *et al.*, 1987).

In investigations of pig laryngeal proteoglycan, preliminary work (A. Ratcliffe & T. E. Hardingham, unpublished work) established that the monoclonal antibody 1-C-6 recognized an epitope in the pig cartilage G1 domain after reduction of disulphide bonds. As other studies showed that the 1-C-6 epitope reacted with proteoglycan from several species, it was clear that at least one copy of the sequence was conserved (Stevens *et al.*, 1981). In general, amino acid sequences are reported to be highly conserved (> 90%) among different species in the globular domains of proteoglycans, but less conserved in the extended regions (Doege *et al.*, 1990).

The monoclonal antibody 1-C-6 has been used as a basis for detecting proteoglycan in an assay for hyaluronan (Kongtawelert & Ghosh, 1989) and also in an e.l.i.s.a. for the proteoglycan G1 domain (Campbell *et al.*, 1989). The presence of the 1-C-6 epitope in more than one proteoglycan domain is thus of some importance in these applications. The isolation of a G1–G2 fragment from the pig cartilage proteoglycan protein core and the preparation of a G2 region from it (Fosang & Hardingham, 1989) provided the opportunity to investigate whether two copies of the 1-C-6 epitope are present in pig cartilage proteoglycan.

MATERIALS AND METHODS

All reagents used were of analytical grade. Trypsin (EC 3.4.21.4) (diphenylcarbamoyl chloride-treated), BSA (fraction V), V_8 proteinase (from *Staphylococcus aureus*, strain 8) and 3,3'-diaminobenzidine tetrahydrochloride were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Endo- β -D-galactosidase (keratanase; *Pseudomonas* sp.) was obtained from Miles Laboratories (Slough, Berks., U.K.). Na^{125}I was obtained from Amersham International (Amersham, Bucks., U.K.). Nylon sheet (Immobilon) was from Millipore (Bedford, MA, U.S.A.). Biotinylated anti-[mouse IgG (H + L)] (where H + L indicates heavy and light chains), biotinylated anti-[rabbit IgG (H + L)] and an avidin–biotinylated horseradish peroxidase reagent kit were from Vector Laboratories (Peterborough, Cambs., U.K.). The monoclonal antibody 1-C-6 was generously supplied by Dr. Bruce Caterson, University of West Virginia, Morgantown, WV, U.S.A. Hyaluronan-binding region (G1 domain) was prepared after trypsin digestion of proteoglycan aggregate as described by Bonnet *et al.* (1985), and double-globe (G1–G2) and G2 domains were prepared as described previously (Fosang & Hardingham, 1989) with mild trypsin digestion of proteoglycan aggregate and V_8 proteinase cleavage of G1–G2.

V_8 proteinase digestion

^{125}I -labelled G1–G2 (1.2×10^6 c.p.m./6 μl) was mixed with 20 μg of unlabelled purified G1–G2 and digested at 37 °C for 80 min in 0.1 M-Tris/HCl (pH 8.0)/10 mM-EDTA with 0.029 units of V_8 proteinase/45 μl of digestion volume. The digestion was stopped by boiling for 5 min.

Keratanase digestion

Aliquots of samples containing 10 μg of purified G1–G2 and ^{125}I -labelled G1–G2 (6×10^5 c.p.m.), which had been digested with V_8 proteinase, were digested at 37 °C for 4 h in 50 mM-Tris/acetate buffer, pH 7.4, with 0.01 unit of keratanase/30 μl . The digestion was stopped by rapid freezing. Purified G1 and G2 proteins (7.5 μg) were digested with 0.005 unit of keratanase/30 μl for 3 h at 37 °C in the same buffer.

Iodination

Iodination of G1–G2 was performed as described by Greenwood *et al.* (1983), with chloramine-T as the oxidizing agent. Protein (5–15 μg) was iodinated with 0.3 mCi of Na^{125}I , and the iodinated product was separated from unincorporated iodide by gel filtration on Sephadex G-50 columns (0.7 cm $\times$ 20 cm) which had previously been equilibrated with phosphate-buffered saline (0.14 M-NaCl/0.01 M-sodium phosphate, pH 7.3), and prewashed with 0.5 ml of 1% (w/v) BSA. The void volume fraction containing the iodinated protein was stored at –20 °C.

SDS/PAGE, immunoblotting and autoradiography

Electrophoresis was performed on 5% (w/v) polyacrylamide gels under reducing conditions as described by Fairbanks *et al.* (1971). Immunoblotting of protein antigens was performed by a modification of the method of DeBlas & Cherwinski (1983). Proteins were electrophoretically transferred from polyacrylamide slab gels on to Immobilon membranes overnight at 80 mA (4 °C) in 40 mM-Tris/HCl (pH 7.4)/20 mM-sodium acetate/2 mM-EDTA (transfer buffer). The membranes were blocked with 1% (w/v) BSA in buffer containing 0.1 M-Tris (pH 7.5)/0.9% NaCl/0.1% Tween 20 for 1 h at 37 °C, and then incubated with anti-G1 antiserum (1:1000 dilution), anti-G2 antiserum (1:100) or 1-C-6 (1:500) for 2 h at room temperature in the Tris buffer (pH 7.5) containing 0.1% (w/v) BSA. After washing, membranes were incubated with either biotinylated anti-mouse or biotinylated anti-rabbit antibody in Tris buffer (pH 7.5) for 1 h at room temperature, followed by washing and incubation with an avidin–biotinylated horseradish peroxidase complex. The peroxidase reaction was developed in the dark using 3,3'-diaminobenzidine tetrahydrochloride as substrate.

Before immunoblotting, dried Immobilon membranes containing ^{125}I -labelled material were exposed for 16 h at –70 °C to X-Omat S X-ray film (Kodak, Hemel Hempstead, U.K.). Developed X-ray films were scanned on a Shimadzu dual-wavelength scanner (model CS-930).

RESULTS

Experiments were carried out to investigate whether the protein core of pig cartilage proteoglycan contained two copies of the peptide sequence recognized by the monoclonal antibody 1-C-6. The sequences predicted to contain the 1-C-6 epitope form part of the PTR motifs of the G1 and G2 domains found at the N-terminal region of the protein core. Techniques recently developed for the isolation of a fragment containing only the globular G1 and G2 domains and for its cleavage by digestion with V_8 proteinase to yield G1- and G2-related products (Fosang & Hardingham, 1989) enabled the presence of 1-C-6 epitopes in both of these domains to be investigated.

The G1–G2 fragment was prepared and purified from pig laryngeal cartilage proteoglycan after mild trypsin digestion of proteoglycan aggregate, as previously described (Fosang & Hardingham, 1989). To samples of the G1–G2 preparation used for V_8 proteinase digestion was added a trace amount of ^{125}I -labelled G1–G2 preparation. Previous studies showed that with mild V_8 proteinase digestion the two major products were a large G2-related fragment and a small G1-related fragment (see Fig. 4a of Fosang & Hardingham, 1989). Present results showed that after separation of the digest products by SDS/PAGE (Figs. 2a, 2c and 2e, broken lines), there were two major peaks containing ^{125}I -labelled fragments. These corresponded to a fragment shown previously to contain the G2 domain of apparent molecular mass 110 kDa and unresolved fragments containing smaller G1 and G2 products (40–60 kDa). When the digest products were further

Fig. 2. Detection of 1-C-6 epitope in V_8 proteinase- and keratanase-digested G1-G2 fragments

Samples containing ^{125}I -labelled and unlabelled G1-G2 were digested with V_8 proteinase, and aliquots were subsequently digested with keratanase as described. Aliquots of each digest were electrophoresed in triplicate on 5% (w/v) polyacrylamide gels under reducing conditions. The gels were transferred on to Immobilon for immunoblotting with anti-G1, anti-G2 or 1-C-6. The dried Immobilon sheets were exposed overnight to X-ray film and the resulting autoradiographs were scanned on a gel scanner to produce profiles for total digest products (broken lines). Profiles for the corresponding immunoblotted samples in the same tracks are shown (solid lines). (a), (c), (e), G1-G2 digested with V_8 proteinase; (b), (d), (f), G1-G2 digested with V_8 proteinase and keratanase; (a), (b), anti-G1 immunoprofiles; (c), (d), anti-G2 immunoprofiles; (e), (f), 1-C-6 immunoprofiles. Approximate molecular masses are marked with arrows.

Fig. 3. Keratanase digestion and immunoblotting of G1 and G2 domains

Samples of G1 (a and c) and G2 (b and d) (2.5 μg) were electrophoresed in triplicate on 5% (w/v) polyacrylamide reducing gels before (solid lines) and after (broken lines) keratanase digestion and the transferred proteins were immunodetected with anti-G1 antiserum (c), anti-G2 antiserum (d) or 1-C-6 (a,b). Densitometric scans of the immunoblots were recorded and the relative staining of control and keratanase-digested samples is shown as a ratio in each figure (calculated from the area under the curve). Approximate molecular masses are marked by the arrows.

digested with keratanase in order to remove keratan sulphate chains, there was a large decrease in the size of the 110 kDa component to approx. 75 kDa and small decreases in the sizes of the 40–60 kDa components (Figs. 2b, 2d and 2f, broken lines).

The digest products separated by SDS/PAGE were transferred to Immobilon sheets and analysed by immunolocalization with polyclonal antisera to the G1 domain and to the G2 domain and with the monoclonal antibody 1-C-6. The main 110 kDa fragment identified by autoradiography was shown by immunoblotting to lack G1 epitopes (Fig. 2a, solid line) and was weakly (and variably) positive for G2 epitopes (Fig. 2c, solid line). It was also only weakly detected by the 1-C-6 antibody (Fig. 2e); however, after keratanase digestion there was strong 1-C-6 localization of similar mobility to the ^{125}I -labelled 75 kDa fragment (Fig. 2f), and this was also strongly detected by anti-G2 antibodies (Fig. 2d) but not by anti-G1 antibodies (Fig. 2b). The large G2 fragment released from the G1-G2 preparation therefore appeared to contain the 1-C-6 epitope, but it was only readily detected after removal of keratan sulphate chains from the fragment.

Immunolocalization of non-keratanase-treated digests showed a major 60 kDa fragment localized by anti-G1 antibodies which was also localized by the 1-C-6 antibody (Figs. 2a and 2e). After keratanase digestion this appeared to run as a 55 kDa fragment and was localized by both anti-G1 antibodies (Fig. 2b) and 1-C-

6 antibody (Fig. 2f), but not by anti-G2 antibodies (Fig. 2d). The main G1 fragment released from G1-G2 therefore appeared to contain a readily detected 1-C-6 epitope.

Fragments of smaller apparent size released from G1-G2 were localized by anti-G2 antibodies at about 40 kDa and also by 1-C-6 antibody, but fragments in this region were also weakly detected by anti-G1 antibodies. Smaller cleavage products of both G1 and G2 resulting from more extensive V_8 proteinase digestion have previously been shown to migrate in this size range (Fosang & Hardingham, 1989). After keratanase digestion the migration of these fragments was not greatly altered and they remained localized by anti-G1, anti-G2 and 1-C-6 antibodies.

The main result of this analysis was that, although cleavage of G1-G2 yielded a major G1 fragment which reacted with 1-C-6, the larger fragment containing the G2 domain required keratanase digestion before it reacted with the 1-C-6 antibody. This suggested that close to the 1-C-6 epitope in G2 a keratan sulphate chain may be attached which obstructs antibody access to the epitope. Furthermore, as the detection of 1-C-6 epitope was on reduced gels in the presence of SDS, the chain must be located particularly close to the epitope. In contrast, the 1-C-6 epitope in the main G1 fragment did not show enhancement of staining after keratanase digestion.

Further experiments were then carried out on purified trypsin-prepared G1 domain (Bonnet *et al.*, 1985) and on G2 domain isolated from a partial V_8 digestion of a G1-G2 preparation

Fig. 4. Immunolocalization of G2 by anti-G1 antiserum after reduction and keratanase digestion

Samples of G1, G2 and G1-G2 (2.5 µg) were electrophoresed on 5% (w/v) polyacrylamide reducing gels before and after keratanase digestion and immunolocalized with anti-G2 antiserum (a) or anti-G1 antiserum (b). Lane 1, G1-G2; lane 2, undigested G1; lane 3, keratanase-digested G1; lane 4, undigested G2; lane 5, keratanase-digested G2.

(Fosang & Hardingham, 1989). These showed characteristic increases in electrophoretic mobility after keratanase digestion (Fig. 3). Both G1 and G2 domains were immunolocalized with the 1-C-6 antibody and the intensity of staining was shown to increase after keratanase digestion for G2 (38% increase), but for G1 it was unchanged. Staining of G2 with the anti-G2 antiserum showed an increase in detection (38%) which was similar to that observed with 1-C-6, indicating that keratanase digestion had revealed epitope(s) which were masked by keratan sulphate chains. There was no evidence of masking of epitopes on G1 by keratan sulphate, as its localization with anti-G1 showed a small decrease after keratanase digestion.

Previous results have shown that the polyclonal anti-G1 and anti-G2 antisera are specific for their native glycosylated G1 and G2 antigens respectively in radioimmunoassays and immunoblotting experiments (Ratcliffe & Hardingham, 1983; Fosang & Hardingham, 1989). In the present work, it was revealed that there was a small degree of cross-reactivity between the anti-G1 antiserum and keratanase-digested reduced G2 (Fig. 4b). This was not apparent with the native G2 domain, which was not recognized by anti-G1 antiserum. This domain also gave no response with anti-G1 antiserum after keratanase digestion. It was only on subsequent reduction that the cross-reaction was evident. The common antigenic determinants present on keratanase-digested and reduced G1 and G2, therefore, are buried epitopes that were unavailable on the native structures. Keratanase-digested reduced G1 was not detected by anti-G2 antiserum (Fig. 4a).

DISCUSSION

The enhanced detection of 1-C-6 epitope in G2 fragments after keratanase treatment provides evidence that the epitope is masked by keratan sulphate substitution. The epitope is contained within the rat G2 sequence Ser-Pro-Glu-Gln-Leu-Gln-Ala-Ala-Tyr-Gly. The serine at position 496 in the sequence is a likely

substitution site. The 1-C-6 epitope has been conserved in rat, bovine, pig and human proteoglycan and the adjacent serine residue, which has been conserved in rat and bovine proteoglycan, is also present in pig G2 (P. J. Neame, A. Fosang & T. E. Hardingham, unpublished work). The identification of this residue in amino acid sequence analyses of G2 tryptic fragments indicates that not every molecule is substituted, and from our results we would estimate that the incidence of keratan sulphate substitution at this site was 50% or less. Indeed, the extent of masking of 1-C-6 epitope by keratan sulphate is variable between different G1-G2 preparations. The results are compatible with the predictions of secondary structure of G2, which indicate that serine at position 496 occurs on the external face of the folded native protein (Perkins *et al.*, 1989). Since there is no evidence of masking of the 1-C-6 epitope in G1, the corresponding residue (Thr) present on rat and bovine G1 is either not present in pig G1, or, if it is, it does not carry a keratan sulphate chain. The sequence of amino acids in and around the 1-C-6 epitope of the G1-G2 domains of rat chondrosarcoma proteoglycan shows that the nearest trypsin sites are situated some distance from the epitopes, and the enzyme would not therefore be expected to destroy them; experiments have confirmed this (A. Fosang & T. E. Hardingham, unpublished work) (see Fig. 1b).

The major antigenic determinants found on reduced proteins were different from those on the native structures. Polyclonal anti-G1 and anti-G2 antisera contain antibodies with specificity for a variety of determinants, some on native and some on reduced antigens. It has been shown previously (Fosang & Hardingham, 1989) that, although there is considerable sequence similarity between these two protein domains, there is no cross-reactivity between the polyclonal antisera with the native G1 or G2 antigens, whether or not the attached keratan sulphate chains are digested. The results presented here show that there is also no cross-reactivity between the antisera with the reduced G1 and G2 antigens. However, keratanase treatment revealed the presence of a common determinant in G1 and G2 which was not present in native structures. It is possible that the major G2 determinant which is detected by anti-G1 antiserum after reduction and keratanase digestion is the 1-C-6 epitope, although there are likely to be other short sequences in common between the two proteins domains.

Digesting keratan sulphate with keratanase shortens the polylactosamine chain, but leaves intact the oligosaccharide structure close to the linkage to protein. The masking of the 1-C-6 epitope therefore appears to be caused by the extended keratan sulphate chain rather than by the region close to its linkage to protein. This is surprising and implies that under denaturing conditions the keratan sulphate chain may interact with the epitope sequence. This might be as a result of ionic interactions between the strongly charged sulphate groups on keratan sulphate and the basic amino acids in the sequence Pro-Glu-Gln-Leu-Gln-Ala-Ala-Tyr, or could involve the hydrophobic Ala-Ala-Tyr sequence and hydrophobic faces noted on the sugar rings within the keratan sulphate structure (Hounsell, 1989).

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