Use of Monoclonal Antibodies to Locate the Chondroitin Sulfate Chain(s) in Type IX Collagen*

(Received for publication, August 26, 1986)

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Recent results show that type IX collagen isolated from chicken cartilage is associated with one or perhaps two chondroitin sulfate chains. To locate the chondroitin sulfate chain(s) along the type IX collagen molecule, rotary shadowing was performed in the presence of monoclonal antibodies which recognize stubs of chondroitin sulfate generated after chondroitinase ABC digestion. Monoclonal antibodies 9-A-2 and 2-B-6 which recognize stubs of chondroitin 4-sulfate were found to bind specifically to the NC3 domain of type IX collagen, and this binding was dependent on prior digestion of the preparation with chondroitinase ABC. Monoclonal antibody 1-B-5, which recognizes unsulfated stubs of chondroitin sulfate, did not show any specific binding to type IX collagen either with or without chondroitinase ABC digestion. As a control, monoclonal antibody 2C2 was used, which in previous work was shown to bind specifically to an epitope located close to or at the NC2 domain. Binding of this antibody to NC2 was unaffected by chondroitinase ABC digestion, and no specific binding of the antibody to the NC3 domain was detected either before or after chondroitinase ABC digestion.

Type IX collagen is present in all hyaline cartilages where it constitutes between 5 and 20% of the total collagen (reviewed in Ref. 1). The structure of the molecule is unique among the different collagen types since the molecule contains three separate triple helical collagenous domains which are interspersed by two noncollagenous domains. In addition, noncollagenous domains are located at the amino and carboxyl termini. The collagenous domains are designated COL1 to COL3 and the noncollagenous domains as NC1 to NC4 (2). Each molecule is assembled from three genetically distinct chains which were previously fractionated either from the two pepsin-resistant fragments of the molecule, called HMW and LMW (3–5), or from the intact molecule (6). The three chains are designated α1(IX), α2(IX), and α3(IX), and all of the available evidence suggests that there is a single type IX molecule containing equimolar amounts of the three chains (reviewed in Ref. 1). Recombinant cDNA clones were recently identified for both the α1(IX) and α2(IX) chains, and nucleotide sequencing of these clones has provided extensive amino acid sequences for both of these chains, as well as defining the location and organization of the pepsin-resistant fragments HMW and LMW within the intact type IX molecule (2, 5, 7). From these results, a model was proposed for the structure of type IX collagen (2), and this model was subsequently largely confirmed by rotary shadowing observations of the intact type IX collagen molecule secreted by a suspension culture of embryonic chick chondrocytes (8).

Type IX collagen is also a proteoglycan and is the same molecule as was previously isolated from embryonic chick epiphyseal cartilage and called proteoglycan light or PG-Lt (9–11). It is now established that one or perhaps two chondroitin or dermatan sulfate chains are covalently linked specifically to the α2(IX) chain (6, 12). In this paper, we will show that the glycosaminoglycan chain or chains are located at the NC3 domain of type IX collagen by electron microscopic observations after rotary shadowing in the presence of monoclonal antibodies which recognize stubs of chondroitin sulfate generated after chondroitinase ABC digestion (13, 14).

MATERIALS AND METHODS

Monoclonal antibodies used in these experiments were (i) 9-A-2 and 2-B-6 which recognize 2-unsaturated disaccharides of unsulfated chondroitin 4-sulfate generated after chondroitinase ABC digestion of proteoglycans containing chondroitin 4-sulfate or dermatan sulfate (13, 15), (ii) 1-B-5 which recognizes C-unsaturated disaccharides of unsulfated chondroitin generated by chondroitinase ABC digestion (13), and (iii) 2C2 which recognizes native type IX collagen at an epitope close to or within the NC2 domain (8).

Antibodies were purified by protein A-Sepharose chromatography as described previously (16) or by use of DEAE-cellulose. In the second method, ascites fluid was first treated with Lipoclean Clearing Agent (Behring Diagnostics) to remove lipid. A mixture of ascites fluid (3 parts) and Lipoclean (2 parts) was vortexed (60 s) and centrifuged (10 min, 3000 × g) and the top layer was collected. Lipid-free ascites fluid was mixed with an equal volume of saturated ammonium sulfate and allowed to stand (1 h, 4 °C) before centrifugation (20,000 × g, 4 °C, 30 min). The pellet was dissolved in 0.1 M phosphate-buffered saline and dialyzed against several changes of 0.02 M phosphate buffer, pH 7.2. The antibody solution was mixed with an equal volume of equilibrated DEAE-32 (Whatman) in 0.02 M phosphate buffer, pH 7.2, and the mixture was shaken overnight before centrifugation (3000 × g, 10 min). Each supernatant was subsequently analyzed by gel electrophoresis (17) and shown to contain a single band of IgG.

The secreted form of type IX collagen was prepared from a suspension culture of chondrocytes isolated from 17-day-old chick embryo sterna (8). After 2-h incubation, collagen was precipitated from the medium with ammonium sulfate (30% of saturation) and isolated by centrifugation. The precipitate was dissolved in chondroitinase-digestion buffer (0.1 M Tris-HCl, 0.04 M sodium acetate, pH 8.0) in the presence of protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 2.5 mM EDTA, and 0.25 mM 6-aminocaproic acid). The sample was divided in half and incubated either with or without chondroitinase ABC (0.25 units/ml, Miles Scientific, Naperville, IL) for 30 min at 35 °C. Each sample was mixed with monoclonal antibody (final concentration 12.5–25 μg/ml) and dialyzed overnight at 4 °C against several changes of 0.2 M ammonium bicarbonate. Samples were sprayed onto freshly cleaved mica, and rotary shadowing with platinum was performed as described previously (18). Lengths of molecules and the location of antibody binding sites were determined using a sonic digitizer as described previously (8). As in previous experiments, the individual molecules secreted by a suspension culture of chondrocytes and observed after rotary shadowing consisted largely of type IX molecules which could be recognized by their overall length, characteristic kink at NC3, and small knob at NC4 (8, 19). In these preparations, the type II procollagen
molecules were observed largely as end-to-end overlapping aggregates, each of which contained many molecules (19).

RESULTS AND DISCUSSION

In previous rotary shadowing experiments observing type IX collagen, an additional, poorly resolved chain was occasionally observed to branch from the kink present in type IX molecules at the NC3 domain (19). These preliminary observations, therefore, suggested that the NC3 domain may represent the location of the chondroitin sulfate chain(s).

To demonstrate this directly, rotary shadowing was now performed in the presence of monoclonal antibodies which recognize stubs of chondroitin sulfate generated after chondroitinase ABC digestion. Previously, it was reported that type IX collagen, isolated from 17-day-old chicken embryo sternum and digested with chondroitinase ABC, gave a ratio of 4-sulfated to 6-sulfated disaccharides of 3:1 (10). In the first series of experiments, therefore, monoclonal antibody 9-A-2 was used which specifically recognizes the disaccharide derived from chondroitin 4-sulfate or dermatan sulfate (13).

Fig. 1 shows a gallery of type IX molecules after chondroitinase ABC digestion in which monoclonal antibody 9-A-2 was specifically bound to the kink at the NC3 domain. Interestingly, the antibody always bound to the outer surface of the kink in every molecule examined regardless of the angle of the kink. Quantitation of the combined results for three separate experiments is shown in Fig. 2 in which binding of antibody 9-A-2 along each type IX molecule was measured and plotted in the form of a histogram. The results show that specific binding of 9-A-2 to the NC3 domain was only observed after prior digestion of the preparation with chondroitinase ABC. The apparent binding of 9-A-2 to the NC4 domain as shown in the histogram is largely a reflection of the concealment of this domain by the antibody. All molecules in which the NC4 domain could not be visualized are included in the bar at 0 nm. Of all molecules examined (total measured = 391), 79.8% showed an antibody binding to the NC3 domain after chondroitinase ABC digestion, whereas only 7.7% (total molecules measured = 236) showed an antibody at this site without chondroitinase ABC digestion. Subsequently, a second antibody (called 2-B-6) was identified with the same specificity to 9-A-2, but with a higher stability on storing.

Rotary shadowing with 2-B-6 gave almost identical results to 9-A-2 (Fig. 3). In these experiments, 79.0% of the molecules (total measured = 363) showed an antibody at the NC3 domain after chondroitinase ABC digestion, whereas without chondroitinase ABC digestion, whereas without chondroitinase ABC digestion, only 10.6% of the molecules showed an antibody at this site (total measured = 236).
Rotary shadowing experiments were also performed in the presence of 1-B-5 which recognizes unsulfated disaccharides (14). Although occasional binding of 1-B-5 to the NC3 domain was observed, there was no consistent increase in binding after chondroitinase ABC digestion. Control experiments were also performed with antibody 2C2 which previously was shown to bind to an epitope close to or at the NC2 domain (8). Fig. 4 shows that binding of 2C2 to NC2 was unaffected by chondroitinase ABC digestion and, moreover, no specific binding of 2C2 to NC3 was observed either with or without chondroitinase ABC digestion.

The present results show that monoclonal antibodies, which were previously identified as recognizing chondroitin sulfate stubs generated after chondroitinase ABC digestion (13, 14), will bind with sufficiently high affinities that the location of each epitope can be visualized by rotary shadowing. These results also confirm our previous rotary shadowing observations which suggested that the chondroitin sulfate chain is located at the NC3 domain (19). Although only one 9-A-2 or 2-B-6 antibody was ever observed to bind to NC3, the results do not clarify if there is more than one chondroitin sulfate chain located at NC3. It may not be sterically possible for more than one antibody molecule to bind to chondroitinase-generated stubs at this site. Also, the results do not establish if the chondroitin sulfate chain is bound to the α2(IX) chain (21).

Previous results showed that chondroitin sulfate is not present in the pepsin-resistant fragments of type IX collagen called HMW and LMW (3, 22). However, the α2 chain of HMW is specifically cleaved at the NC3 domain probably during pepsin digestion (7, 22). This must result in the release of the chondroitin sulfate chain so that there must be two sites available for pepsin cleavage at NC3. The possible existence of a polypeptide loop specifically from the α2(IX) chain at NC3 and the location of these cleavage sites are at present unknown.

Acknowledgments—We thank Pauline M. Mayne, N. Keith Waddell, and Fran Allen for their excellent technical assistance. Also, we thank Drs. Bruce Caterson, James Christner, and Mary Ann Accavitti for providing antibodies and for their good advice. Ascites fluid was provided by the Hybridoma Core Facility, Multipurpose Arthritis Center, University of Alabama at Birmingham.

REFERENCES


FIG. 4. Histograms comparing the binding of antibody 2C2 to type IX collagen either with (panel A) or without (panel B) chondroitinase ABC digestion. Note that binding to the NC2 region is unaffected by chondroitinase ABC digestion and that no specific binding occurs at the NC3 either with or without chondroitinase ABC digestion. Panel A, 116 molecules measured; panel B, 190 molecules measured.