

Monoclonal Antibody Against Chicken Type IX Collagen: Preparation, Characterization, and Recognition of the Intact Form of Type IX Collagen Secreted by Chondrocytes

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ABSTRACT A series of monoclonal antibodies was prepared against the pepsin-resistant fragment of type IX collagen designated HMW. One of these antibodies (called 2C2) was selected for further analysis. Antibody 2C2 showed no cross-reactivity with other collagen types by inhibition enzyme-linked immunosorbent assays. It recognized an epitope present in native HMW, but failed to recognize any of the three chains of HMW fractionated after denaturation followed by reduction and alkylation of interchain disulfide bridges. Electron microscopic observations after rotary shadowing showed that the location of the epitope for antibody 2C2 was close to the carboxy-terminus of HMW. Immunofluorescent staining of sections of embryonic and adult cartilage with antibody 2C2 after removal of proteoglycans by testicular hyaluronidase digestion showed that type IX collagen is distributed throughout the cartilage matrix, and is not present in other connective tissues or skeletal muscle. The intact type IX collagen molecule, which was secreted by a suspension culture of freshly isolated embryonic chick chondrocytes, was recognized by rotary shadowing in the presence of antibody 2C2 after first precipitating the procollagens from the culture medium with ammonium sulfate (30%). Two different collagenous molecules were present in the precipitate: a longer molecule of type II procollagen (average length, 335 nm) with both amino- and carboxy-propeptides still remaining uncleaved, and a shorter molecule (average length, 190 nm) which was identified as type IX collagen. Antibody 2C2 consistently bound to the shorter molecules at a site located 136 nm from a distinctive knob at one end of the molecule, and did not bind to any specific site on the type II procollagen molecules. The structure of the intact type IX collagen molecule with the location of both collagenous and noncollagenous domains was as predicted after converting the nucleotide sequence of a cDNA clone encoding for one of the chains of type IX collagen to an amino acid sequence (Ninomiya, Y., and B. R. Olsen, 1984, *Proc. Natl. Acad. Sci. USA.*, 81:3014–3018).

Type IX collagen was initially isolated from chicken sternum as two pepsin-resistant fragments designated HMW and LMW (1, 2) or M1 and M2 (3). Subsequent studies showed that the intact molecule from which these fragments are derived is much larger (4–7), and that both fragments may be derived from a single molecule (6). Three genetically distinct chains were fractionated from both LMW (8, 9) and HMW (10). The three chains were consistently present in a 1:1:1 proportion for both HMW and LMW, and it was proposed that there is a single type IX molecule of chain organization

$\alpha 1(\text{IX})\alpha 2(\text{IX})\alpha 3(\text{IX})$ (8–10). A cDNA clone (called pYN 1738) was prepared from chick chondrocyte RNA and shown to encode for one of the chains of both HMW and LMW (11). The DNA sequence of this clone was converted to an amino acid sequence and this sequence compared to the amino acid compositions of the tryptic peptides and the amino-terminal sequences of the three chains of both HMW and LMW (10). By this approach, it was possible to determine which of the three chains of HMW and LMW are encoded for by the cDNA clone pYN 1738, and to determine the

arrangement of HMW and LMW within the intact type IX molecule. This chain was designated the $\alpha 1(\text{IX})$ chain (10), and subsequently a second cDNA clone (called pYN 1731) was identified which encodes for approximately one-half of the $\alpha 2(\text{IX})$ chain (12).

Recently, genomic clones were isolated for the $\alpha 1(\text{IX})$ and $\alpha 2(\text{IX})$ chains (13), and it is anticipated that the complete amino acid sequences of the chains of type IX collagen will eventually be determined with DNA sequencing studies. However, the function of type IX collagen in cartilage matrix still remains unknown, and to examine the location of this collagen in cartilage matrix, we now describe the preparation and characterization of a monoclonal antibody (called 2C2) of high affinity for type IX collagen. The location of the epitope for antibody 2C2 was determined by rotary shadowing of the antibody in the presence of both HMW and the intact type IX collagen molecule as secreted by a suspension culture of freshly isolated chondrocytes.

MATERIALS AND METHODS

Preparation of Collagens: Type I collagen was prepared from an acid extract of lathyritic chicken skin (14). Type II collagen, HMW, and LMW were isolated from a pepsin digest of adult chicken sterna and further fractionated by differential salt precipitation in acid conditions as previously described (1). Further purification of type II collagen was achieved as the precipitate obtained during differential salt precipitation between 0.7–0.8 M NaCl, 0.5 M acetic acid. HMW and LMW were fractionated by molecular sieve chromatography (Bio-Gel A-5m) (Bio-Rad Laboratories, Richmond, CA) without prior denaturation of the sample (2). Further purification of HMW was achieved by CM-cellulose chromatography in non-denaturing conditions (15), and of LMW by an additional passage over the molecular sieve column. Types III, IV, and V collagens were obtained by limited pepsin digestion of adult chicken gizzard and separated by differential salt precipitation in acid and neutral conditions (15, 16). Type X collagen (59 kD form) was isolated from the medium of long-term cultures of chondrocytes from chick tibiotarsus (17), and was a gift from Dr. Thomas M. Schmid, Department of Biochemistry, Rush-Presbyterian-St. Luke's Medical Center, Chicago. For some experiments, HMW was reduced with 2-mercaptoethanol (0.1 M, 100°C, 2 min) and alkylated with iodoacetic acid (0.2 M) at room temperature before being separated into three fractions (called C-2 + C-5, C-3, and C-4) by molecular sieve chromatography (Bio-Gel A-1.5m), as described previously (2). In earlier work C-2 was regarded as a single chain of HMW (2), but further experiments have shown that C-2 can be fractionated by reverse-phase high performance liquid chromatography into two chains now designated C-2 and C-5 (10). However, the separation of C-2 and C-5 was not carried out in the present series of experiments. The fragments C-3 and C-4 represent the third chain of HMW, and arise from the cleavage of this chain in a noncollagenous domain called NC3 between two triple helical domains called COL2 and COL3 (2, 10, 11).

Hybridoma Production: Female BALB/c mice (6–8 wk old) were initially immunized with 100 μg of HMW emulsified in complete Freund's adjuvant; the injections were made subcutaneously into the rear footpads, inguinal and axial regions. A similar injection in incomplete Freund's adjuvant was given 3 d later, and then at 3–4-d intervals four more injections were given with the antigen dissolved in physiological saline. 1 d after the last injection, the local lymph nodes draining the subcutaneous areas of injection were removed and a cell suspension was prepared before fusion.

The preparation of hybridomas was described in detail elsewhere (18) and was performed by the staff of the Hybridoma Core Facility, Multipurpose Arthritis Center, University of Alabama at Birmingham. Briefly, lymph node cells and myeloma cells (P3 \times 63A8.653) were both washed in serum-free RPMI-1640 medium and then combined in a 2:1 ratio and pelleted. The cells were agitated with 1 ml PEG-4000 at 37°C for 30 s before being slowly diluted with serum-free RPMI-1640 medium at 37°C. Cells were briefly centrifuged and resuspended in hypoxanthine/aminopterin/thymidine medium. Peritoneal exudate feeder cells were added and the suspension plated (1 ml/well) into 24-well Costar plates to give 5×10^5 lymph node cells/well. After 2–3 wk, hypoxanthine/aminopterin/thymidine medium was replaced by normal medium. Hybridomas which grew and produced antibodies against HMW were cloned several times by limiting dilution. Ascites fluid was prepared by injecting 10^7 hybridoma cells into BALB/c female mice primed with Pristane (0.5 ml,

Aldrich Chemical Co., Milwaukee, WI). After 2–3 wk, ascites fluid was harvested, briefly centrifuged, and protease inhibitors added to the supernatant.

Purification and Characterization of Monoclonal Antibodies: Immunoglobulins were precipitated from ascites fluid with ammonium sulfate (50% of saturation, 4°C), and the precipitate redissolved in 0.01 M phosphate-buffered saline (PBS), pH 7.2. After extensive dialysis against PBS, further purification of each antibody was achieved by passage over an HMW affinity column prepared by coupling HMW to Sepharose-4B (Pharmacia Fine Chemicals, Piscataway, NJ) using published procedures (19, 20). Each antibody was eluted with 3 M KSCN, 0.05 M PBS, pH 6.0, dialyzed immediately against PBS, and concentrated by ultrafiltration before being stored in the presence of protease inhibitors. Enzyme-linked immunosorbent assays (ELISAs)¹ for each antibody were performed with a Hybridoma Screening Kit specific for the mouse IgG class (Bethesda Research Laboratories, Gaithersburg, MD) as described previously (21). Each antibody preparation was also analyzed by SDS PAGE (5–10% gradient slab gel), and the subclass of each antibody was determined by ELISAs. The anti-isotype reagents were purchased from Southern Biotechnology Associates, Birmingham, AL.

Electron Microscopic Observations of Collagen/Antibody Binding: The procedures have been described in detail elsewhere (20, 22). A mixture of the collagen and antibody (25 $\mu\text{g}/\text{ml}$ each) were dialyzed against 0.2 M ammonium bicarbonate (pH 7.9) before spraying. Lengths of molecules and the location of antibody binding sites were measured using a Science Accessories Graf Pen 3 Sonic digitizer interfaced with a Hewlett-Packard 9825A (Hewlett-Packard Co., Palo Alto, CA) programmable calculator. Calibration of the electron microscope was achieved using a grating replica of silicon monoxide (21,600 lines/cm, Ernest F. Fullam, Inc., Schenectady, NY).

Immunofluorescence Observations: The procedures used were described in detail previously (23). Frozen sections (5 μm) were prepared from the sterna of 18-d chick embryos and from the sterna of adult chicken and quail. All sections included the connective tissue of the outer perichondrium and some skeletal muscle. Sections were dried onto slides (30 min, 4°C) and some were treated with bovine testicular hyaluronidase (1%, Type I, Sigma Chemical Co., St. Louis, MO) dissolved in 0.01 M PBS, pH 7.2, for 30 min at room temperature (24). After washing (0.01 M PBS, pH 7.2), sections were treated with primary antibody (30 min, room temperature). The primary antibodies were present in the undiluted culture supernatants from hybridoma 2C2 against HMW and, as a control, from hybridoma II₆B₃(15A₄) against chicken type II collagen (25). After washing in PBS, sections were exposed to a fluorescein-conjugated IgG fraction of goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) diluted 1:40 in PBS. After 30 min, sections were washed extensively in PBS, mounted with a coverslip and a drop of 90% glycerol, and observed in a Leitz Ortholux II microscope equipped for epifluorescence. Photographs were taken with Kodak Tri-X film rated at ASA 1,600. Control sections either did not receive a primary antibody, or antibody IA8 was used which recognizes an epitope in the (F1)₂F2 domain of type IV (basement membrane) collagen (21). Controls consistently gave no fluorescent staining of hyaline cartilage.

Rotary Shadowing of Collagens Secreted by Suspension

Cultures of Chick Chondrocytes: Approximately 80–100 sterna were dissected from 17-d chick embryos and incubated for 2 h at 37°C in basal salts solution (50 ml) containing collagenase (CLS, 1 mg/ml, Worthington Biochemical Corp., Freehold, NJ) and trypsin (2.5%, 5 ml, Gibco Laboratories, Grand Island, NY) in an atmosphere of 95% O₂:5% CO₂. Cells were liberated from the matrix by repeated pipetting and isolated by filtration through a double layer of lens paper. The cells were washed with Eagle's minimum essential medium (four times) containing 10% fetal calf serum, 1% antibiotic/antimycotic mixture and 1% L-glutamine (all from Gibco Laboratories). The cells were washed once in the same solution with the serum reduced to 1%, resuspended in the low serum solution (20 ml), counted, and incubated at 37°C for 2 h in an atmosphere of 95% O₂:5% CO₂. Cells were centrifuged from the medium, the medium cooled to 4°C, and protease inhibitors added. The final concentration of protease inhibitors was 0.2 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 2.5 mM EDTA and 0.25 mM ϵ -aminocaproic acid. A saturated solution of ammonium sulfate was added to give a final concentration of 30% saturation and the mixture allowed to stand at 4°C overnight. The precipitate obtained after centrifugation (48,000 g, 1 h) was dissolved in 0.2 M ammonium bicarbonate (2 ml) and dialyzed against several changes of this solution before spraying for rotary shadowing. In some experiments, a solution of monoclonal antibody 2C2 was added before dialysis to give a final concentration of 12.5 $\mu\text{g}/\text{ml}$.

¹ *Abbreviation used in this paper:* ELISA, enzyme-linked immunosorbent assay.

RESULTS

Antibody Isolation and Characterization

Initially, two hybridomas (designated 2C2 and 4D6) were selected which secreted monoclonal antibodies of high titer against HMW in ELISAs. Ascites fluid was prepared from each hybridoma and the secreted antibodies were purified by ammonium sulfate precipitation (50% of saturation), followed by elution from an HMW affinity column. Further characterization of the antibodies showed that 2C2 and 4D6 were both of the subclass IgG1.

Fig. 1 shows an inhibition ELISA performed with 2C2 and a variety of collagen types. Inhibition was only observed with HMW and not with collagen types I, II, III, IV, and V or with LMW. In separate ELISAs type X collagen also did not inhibit the binding of 2C2 to HMW (data not shown). For antibody 4D6, inhibition ELISAs could only be performed successfully at high concentrations of HMW. These results suggest that 4D6 has a relatively weak affinity for HMW, and subsequent experiments were performed largely with 2C2.

Location of the Epitope for 2C2 in HMW

Fig. 2A shows an inhibition ELISA performed with HMW which had first been heated to 55°C for 30 min in inhibition buffer. This temperature was previously shown to denature the collagen triple helix of HMW (5). However, the ability of HMW to inhibit remained unaffected. One possible explanation for this result is that the epitope for 2C2 is not dependent on the native conformation of HMW. The individual chains of HMW were then isolated and tested by inhibition ELISAs, but little or no inhibition was observed (Fig. 2B). A second, more likely, possibility is that during cooling of HMW after denaturation, the triple helix of HMW renatured and at the same time the epitope was restored. Previously, it was shown by both rotary shadowing (2) and by optical rotation (5) that only the long arm of HMW renatures after cooling HMW. These results therefore suggest that the epitope for 2C2 may

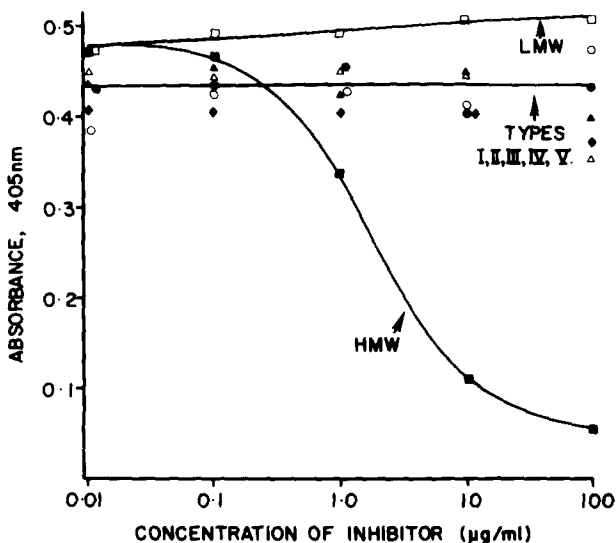


FIGURE 1 An inhibition ELISA using antibody 2C2 and various concentrations of native collagens (abscissa) as inhibitors. The upper solid line shows the lack of inhibition by collagen types I (Δ), II (\blacktriangle), III (\circ), IV (\bullet), and V (\blacklozenge). LMW (\square), which is a separate component of the type IX molecule (10), also showed no inhibition. Strong inhibition was, however, observed with HMW (\blacksquare).

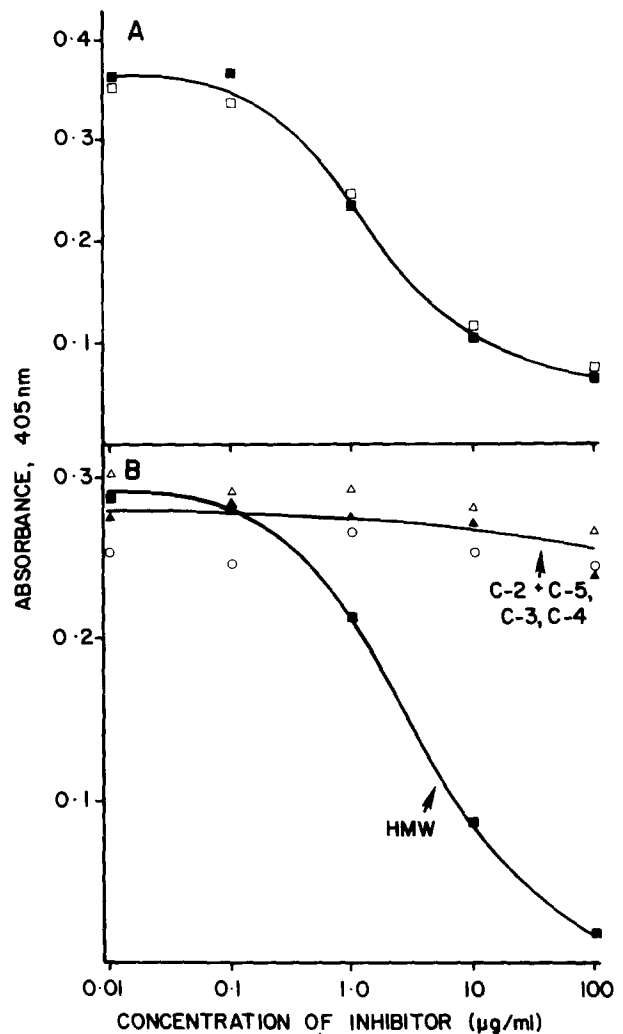


FIGURE 2 (A) Inhibition ELISA performed with HMW in which one-half of the sample was heated to 55°C for 30 min prior to incubation with 2C2. HMW unheated (\blacksquare), HMW heated (\square). Note that prior denaturation of HMW did not affect its ability to inhibit during ELISA. (B) Inhibition ELISA performed with peptides of HMW isolated after reduction and alkylation of interchain disulfide bridges, and subsequently fractionated by molecular sieve chromatography. The peptides used were a mixture of C-2 and C-5 chains (Δ), C-3 (\blacktriangle), and C-4 (\circ). Little or no inhibition was observed with any of the peptides.

be located in the long arm of HMW.

Direct evidence for the location of the epitope for 2C2 was obtained by rotary shadowing of mixtures of HMW and 2C2 purified from ascites fluid (Fig. 3A). Antibody 2C2 was consistently observed binding close to or at the end of the long arm of HMW (arrows). To demonstrate that this binding did not arise from a nonspecific interaction of antibody 2C2 with HMW, rotary shadowing was also performed with antibody AB12 previously prepared against type V collagen (23). Antibody AB12 was chosen as a control since it is also of the IgG1 subclass (23). No specific binding of AB12 to any site along the HMW molecule could be observed (data not shown). Rotary shadowing was also performed with antibody 4D6 which was isolated from ascites fluid and subsequently eluted from an HMW affinity column. For antibody 4D6, specific binding occurred to the end of the short arm of HMW (Fig. 3B, arrows). A second, shorter population of molecules

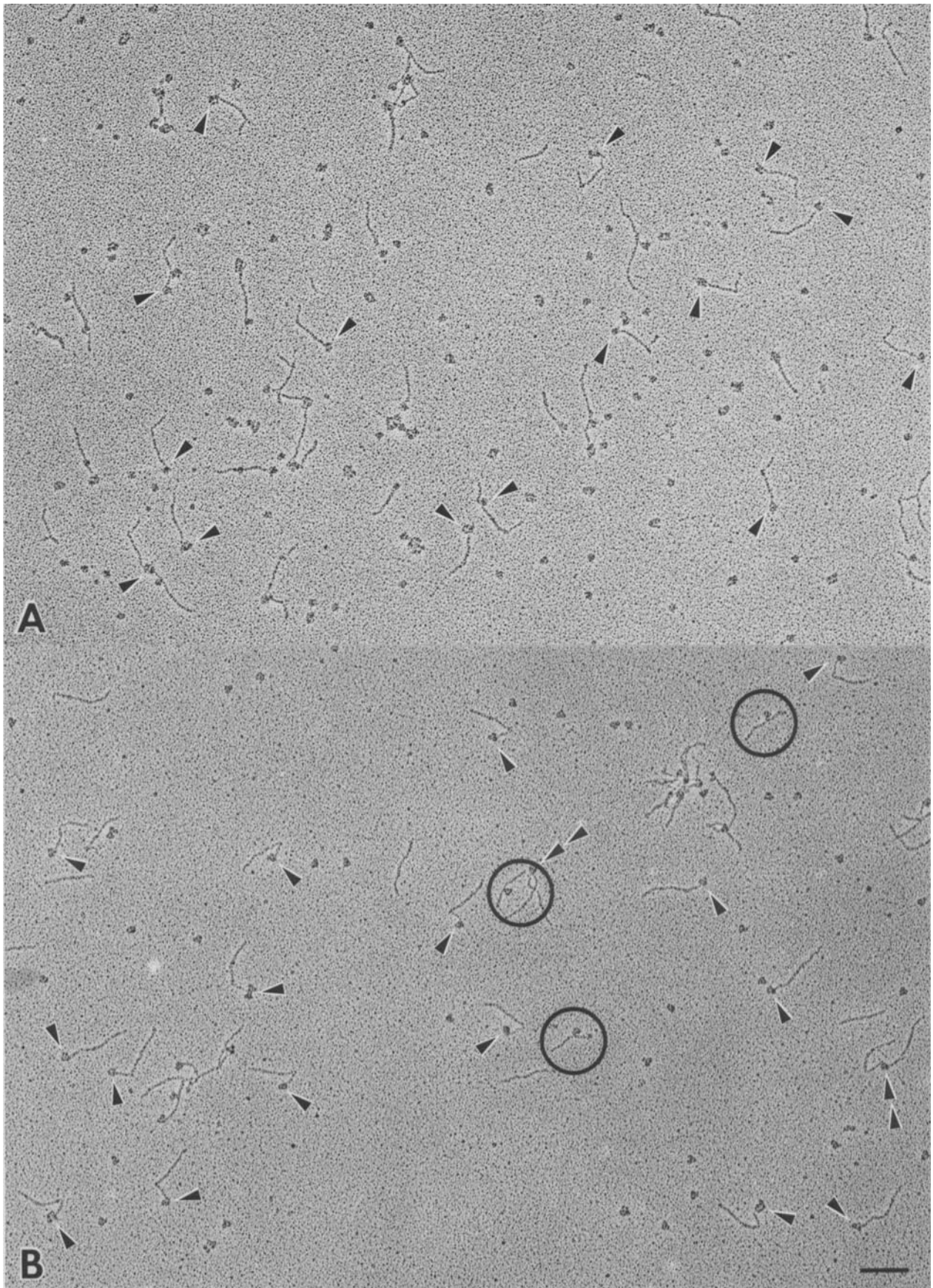


FIGURE 3 Electron micrographs after rotary shadowing of antibodies 2C2 (A) and 4D6 (B) interacting with the fragment of type IX collagen called HMW. The antibody ($25 \mu\text{g/ml}$) and the antigen ($25 \mu\text{g/ml}$) were mixed together and dialyzed overnight against 0.2 M ammonium bicarbonate ($\text{pH } 7.9$) before addition of glycerol (40%). The mixture was sprayed onto freshly cleaved mica and shadowed with platinum at 9° followed by carbon at 90° . Note that in A (single arrowheads) antibody 2C2 binds to the end of the long arm of HMW whereas in B (single arrowheads) antibody 4D6 binds to the end of the short arm of HMW. Circles indicate three molecules in B for which the short arm of HMW has apparently bent back onto the long arm. Double arrows indicate antibodies which appear to be cross-linking two HMW molecules. Bar, 100 nm . $\times 89,000$.

was also observed which exhibited antibody binding close to the center of the molecule (Fig. 3*B*, circles). The probable explanation for the structure of these latter molecules is that the short arm of HMW has folded back onto the long arm of HMW. Occasionally, antibody molecules were also observed which appeared to cross-link two HMW molecules (double arrows). These results show that, although antibody 4D6 binds weakly to HMW as judged by the inhibition ELISA, the affinity of 4D6 for HMW nevertheless is such that the location of its epitope can be visualized by rotary shadowing.

A total of three antibodies (2C2, 4D6, and AB12) were therefore examined by rotary shadowing for binding to HMW. Of these, only antibody 2C2 bound to the end of the long arm of HMW, and this result therefore strongly suggests that this is the location of the epitope for 2C2, and that the result is not due to nonspecific binding. Quantitation of the binding of 2C2 to HMW was achieved by analysis of the individual molecules of HMW that could be distinguished after rotary shadowing. Examples of four major populations of molecules that were observed are illustrated in Fig. 4. Row *A* illustrates a population of molecules that lacked the presence of an antibody. Sometimes a kink could be recognized in these molecules between the short and long arms of HMW. In row *B*, molecules of similar length are illustrated but with an

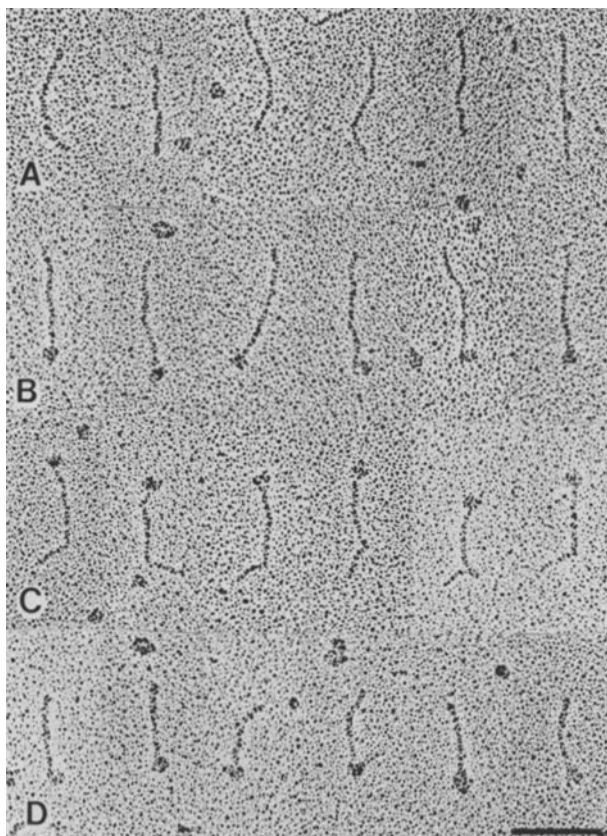


FIGURE 4 Gallery of selected molecules of HMW after rotary shadowing in the presence of monoclonal antibody 2C2 as described for Fig. 3. Row *A* shows molecules without attached antibody; Row *B* shows molecules with antibody bound at one end but without a prominent kink; Row *C* shows molecules with a kink and with an antibody molecule bound at the end of the long arm of HMW; Row *D* shows shorter molecules with antibody bound at one end. Close examination of several of the molecules in row *D* suggests that the short arm of HMW is bent back onto the long arm. Bar, 100 nm. $\times 119,000$.

antibody bound to one end, and in row *C* molecules are shown with a kink and an antibody bound near the end of the long arm of HMW. In row *D*, a second shorter population of molecules is shown with an antibody bound to one end. It is probable that for many, if not all, of these shorter molecules the short arm of HMW has folded back onto the long arm of HMW. Indications of this can be observed for some of the molecules illustrated in row *D*. This conclusion is also supported by the shorter molecules observed in Fig. 3*B* (circled molecules) in which the binding of antibody 4D6 is at the expected location if the short arm of HMW has folded back onto the long arm.

Of a total of 176 molecules examined, two populations of molecules could be recognized regardless of their ability to bind antibody: long molecules of 101–146 nm (72% of total, mean length = 125 nm, SD, ± 8 nm) and shorter molecules of 70–100 nm (28% of total, mean length = 87 nm, SD, ± 8 nm). The differences between the length of the long and short molecules is 38 nm and this is the same as the length previously determined for the short arm of HMW (2). This result, therefore, provides additional evidence that the short molecules of HMW arise from the folding back of the short arm onto the long arm. Of the total molecules examined (long and short), 136 (77%) possessed an antibody located at one end of the molecule, whereas only 32 (18%) possessed an antibody located anywhere else along the molecule. Of the long molecules, 86 (68%) possessed a recognizable kink and, of these 86 molecules, 69 (80%) possessed an antibody located at the end of the long arm. Only 17 (20%) possessed an antibody located elsewhere along the molecule. These results demonstrate that a very high proportion of HMW molecules bind 2C2 at only one site and, by examining molecules which display a kink, the binding site can be localized close to or at the end of the long arm of HMW.

The lengths of several different populations of long molecules as illustrated in Fig. 4 were also measured. For those molecules which did not possess an antibody binding at one end (20 molecules, Fig. 4*A*), the average length was 136 nm (SD, ± 8 nm), this value agreeing closely with our previous value of 134 nm for the length of HMW (2). This measurement was made regardless of whether or not a kink could be recognized for each molecule. Measurements of long molecules without a kink but with an antibody located at one end (25 molecules, Fig. 4*B*) gave a value of 123 nm (SD, ± 6 nm), this being shorter than the measurement of molecules without an antibody at the end (136 nm). It therefore appears that the antibody may shield a part of HMW during rotary shadowing as measurements were made only up to where the binding of the antibody to HMW could first be recognized. Measurements of molecules with a kink and with an antibody at the end of the long arm (86 molecules, Fig. 4*C*) gave values of 86 nm (SD, ± 10 nm) and 39 nm (SD, ± 7 nm) for the long and short arms, respectively. The value of 39 nm agrees well with the value of 38 nm previously determined for the length of the short arm of HMW (2). However, the value of 86 nm is less than the value of 96 nm previously determined for the length of the long arm (2) and again suggests that the antibody may be shielding a small part of the long arm. The sum of the long and short arms (125 nm) agreed well with the length of 123 nm determined for molecules without a kink but with an antibody binding to one end. These measurements therefore all suggest that the epitope for 2C2 may be located at a short distance (10–15 nm) from the noncolla-

genous carboxy-terminus of HMW.

Fig. 5 shows immunofluorescent staining of 18-d chick embryo sternum with monoclonal antibody II₆B₃(15A₄) against type II collagen (*A* and *B*) and monoclonal antibody 2C2 against type IX collagen (*C* and *D*). *A* shows that without hyaluronidase treatment, immunofluorescent staining for type II collagen was present only at the outer edges of the cartilage matrix close to the perichondrium. However, after hyaluronidase treatment (*B*), fluorescent staining was observed throughout the cartilage matrix. These results were the same as previously reported for immunofluorescent staining of chick cartilage with a polyclonal antibody against type II collagen (24). For antibody 2C2, without hyaluronidase treatment, some immunofluorescent staining for type IX collagen was observed throughout the cartilage matrix (*C*). However, after hyaluronidase treatment, fluorescent staining was much brighter and of equal intensity throughout the matrix (*D*). In all sections, fluorescent staining was not present in the outer connective tissue of the perichondrium, nor in the skeletal muscle associated with the sternal cartilage.

Monoclonal antibodies against type II and type IX collagens were both found to cross-react with quail cartilage. Fig. 6 shows immunofluorescent staining of adult quail sternum with antibody II₆B₃(15A₄) against type II collagen (*A* and *B*) and antibody 2C2 against type IX collagen (*C* and *D*). Sections

shown in *B* and *D* were treated with hyaluronidase prior to immunofluorescent staining. For type II collagen, immunofluorescent staining was observed only in the outer cartilage unless the sections were first treated with hyaluronidase (comparison of *A* and *B*). For type IX collagen, without hyaluronidase treatment, fluorescent staining occurred both in the outer cartilage and also in a pericellular location around the inner chondrocytes (*C*). However, after hyaluronidase digestion, fluorescent staining occurred with equal intensity throughout the cartilage matrix and was the same as the staining pattern for type II collagen. No staining for type II or type IX collagen was observed for the outer connective tissue or for the skeletal muscle attached to the sternum. Very similar results with both antibodies were also obtained for adult chicken sternum. However, antibody 2C2 did not show fluorescent staining of adult rat or guinea pig xiphoid process or the matrix of a transplantable rat chondrosarcoma (data not presented). With antibody 4D6, immunofluorescent staining patterns identical to those for 2C2 were observed with and without hyaluronidase digestion for chick embryo sternum and adult quail sternum.

The results for both embryonic and adult sterna suggest that without hyaluronidase digestion type IX collagen is more readily accessible to antibody than type II collagen, especially in the central region of the embryonic sternum and in a

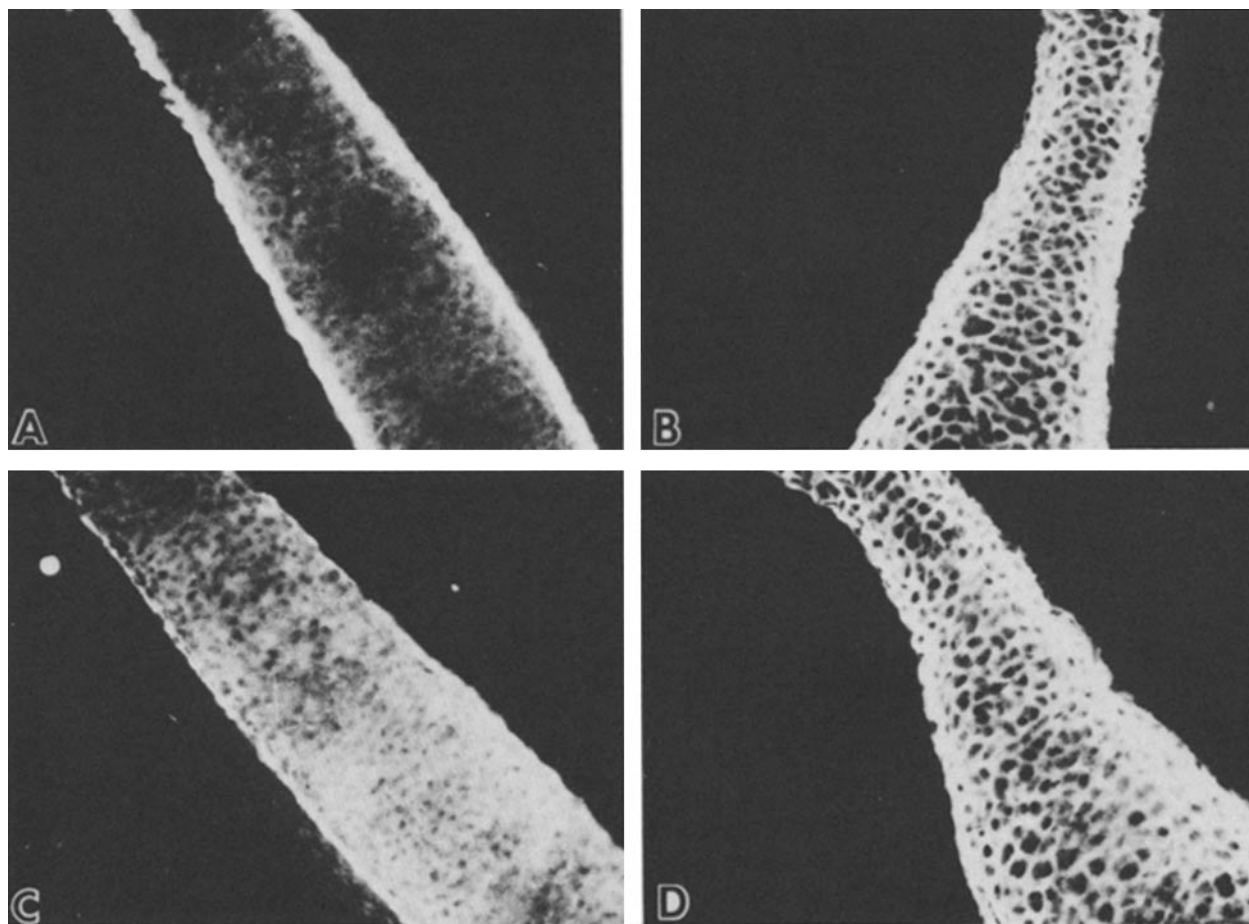


FIGURE 5 Indirect immunofluorescent staining of sections of chick sternal cartilage from 18-d embryos with monoclonal antibody II₆B₃(15A₄) against type II collagen (*A* and *B*) and monoclonal antibody 2C2 against type IX collagen (*C* and *D*). Sections in *B* and *D* were treated with hyaluronidase (1%) prior to immunofluorescent staining. Note that fluorescent staining for both type II and type IX collagen was present throughout the sternum only if the glycosaminoglycans were first removed with hyaluronidase. $\times 234$.

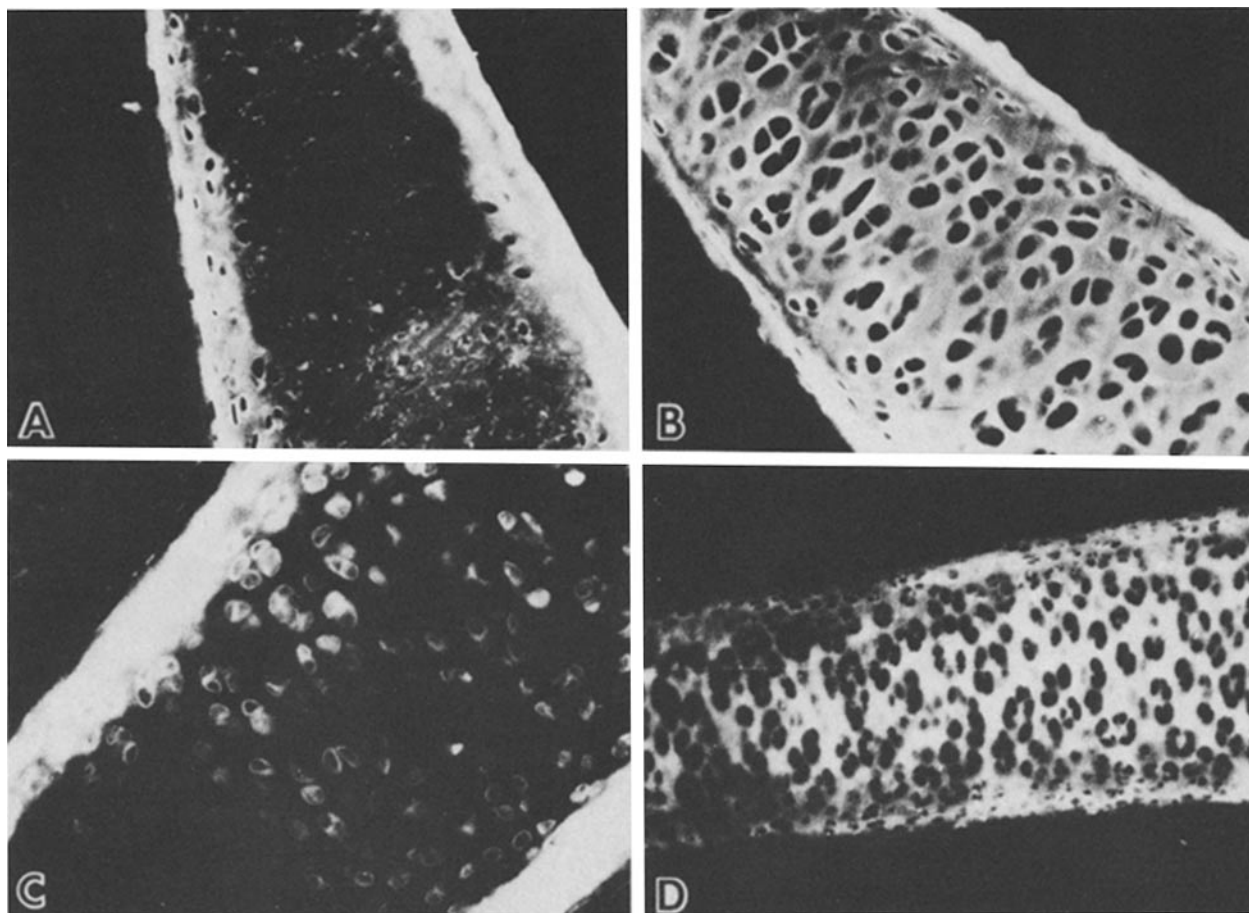


FIGURE 6 Indirect immunofluorescent staining of sections of adult quail sternal cartilage with antibody $11_6B_3(15A_4)$ against type II collagen (A and B) and antibody 2C2 against type IX collagen (C and D). Sections in B and D were treated with hyaluronidase (1%) prior to immunofluorescent staining. Note that without hyaluronidase treatment immunofluorescent staining for both type II and type IX collagen was predominantly at the outer surface of the sternum (A and C). For type IX collagen (C), some pericellular fluorescent staining was also observed. After hyaluronidase treatment, fluorescent staining for both type II and type IX collagen was present throughout the cartilage matrix. (A–C) $\times 375$; (D), $\times 234$.

pericellular location around the chondrocytes of the adult sternum. However, after hyaluronidase digestion, the fluorescent staining patterns for type II and type IX collagens were the same and indicate an equal distribution of the two collagens throughout the cartilage matrix.

Experiments were performed to examine the binding of 2C2 to the intact type IX collagen molecule secreted by a suspension culture of freshly isolated chondrocytes. Two populations of molecules were recognized in the ammonium sulfate precipitate of the medium and selected examples of these molecules are illustrated in Fig. 7. The longer molecules (rows A and B) possessed a prominent knob at one end and a sharp kink at the opposite end (arrows) and were identified as type II procollagen, although a few of these molecules may also be a procollagen form of $1\alpha,2\alpha,3\alpha$ collagen (1). A similar sized knob was previously observed at the carboxy-terminus of type I procollagen after rotary shadowing (26), and it is known that the carboxy-terminal propeptide of type II procollagen is similar in size and structure to the propeptides of type I procollagen (27, 28). The overall length of the type II procollagen molecules including the knob was 335 nm (SD, ± 12 nm, 91 molecules measured); this compares closely with the published value of 330 nm for type I procollagen (26). The kink is probably located at a short noncollagenous do-

main containing the amino-terminal (NH_2 -terminal) propeptidase cleavage site. A kink at a similar location was previously observed for type I pN-collagen (29, 30). However, the NH_2 -terminal propeptide of chick and rat type II procollagen differ from the NH_2 -terminal propeptide of type I collagen in lacking a large globular domain and containing a longer collagenous domain (31, 32). This explains why we were unable to observe a globular domain at the amino terminus of type II procollagen after rotary shadowing as can be observed for type I pN-collagen (29, 30). The presence of a longer collagenous domain for type II procollagen also explains why the length we observe from the kink to the amino-terminus appears longer than for the type I pN-collagen molecules previously examined by rotary shadowing (29).

For type II procollagen molecules, the possible binding of antibody 2C2 was analyzed after rotary shadowing; of 42 type II procollagen molecules examined only 12 showed an antibody apparently binding anywhere along the molecule. The length from each antibody to the knob of the carboxy-terminus was measured, and the results showed that there was no consistent binding of antibody 2C2 to any site along the procollagen II molecule (data not presented).

A second population of shorter molecules was also prominent among the collagens secreted by short-term cultures of

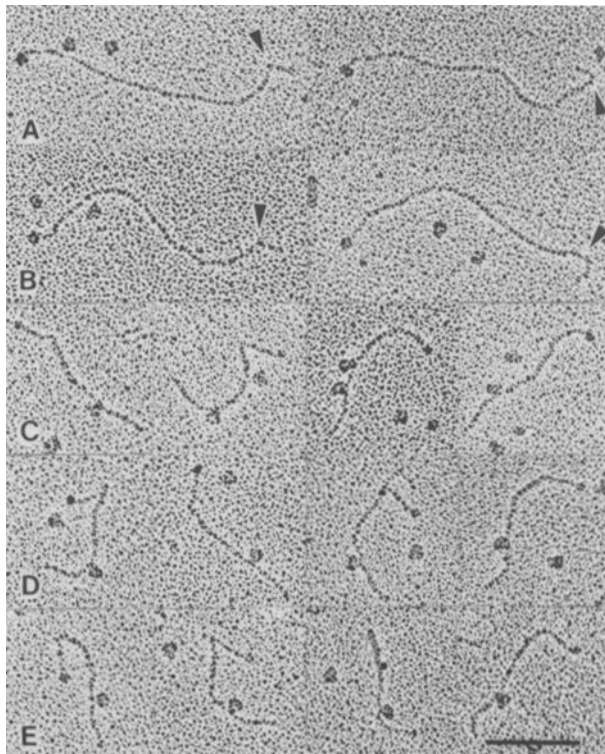


FIGURE 7 Electron micrographs of representative collagen molecules secreted by embryonic chick chondrocytes and observed after rotary shadowing in the presence of monoclonal antibody 2C2. Freshly isolated embryonic chondrocytes were incubated for 2 h and the collagens precipitated from the medium with ammonium sulfate. The precipitate was dissolved in 0.2 M ammonium bicarbonate and rotary shadowing performed in the presence of antibody 2C2 (12.5 $\mu\text{g/ml}$) after extensive dialysis against 0.2 M ammonium bicarbonate. Rows A and B show type II procollagen molecules with a kink (arrows) at the amino-terminus and a knob at the carboxy-terminus. Rows C–E show type IX molecules with a smaller knob at the amino-terminus and a characteristic kink located 47 nm from the knob. Note that monoclonal antibody 2C2 consistently bound to a site which was located 136 nm from the knob. Occasionally, antibody molecules were randomly located at other sites along the molecule. The angle of the kink was not fixed, and was not observed in all molecules. Bar, 100 nm. $\times 124,000$.

chick chondrocytes. A series of representative molecules is shown in Fig. 7, rows C–E. Each molecule possessed a small knob at one end, and the total length of the molecules including the knob was 190 nm (SD, ± 8 nm, 112 molecules measured). In addition, a prominent kink was consistently present in 95% of the molecules at a distance of 47 nm (SD, ± 5 nm, 106 molecules examined) from the knob. In a preparation which contained antibody 2C2, of 92 molecules examined, 81 (88%) showed an antibody molecule binding at a site located 136 nm (SD, ± 6 nm) from the knob. Only 20 of the molecules (22%) showed a 2C2 antibody molecule located elsewhere in a random manner along the type IX molecule. For the intact type IX molecule, the length from the kink to the antibody was 89 nm, this comparing closely to the previous value of 86 nm for the binding of antibody to HMW. The length from the antibody to the carboxy-terminus of the intact type IX molecule was 54 nm of which it can be calculated that only 30–35 nm is represented by LMW. These results therefore also suggest that antibody 2C2 may not bind

to the carboxy-terminus of HMW but binds at a short distance (10–15 nm) from the terminus.

Rotary shadowing of the intact type IX molecule was also performed in the presence of antibody 4D6 but only a few antibody molecules were observed at the expected site close to the knob (unpublished observations). It therefore appears that 4D6 may be recognizing an epitope present within the pepsin-generated amino terminus of HMW, but this epitope may not be readily available in the intact type IX molecule.

These results demonstrate that the structure of the type IX molecule as predicted from the nucleotide sequence of the cDNA clones pYN1738 (9–11) and pYN1731 (12) is entirely consistent with the structure of the type IX molecule as secreted by chick chondrocytes. The knob must represent the noncollagenous amino-terminal domain called NC4 and the kink is located at the NC3 domain between the collagenous domains COL3 and COL2, or between the short and long arms of HMW. The extension of the intact type IX molecule beyond the binding site for antibody 2C2 as observed in Fig. 7 must be largely represented by the collagenous domain COL1 or LMW. From the nucleotide sequences of the cDNA clones pYN1738 and pYN1731 (11, 12) it can be deduced that there is only a small noncollagenous domain at the carboxy-terminus of the intact type IX molecule and this would not be large enough to be visualized after rotary shadowing. The angle of the kink between COL3 and COL2 at NC3 was variable and molecules were often observed in which COL3 was bent back completely onto COL2 (Fig. 7, row E, third molecule from left). At no time were type IX molecules observed which interacted with each other to form a structured aggregate.

DISCUSSION

Although the overall structure of type IX collagen is now well understood (1–12), the function of this collagen in cartilage matrix is still unclear. To achieve high resolution electron microscopic immunolocalization of type IX collagen in cartilage matrix, we have begun to prepare a series of monoclonal antibodies directed against different epitopes of the type IX molecule. Of the two antibodies initially selected, only 2C2 bound to HMW with high affinity, and only with this antibody could the location of its epitope be recognized after rotary shadowing of both HMW and the intact type IX molecule. Antibody 2C2 is therefore the only one which fulfills all of our desired properties, and it will be used predominantly for future experiments.

Our present rotary shadowing observations (Fig. 3A) show that the epitope for 2C2 is located at or close to the carboxy-terminus of HMW and could therefore be located within the noncollagenous NC2 domain of the intact type IX collagen molecule. At present, we do not know how much of the NC2 domain remains after pepsin digestion to produce HMW. Sequencing of cDNA clones pYN1738 and pYN1731 shows that the total number of amino acids in NC2 is thirty for both the $\alpha 1(\text{IX})$ and $\alpha 2(\text{IX})$ chains (10, 12). However, amino acid sequencing shows that some of these amino acids are located at the amino termini of the three chains of LMW (9). Within the NC2 domain the $\alpha 1(\text{IX})$ chain contains a single cysteine (10), whereas the $\alpha 2(\text{IX})$ chain is without cysteine (12). It is not known if this cysteine forms a disulfide bridge to the $\alpha 3(\text{IX})$ chain or if it is present in HMW. The other possibility is that the epitope for 2C2 is located within the triple helix of

COL2 but at a site very close to the carboxy-terminus of HMW. The antibody may then shield the carboxy-terminus of HMW during rotary shadowing. This would explain why, after denaturation of HMW, the epitope returned completely as judged by the inhibition ELISA (Fig. 2A). Previously, it was shown by both rotary shadowing (2) and by optical rotation (5) that the triple helix of the long arm of HMW rapidly renatured after HMW was denatured and subsequently brought to room temperature.

Several studies using polyclonal antibodies to fragments of type IX collagen have reported immunolocalization to the pericellular matrix of chondrocytes in adult pig articular cartilage (33), the epiphyseal cartilage of developing fetal calf long bones (34), and bovine nasal cartilage (35). Furthermore, electron microscopic immunolocalization with a secondary antibody coupled to peroxidase suggested that type IX collagen is present in fine nonstriated fibrils of the pericellular matrix (36). Similar fibrils have been observed in the pericellular capsule of adult canine articular cartilage and suggested to contain type IX collagen (37). In all of the above studies, proteoglycans were removed from the sections by digestion with hyaluronidase prior to staining. In our experiments, we performed similar digestion with hyaluronidase using sections of embryonic and adult sterna, and observed immunofluorescent localization of type IX collagen throughout the cartilage matrix (Figs. 5 and 6). The same distribution was also observed for type II collagen after hyaluronidase digestion but, without hyaluronidase treatment, no staining for type II collagen occurred in the center of the sternum. However, for embryonic sterna without hyaluronidase digestion, some immunofluorescent staining for type IX collagen was observed throughout the matrix (Fig. 5, C), and in adult sterna the staining was present in a pericellular location (Fig. 6, C). It therefore appears that type IX collagen may be more readily available for immunofluorescent staining than type II collagen. The pericellular staining observed previously by other investigators may be due to staining only of the fraction of the type IX collagen to which the antibody can penetrate, while the remainder of the type IX collagen continues to be masked.

Recently, a type IX collagen preparation was isolated from rat chondrosarcoma and examined by rotary shadowing (38). The molecule appeared to be somewhat shorter than reported here (171 vs. 190 nm) and sometimes showed a prominent kink towards one end. The noncollagenous knob of the amino-terminus was not present, and may have been lost either by an NH₂-terminal processing event or by nonspecific protease activity. For chick type IX collagen, the amino-terminus appears to be the most easily cleaved by pepsin (7), and may also be sensitive to other proteases. We have not observed the lateral end-to-end associations of two type IX molecules as were observed previously for rat chondrosarcoma type IX collagen (38).

The use of monoclonal antibodies combined with rotary shadowing may be applicable to analyses of the structure of several large collagenous molecules which cannot be readily isolated or fractionated. Previously, we prepared a series of monoclonal antibodies against different pepsin-resistant fragments of chicken type IV collagen, and used these antibodies combined with rotary shadowing to immunomap the pepsin-resistant fragments of tetramers of type IV collagen (20). In the present study, we have used a monoclonal antibody prepared against a fragment of type IX collagen to identify

the structure of the intact type IX molecule as secreted by freshly isolated chondrocytes. In the future, it seems probable that monoclonal antibodies prepared against protease-resistant fragments of other poorly characterized collagens will be used to analyze the structure of the intact molecule from which these fragments were derived.

The intact type IX molecule, as observed by rotary shadowing, fulfills all of the predictions of its structure from nucleotide sequencing of cDNA clones. Thus, from the sequence of pYN 1738, type IX would be expected to have a prominent noncollagenous domain only at the amino-terminus and not at the carboxy-terminus (11). In addition, kinks might be anticipated at specific sites along the molecule and at least one of the kinks at the NC3 domain can be observed in a high proportion of the molecules. The angle of this kink does not appear to be fixed, and it is still not known if the kink arises from cleavage of one of the chains by a specific processing event, or is due to a natural flexibility of the molecule at this site. For HMW, cleavage of only one of the chains is known to occur at this site (2, 10), but it is not known if this occurred during pepsin digestion or by an earlier cleavage *in vivo*.

Type IX collagen is clearly a unique member of the collagen family with several distinctive characteristics. Within the molecule there are three separate collagen triple helices, one of which (COL3 or the short arm of HMW) forms a very stable helix with a high content of proline and hydroxyproline (2, 11), and an elevated melting temperature (5). The functional significance, if any, of the COL3 domain is at present unknown. Another unique feature of type IX collagen is the apparent covalent linkage of chondroitin sulfate to at least one of the chains (39), and type IX collagen is probably related to proteoglycan light (40, 41). It is tempting to speculate that type IX collagen may interact with type II collagen and be involved in the organization of the branching network of type II collagen fibrils present in hyaline cartilage. It is easy to see how such a molecule with several collagenous domains between which are very flexible noncollagenous domains could easily be involved in organizing such a network. In addition, the chondroitin sulfate chains present in type IX collagen may be able to interact with the large cartilage proteoglycans present between the collagen fibrils. With the preparation of a monoclonal antibody against type IX collagen for which the location of the epitope is precisely known, it may be possible to localize type IX collagen in cartilage matrix at high resolution, and to obtain some evidence in support of the above speculations.

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