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The Structure of a Small Collagenous Fragment Isolated From Chicken Hyaline Cartilage

Richard Mayne, Michel van der Rest, Darrel C. Weaver, and William T. Butler

Departments of Anatomy (R.M., D.C.W.) and Biochemistry (R.M., W.T.B.), University of Alabama Medical Center, Birmingham, Alabama 35294, and Genetics Unit, Shriners Hospital for Crippled Children (M.v.d.R.), Montreal, Quebec, Canada H3G 1A6

In previous experiments, two collagenous fragments were isolated from pepsin digests of chicken hyaline cartilage and called the high molecular weight, (HMW) and low molecular weight (LMW) fractions [3]. In the present experiments, the chains of LMW were isolated after denaturation and subsequent reduction and alkylation of interchain disulfide bridges and were further fractionated by carboxymethyl-cellulose chromatography. Four peaks were resolved during chromatography and were designated LMW 1, 2A, 2B, and 3. Amino acid analyses and peptide mapping after cleavage with trypsin, V8 protease, and cyanogen bromide showed that three genetically distinct chains must be present in LMW. Fractions 2A and 2B were very similar, but not identical, in structure. LMW 1, 2A plus 2B, and 3 were consistently isolated in approximately equal proportions, suggesting that the probable chain organization of LMW is [1][2A+2B][3].This suggestion was supported further by experiments that attempted to fractionate LMW by carboxymethyl-cellulose chromatography after denaturation but without reduction and alkylation of interchain disulfide bridges. No fractionation of LMW was achieved, the single peak subsequently being shown to contain LMW 1, 2A plus 2B, and 3.

Key words: HMW, LMW, minor cartilage collagens, chain composition

Recent studies show that hyaline cartilages contain a variety of minor collagens in addition to the major component of type II collagen [reviewed in 1]. Among these include the native form of the 1α, 2α, and 3α chains [2,3,4] and a low molecular weight collagen that is synthesized exclusively by hypertrophic chondrocytes and is called G collagen [5,6] or short chain (SC) collagen [7,8]. In addition, two collagenous components were isolated from pepsin digests of *chicken* sternal cartilage and called HMW and LMW [3,9], or M1 and M2 [10]. These fractions both contain interchain disulfide bridges, and a detailed model was presented earlier for the

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structure of HMW [9]. It appears that one of the chains of HMW is cleaved during pepsin digestion, this giving rise to a characteristic kink in the molecule as observed after rotary shadowing. Somewhat different fragments were isolated from pepsin digests of *mammalian* cartilage, and it appears that type M collagen [11] or C-PS1 [12] probably represent the long arm of HMW in which an additional cleavage by pepsin has occurred at the kink region [9]. The fragment LMW appears very similar to fragments isolated from mammalian cartilages and called CF2 [13], C-PS2 [14], or X4 [15]. This paper presents a further analysis of the structure of LMW and reports the isolation and characterization of three genetically distinct chains.

MATERIALS AND METHODS

Isolation and Fractionation of LMW

Chicken sterna (200g) were extracted initially with 4 M guanidine, 50mM Tris-HCl, followed by limited pepsin digestion, as described previously [3]. Differential salt precipitation was performed initially at 0.9 M NaCl, 0.5 M HAc, to precipitate type II collagen; subsequently HMW, LMW, and the native form of the 1α, 2α, and 3α chains were precipitated at 2.0 M NaCl, 0.5 M HAc [2,3]. Separation of LMW from HMW and the small amounts of the 1α, 2α, and 3α chains present in the 2.0 M NaCl precipitate was achieved by molecular sieve chromatography (Bio-Gel A-15m) without denaturation of the sample prior to chromatography, as described previously [9]. The LMW fraction after desalting and lyophilization was reduced with 2-mercaptoethanol (0.1 M) at 100°C for 2 min in 5 M urea 0.05 M Tris-HCl, pH 8.0, and alkylated with iodoacetic acid (0.2 M) at room temperature. The individual chains of LMW subsequently were isolated by molecular sieve chromatography on a column (2.5 × 150 cm) of agarose beads (Bio-Gel A-1.5m, 200-400 mesh). The column was eluted with 1 M $CaCl_2$, 50 mM Tris-HCl, pH 7.5, at a flow rate of 25.9 ml/hr.

Carboxymethyl-Cellulose Chromatography

Fractionation of the chains of LMW was achieved using a 1.5 × 10 cm column of CM cellulose (Whatman CM32), which was eluted at a flow rate of 100 ml/hr in 0.01 M (Na^+) sodium citrate (pH 3.6), with a linear gradient of 0.0 to 0.2 M NaCl over a total volume of 400 ml. In some experiments, CM-cellulose chromatography was performed with denatured LMW without reduction and alkylation. In these conditions, the column (1.5 × 10 cm) was eluted at a flow rate of 100 ml/hr in 0.01 M (Na^+) sodium citrate (pH 3.6) in the presence of 4 M urea with a linear gradient of 0.0 to 0.2 M NaCl over a total volume of 400 ml.

Polyacrylamide Gel Electrophoresis

Characterization of the chains of LMW initially fractionated by CM-cellulose chromatography was performed by $NaDodSO_4$-polyacrylamide gel electrophoresis using a 10–15% gradient slab gel, as described previously [3].

Amino Acid Analyses

Samples were hydrolyzed in 2 ml of constant-boiling HCl at 108°C for 24 hr and were analyzed with a Beckman 121M automatic analyzer using a dual column procedure, as described previously [16].

Cleavage With Trypsin and Fractionation of the Peptides

Peptides were dissolved in 0.2 M ammonium bicarbonate, pH 7.9, at a concentration of 1 mg/ml, and a solution of TPCK-trypsin (Worthington) in water was added to give a 1:30 enzyme:substrate ratio. After digestion (37°C, 4 hr), a drop of glacial acetic acid was added and the solutions were dried in a Speedvac concentrator (Savant). Fractionation of the tryptic peptides was performed by reversed phase HPLC, as described previously [17], the apparatus consisting of two 110-A pumps, a gradient controller model 421, a UV monitor model 160 equipped with a zinc lamp, and a C-RIB data system (all from Beckman). The column was a C18 Vydac TP 201 (4.6 × 250 nm) protected by a guard column of pellicular C18 resin (both from Waters). Each sample was dissolved in 9 mM trifluoroacetic acid (1mg/ml) before being injected onto the column, and elution was achieved with an aqueous linear gradient of acetonitrile (0–32%) over 90 min at 1 ml/min.

Cleavage With Staphylococcus Aureus V8 Protease and Fractionation of the Peptides

Peptides were dissolved in 0.2 M ammonium bicarbonate at 1 mg/ml and digestion (18 hr, 37°C) was performed with S aureus V8 protease (Miles) with an enzyme:substrate ratio of 1:20. After drying the reaction mixture, fractionation of the peptides was performed by HPLC as described above for tryptic peptides.

Cleavage With CNBr and Fractionation of the Peptides

The methionine sulfoxide residues of LMW first were reduced by the method of Adelstein and Kuehl [18]. Each chain was dissolved in 0.2 M NH_4HCO_3, pH 7.9, and incubated with 2-mercaptoethanol (25%, 22 hr, 45°C) in an atmosphere of nitrogen. After desalting and lyophilization, cleavage with CNBr was performed as described previously [3]. Fractionation of the peptides was performed by molecular sieve chromatography on a column (1.5 × 150 cm) of G-75 (Pharmacia) that was eluted with 0.2 M NH_4HCO_3, pH 7.9, at a flow rate of 7.3 ml/hr. Calibration of the column was achieved with a mixture of CNBr peptides derived from chicken type II collagen.

RESULTS

Initially, HMW and LMW were obtained from a pepsin digest of chicken sternal cartilage and fractionated from type II collagen by differential salt precipitation first at 0.9 M NaCl-0.5 M HOAc and subsequently at 2.0 M NaCl-0.5 M HOAc. Separation of HMW from LMW was achieved by molecular sieve chromatography (Bio-Gel A-15m) without prior denaturation of the sample [9], followed by desalting of the peaks of HMW and LMW and lyophilization. Figure 1 shows the further isolation of the individual chains of LMW by molecular sieve chromatography (Bio-Gel A-1.5m) after initial reduction and alkylation of disulfide bridges. In addition to the major peak of the individual chains of LMW, a minor peak also eluted earlier from the column. This latter peak was found to consist predominantly of dimers of the chains of LMW held together by nonreducible bonds (R. Mayne, unpublished observations). These bonds probably represent lysine-derived cross-links that have been reported to occur in type M collagen [19].

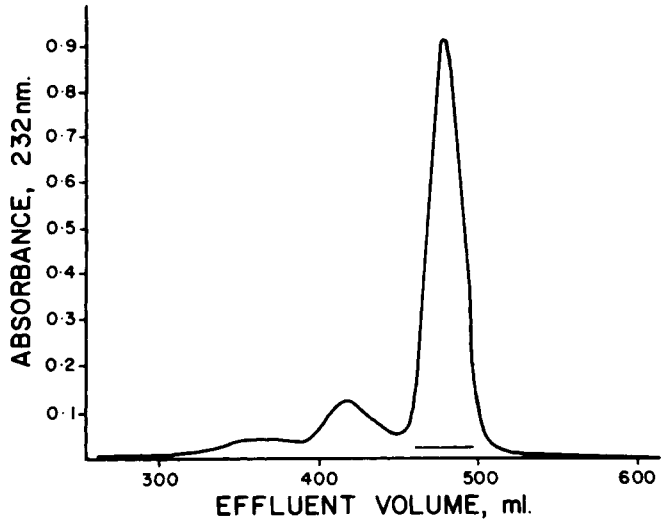

Fig. 1. Agarose (Bio-Gel A-1.5m) molecular sieve elution pattern of LMW after reduction and alkylation following an initial separation from HMW by molecular sieve chromatography (Bio-Gel A-15m). The column (2.5 × 150 cm) was eluted with 1 M $CaCl_2$ in 50 mM Tris-HCl, pH 7.5, at a constant flow rate. The sample (40 mg) was dissolved in 6 mL of 5.0 M urea, 0.05 M Tris HCl pH 8.0, reduced with 2-mercaptoethanol (0.1 M) at 100°C for 2 min and alkylated with iodoacetic acid (0.2 M) at room temperature before being applied directly to the column. Bar indicates the fraction of LMW which was desalted, lyophilized and further separated by CM-cellulose chromatography.

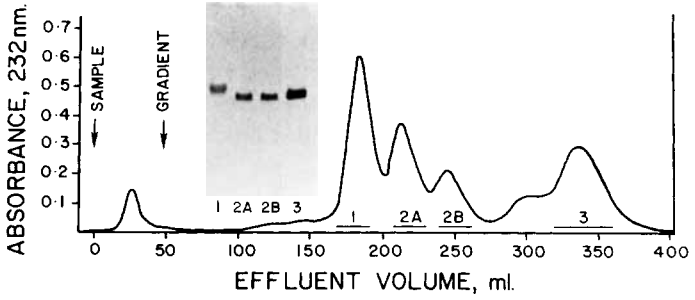

Fig. 2. CM-cellulose chromatography of the chains of LMW obtained after reduction and alkylation followed by agarose gel filtration.The column (1.5 × 10 cm) was equilibrated at 42°C with 0.01 M (Na^+) sodium citrate pH 3.6, and elution was achieved with a linear gradient of 0.0–0.2 M NaCl at a flow rate of 100 ml/hr over a total volume of 400 ml. Bars show the four fractions that were pooled for further analysis. The inset shows polyacrylamide gel electrophoresis of these fractions (10–15% gradient gel).

Figure 2 shows the further fractionation of reduced and alkylated chains of LMW by CM-cellulose chromatography. Four major peaks were obtained and designated LMW 1, 2A, 2B, and 3. A fifth smaller peak, which eluted between LMW 2B and 3, was found by amino acid composition and CNBr peptide mapping (see below) to be the same as LMW 3 and was not investigated further. Each fraction, after desalting and lyophilization, was characterized by polyacrylamide gel electrophoresis

(10–15% gradient gel) and found to consist of a single band (Figure 2, inset). LMW 1 was found consistently to migrate slightly slower than LMW 2A, 2B, and 3, which all migrated to the same position. This result suggests that LMW 1 may possess a slightly higher molecular weight than the other fractions, and this was supported by estimates of the apparent molecular weight of each fraction by gel filtration using a calibrated column of G-75. The estimated molecular weights for each fraction were:- LMW 1, M_r 11,100; LMW 2A, M_r 10,600; LMW 2B, M_r 10,500; LMW 3, M_r 10,600.

In different preparations of LMW, it was observed consistently that LMW 1, LMW 2A plus 2B, and LMW 3 were isolated in a proportion close to 1:1:1. This result suggests that LMW may exist predominantly as a single native molecule of chain organization [1] [2A+2B] [3]. Other evidence supporting such a chain organization includes a single sharp melting curve for LMW with a t_m of 35°C [20], and the migration of LMW as a single band on polyacrylamide gel electrophoresis without reduction [3]. In addition, LMW eluted as a single peak during CM-cellulose chromatography in denaturing conditions (42°C) but without reduction of interchain disulfide bridges (Fig. 3). This peak was desalted, reduced and alkylated, and shown to give rise to LMW 1, 2A, 2B, and 3 after CM-cellulose chromatography as described for Figure 1.

Amino acid analyses of each fraction of LMW are presented in Table I. All four fractions showed characteristic analyses for collagenous peptides with slightly less than one third of the residues being glycine. Marked differences were observed for the four fractions in the contents of aspartic acid, threonine, alanine, and arginine. LMW 2A and 2B showed similar contents of all amino acids except that LMW 2B consistently showed a slightly higher arginine content.

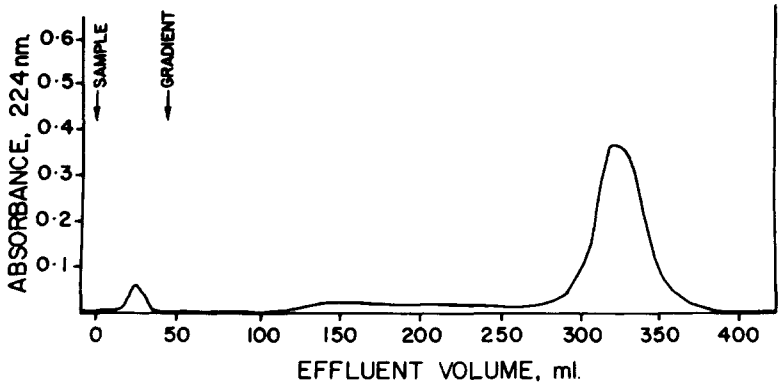

Fig. 3. CM-cellulose chromatography of LMW after denaturation but without reduction and alkylation. The column (1.5 × 10 cm) was equilibrated at 42°C with 0.01 M (Na^+) sodium citrate, pH 3.6 containing 4 M urea, and elution was achieved with a linear gradient of 0.0–0.2 M NaCl at a flow rate of 100 ml/hr over a total volume of 400 ml.

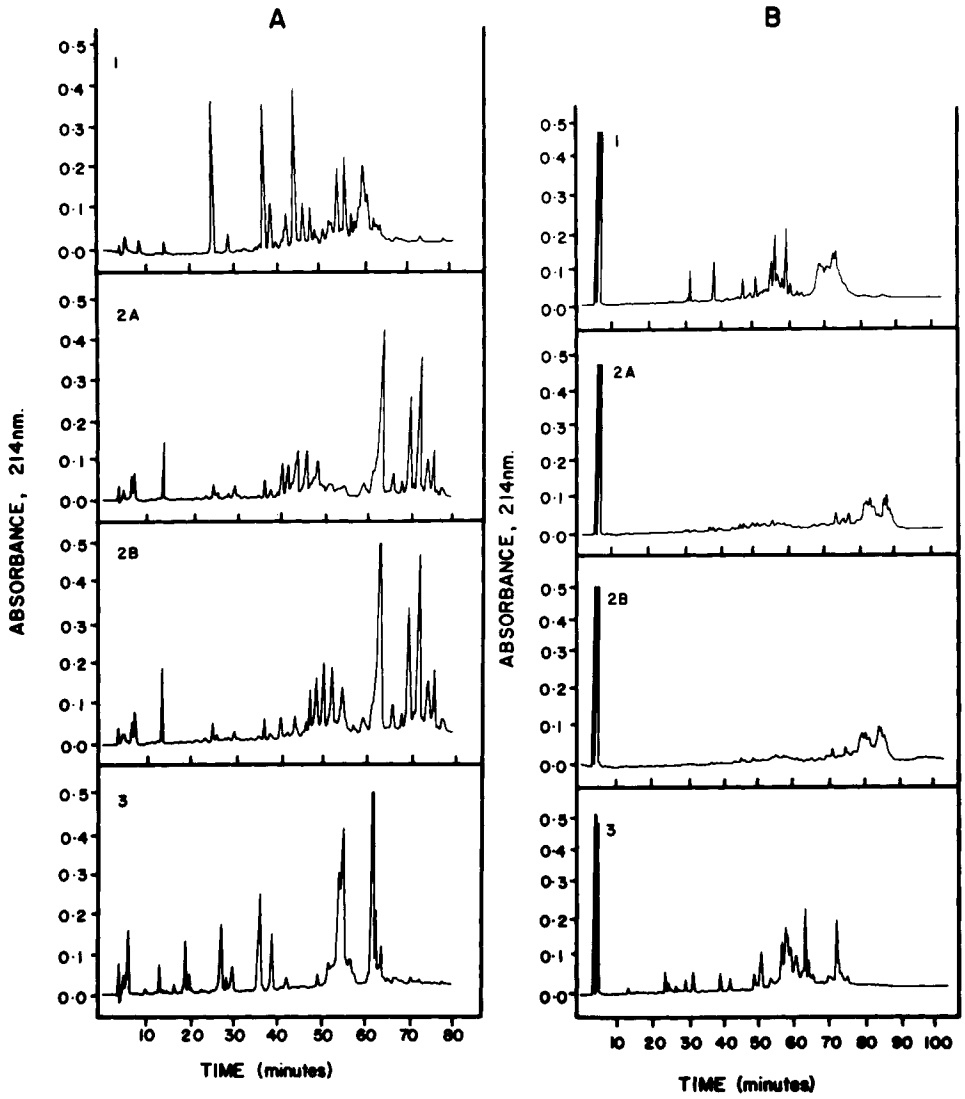

Fig. 4. Fractionation by reversed phase HPLC of (A) tryptic peptides. B) V8 protease peptides obtained from fractions 1, 2A, 2B, and 3 derived from LMW. For tryptic peptides digestion was performed at 37°C for 4 hr in 0.2 M NH_4HCO_3 (pH 7.9) with an enzyme:substrate ratio of 1:30. For V8 protease peptides, digestion was performed at 37°C for 18 hr in 0.2 M NH_4HCO_3 (pH 7.9) with an enzyme:substrate ratio of 1:20. After drying, each sample was dissolved in trifluoroacetic acid (9 mM) and fractionation performed on a column of C18 Vydac TP201 (4.6 × 250 nm) using an aqueous linear gradient of acetonitrile (0–32%) over 90 min at 1 ml/min. Note the marked differences in tryptic or V8 protease peptide patterns for LMW 1, 2A or 2B, and 3.

Figure 4A shows the tryptic peptides of LMW 1, 2A, 2B, and 3 after fractionation by reversed phase HPLC. Very different peptide patterns were observed for LMW 1, LMW 2A or 2B, and LMW 3, this result strongly suggesting that LMW contains at least three genetically distinct chains. For LMW 2A and 2B, the peptide

TABLE I. Amino Acid Compositions of Fractions Recovered After CM-Cellulose Chromatography*

Amino acid	Residues/100			
	LMW 1	LMW 2A	LMW 2B	LMW 3
3-Hyp	—	—	—	—
4-Hyp	9.6	13.5	12.3	15.6
Asp	8.4	5.0	4.4	1.2
Thr	5.2	1.5	1.2	—
Ser	3.2	2.0	1.8	2.1
Glu	6.7	6.5	6.0	10.0
Pro	10.1	6.3	8.0	10.7
Gly	30.2	32.7	31.7	30.0
Ala	7.3	8.3	9.1	4.3
1/2-Cys[a]	1.4	1.9	1.4	1.3
Val	2.6	2.3	2.4	1.9
Met	1.5	1.8	2.0	0.7
Ile	2.4	3.1	3.4	1.8
Leu	4.0	5.6	5.8	5.3
Tyr	0.6	0.2	0.2	1.3
Phe	—	0.7	0.6	1.9
Hyl	0.6	1.3	1.3	2.5
Lys	2.2	1.6	1.9	1.1
His	0.7	1.9	1.9	—
Arg	3.5	3.8	4.6	8.2

*Each analysis is expressed as residues/100. No corrections were made for loss of threonine or serine, or the incomplete release of valine.

[a]Determined as S-(carboxymethyl)cysteine. The values are the average of determinations made on four separate preparations.

patterns were very similar at both the start and the end of the gradient, but in the center of the gradient a family of five peptides was observed which for LMW 2B eluted slightly later in the gradient. Amino acid analyses were performed on each of the peaks obtained after reverse phase HPLC and, with this procedure, it was possible to identify many of the tryptic peptides for each of the four fractions (van der Rest M, Mayne R., unpublished experiments). Fractionation of the peptides obtained after cleavage with Staphylococcus aureus V8 protease also gave very different peptide patterns for LMW 1, 2A or 2B, and 3 (Figure 4B). However, for LMW 2A and 2B, the same peptide patterns were observed.

Figure 5 shows the separation of CNBr peptides from LMW 1, 2A, 2B, and 3 by molecular sieve chromatography using a column of G-75. For LMW 1 and 3, little if any cleavage was observed, although both of these peaks contain methionine (Table I). This result suggests either that the methionine residues in these peaks are located close to the amino- or carboxyl-termini or are poorly cleaved by CNBr even after prior reduction of methionine sulfoxides. For LMW 2A and 2B, extensive cleavage with CNBr occurred with the formation of three major peaks of apparent M_r 5400 (peak a), 3200 (peak b), and 2200 (peak c). It therefore appears that LMW 2A and 2B are similar in primary structure and contain at least two methionine residues that are centrally located.

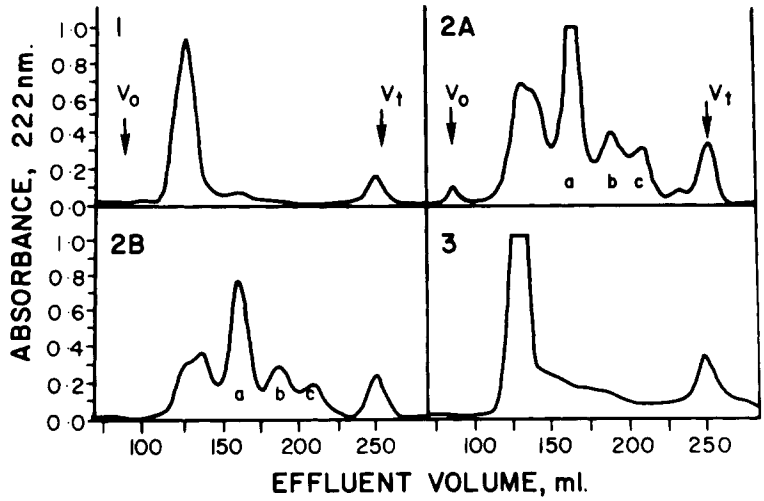

Fig. 5. Molecular sieve elution pattern (G-75) of the four fractions obtained from LMW after CM-cellulose chromatography followed by cleavage with CNBr. The column (1.5 × 150 cm) was eluted with 0.2 M NH_4HCO_3, pH 7.9, at a constant flow rate and the sample (1-2 mg) was dissolved in this solution. The peaks designated *a*, *b*, and *c* were derived from CNBr cleavage of peaks 2A and 2B and possessed apparent molecular weights of 5,400 3,200, and 2,200 respectively. Calibration of the column was achieved with a mixture of CNBr peptides derived from chicken type II collagen.

DISCUSSION

The present results show that after reduction and alkylation of disulfide bridges, it is possible to separate and partially characterize at least three genetically distinct chains from LMW. Each chain possessed an apparent molecular weight of 10,500–11,100. The individual chains were characterized by amino acid composition and by peptide mapping after cleavage with trypsin, V8 protease, and CNBr. In separate experiments, cleavage with clostripain also gave rise to different peptide patterns for LMW 1, 2A or 2B, and 3 (M. van der Rest and R. Mayne, unpublished observations). The difference between LMW 2A and 2B during CM-cellulose chromatography probably arises from differential cleavage with pepsin during the isolation procedures. Sequencing studies of LMW 2A and 2B recently have shown that the amino terminus of LMW 2B contains six additional amino acids, one of which is an arginine residue (Ninomiya Y, van der Rest M, Mayne R, Lozano G, Olsen BR, submitted for publication).The remainder of the amino-terminal sequences were identical.

Comparison of the present results with the fragment called CF2 [13], C-PS2 [14], and X4 [15] has proven difficult. Both Shimokomaki et al [13] and Ricard-Blum et al [15] reported the presence in this fragment of a single chain. However, Ayad et al [14], after reduction and alkylation of C-PS2, were able to isolate by CM-cellulose chromatography three fractions that possessed molecular weights of 16,000, 10,000, and 8,000. How these fractions relate to the three chains of LMW is at present unclear. Considerable differences were noted when attempts were made to compare our amino acid analyses with analyses of the three fractions isolated from C-PS2.

Several attempts have been made to isolate and identify the higher molecular weight species from which HMW, and possibly LMW,are derived after pepsin digestion [6,20,21]. From cultured chick chondrocytes, an apparent precursor mole-

cule to HMW was isolated that contains two different chains of apparent M_r 69000 and 84000 and called H and J chains [6]. The relationship of these two chains to the results presented in this paper remains uncertain. Recently, Ninomiya and Olsen [22] succeeded in preparing a cDNA clone from a mRNA encoding for a minor cartilage-specific collagen. Subsequent sequence analysis showed that this clone is derived from a mRNA encoding for a collagen chain containing sequences derived from one of the chains of both HMW and LMW [23]. This result strongly suggests that HMW and LMW are the pepsin-resistant fragments of a larger collagen molecule, which has been designated type IX collagen [23]. It therefore follows that HMW must contain also three genetically distinct chains. In previous experiments HMW was shown to contain at least two different chains [9], and more recently the present authors have been able to fractionate and characterize by peptide mapping three different chains from HMW. These three chains were present in equal proportions (Ninomiya Y, van der Rest M, Mayne R, Lozano G, Olsen BR, submitted for publication).

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