Chondrocalcin is identical with the C-propeptide of type II procollagen

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The primary structure of the cartilage matrix molecule chondrocalcin has been found to be identical with that of the C-propeptide of type II procollagen by comparing sequence analyses of the *N*-terminal regions and of tryptic peptides derived from chondrocalcin. This implies that in type II collagen the C-propeptide of type II collagen is employed not only in the assembly of the triple helix of type II collagen, as demonstrated previously, but in calcifying cartilage it may also be involved in those events leading to cartilage calcification, as earlier indicated.

INTRODUCTION

Cartilage matrix is composed primarily of type II collagen and a large proteoglycan of high buoyant density which aggregates with hyaluronic acid. In addition, other matrix proteins have been identified, such as link proteins [1, 2], which stabilize proteoglycan aggregates [2, 3], other collagens (type IX [4, 5], type X [6, 7], $1\alpha 2\alpha 3\alpha$ [8]) and a recently described molecule which we called chondrocalcin [9, 10]. This was characterized by SDS/polyacrylamide-gel electrophoresis as a protein of apparent M_r 69000. Upon reduction the epiphyseal molecule gives rise to two polypeptides of M_r 35500 and 34500 (A. R. Poole & L. C. Rosenberg, unpublished work), of which the smaller is the dominant species [9]. Chondrocalcin is a calcium-binding protein (P. Hauschka & L. C. Rosenberg, unpublished work) and is found in developing fetal cartilage matrix [10]. In growth plate cartilage it is focally concentrated when and where mineralization occurs in the lower hypertrophic zone [10]. We believe that it may play a role in endochondral ossification. We now report that the primary structure of this protein is identical with the primary structure of the C-propeptide of type II procollagen, suggesting a new function for this peptide.

MATERIALS AND METHODS

Sequencing

Chondrocalcin (1.5 nmol) in 2 M-guanidinium chloride was dialysed against 0.1 M-acetic acid and applied to a Biobrene-coated glass filter of an Applied Biosystem model 470A gas phase protein sequencer. The released anilinothiazolinone amino acids were converted to phenylthiohydantoin amino acids by using the trifluoroacetic acid conversion program. The phenylthiohydantoin amino acids were analysed on a Beckman 346 h.p.l.c. chromatograph by using the procedure described

by Lazure *et al.* [21]. The residues are numbered from the cleavage site of the C-proteinase.

Preparation of tryptic peptides and h.p.l.c. separation

Chondrocalcin (500 μ g in 1 ml of 2 M-guanidinium chloride) was precipitated with an equal volume of 75% ethanol/1.3% potassium acetate and washed several times with the same solution. It was then resuspended in 1 ml of 0.2 M- NH_4HCO_3 and digested with tosylphenylalanylchloromethane ('TPCK')-treated trypsin (Millipore) (1 mg/ml in water) with an enzyme/substrate ratio of 1:25. The reaction was stopped with one drop of glacial acetic acid and the reaction mixture was directly injected on a reverse-phase C_{18} TP 201 column (Vydac) and eluted with an aqueous acetonitrile gradient (0–64%) in 9 mM-trifluoroacetic acid over 120 min.

RESULTS AND DISCUSSION

The purified chondrocalcin isolated from fetal epiphyseal cartilage [9] (1.5 nmol) was submitted to sequential Edman degradation for 14 cycles. The analysis of the released phenylthiohydantoin amino acids showed clearly that the preparation contained two *N*-terminal sequences in a 2:1 ratio. Assignments to the major and minor sequences were thus made on the basis of the relative yield of the two residues found in each cycle. Cycle 1 presented a special problem since it contained aspartate as the major residue but also five other amino acids in significant quantities. Thus no residue was assigned to this cycle. No residue could be assigned in cycle 8 to the minor sequence. The sequences obtained were found to match closely the published sequences of chicken [11] and human [12] type II collagen C-propeptide and are presented in Fig. 1. The major sequence overlaps the minor one, which is longer by 10 residues. The M_r added by these 10 residues (1092) fully accounts for the difference of 1000 in apparent M_r

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- (1) Asp-Glu-Ala-Ala-Gly-Gly-Leu-Arg-Gln-His-Asp-Val-Glu-Val-Asp-Ala-Thr-Leu-Lys-Ser-Leu-Asn-Asn-Gln-
 (2) Asp-Gln-Ala-Ala-Gly-Gly-Leu-Arg-Gln-His-Asp-Ala-Glu-Val-Asp-Ala-Thr-Leu-Lys-Ser-Leu-Asn-Asn-Gln-
 (3) ? -Ala-Glu-Val-Asp-Ala-Thr-Leu-Lys-Ser-Leu-Asn-Asn-Gln-
 (4) ? -Glu-Ala-Ala-Gly-Asn-Leu- ? -Gln-His-Asp-Ala-Glu-Val-

Fig. 1. *N*-Terminal sequence analysis of chondrocalcin (line 3, major sequence; line 4, minor sequence) and its comparison with chicken [11] (line 1) and human [12] (line 2) type II procollagen C-propeptides

Fig. 2. H.p.l.c. of the tryptic fragments of chondrocalcin

observed between the major and the minor species of chondrocalcin. The residues that could not be assigned with certainty from the sequence analysis (no. 1 and no. 8 from the minor sequence and no. 1 from the major sequence) are probably identical with the ones found in the chicken and human propeptides. Close examinations of the phenylthiohydantoin amino acid analyses performed for these cycles are entirely consistent with such an identification.

To extend the structural comparison between chondrocalcin and the type II collagen C-propeptide and to cover the region where the bovine sequence had been determined by cDNA sequencing [13, 14], chondrocalcin was digested with TPCK-trypsin and the resulting fragments were separated by h.p.l.c. (Fig. 2) [15]. Aliquots (50%) of the major peaks were hydrolysed and their amino acid compositions were established. Comparison of these compositions with the one predicted from the published cDNA sequences for bovine type II collagen C-propeptide and, where not available, for its

Table 1. Identified tryptic fragments of chondrocalcin and their elution properties

Tryptic fragment (residue numbers)	Acetonitrile concentration (%)	Elution time (min)
9-19	20.3	45.1
11-19	18.9	42.5
20-29	25.0	53.8
47-53	22.7	49.5
54-71	30.3	63.9
54-92	28.3	60.1
94-99	24.4	52.7
105-138	38.9	79.9
139-153	21.4	47.1
154-167	29.0	61.4
188-194	25.7	55.1
204-206	13.4	32.2
207-212	20.7	45.8
219-246	42.3	86.4

human and chicken equivalents, has permitted the recognition of many common tryptic fragments covering all the regions of the molecule (Table 1). It should be stressed that this analysis is not complete, since some h.p.l.c. peaks correspond to unresolved tryptic fragments and are not included in the table. Partial *N*-terminal sequences of two tryptic peptides were obtained and confirmed the identification (Fig. 3).

Our data clearly establish that the two subunits reported for chondrocalcin are related to each other and to the C-propeptide of type II procollagen. The observed sequences are identical between chondrocalcin and the peptide encoded by a recombinant cDNA (Bc7) reported to correspond to the bovine type II C-propeptide [13].

	54	60
C-propeptide:	Ser-Gly-Asp-Tyr-Trp-Ile-Asp-Pro-Asn-Gln-Gly-	
Chondrocalcin:	Ser-Gly-Asp-Tyr-Trp-Ile-Asp-Pro-Asn-Gln-Gly-	
	154	160
C-propeptide:	Asn-Ser-Ile-Ala-Tyr-Leu-Asp-Glu-Ala-Ala-Gly-Asn-Leu-Lys	
Chondrocalcin:	Asn-Ser-Ile-Ala-Tyr-Leu-Asp-Glu-Ala-Ala-Gly-Asn-Leu-Lys	

Fig. 3. Sequence analyses of two tryptic fragments of chondrocalcin (residues 54-71 and 154-167) and comparison with chicken cDNA [11] and human genomic DNA [12] derived sequences for the 54-71 fragment and, for the 154-167 fragment, with the cDNA-derived bovine sequence [13] as amended [14]

Samples (1 ml) of h.p.l.c. fractions containing the peptides (Fig. 2) were dried in a centrifuge evaporator, resuspended in 0.1 M-acetic acid and sequenced as described in the text.

For other regions, they show a high degree of homology with the previously reported protein and cDNA chicken sequences [11] and with the published human genomic DNA sequence of type II collagen C-propeptide [12]. Sangiorgi *et al.* [13] reported that Bc7 hybridizes to a unique locus in the bovine haploid genome, suggesting that chondrocalcin is a product of the same gene as the C-propeptide of type II procollagen.

The C-propeptide is released from the type II procollagen molecule by a specific peptidase ('C-proteinase') that cleaves a unique Ala-Asp bond between the telopeptide and the C-propeptide of the procollagen molecules [11, 16]. The minor (larger) species of chondrocalcin corresponds exactly to such a cleavage product. The lower M_r major sequence, starting 10 residues inside the C-propeptide, does not correspond to the cleavage product of any known C-proteinase or endopeptidase but it also produces an N-terminal Asp. Whether the terminal Asp at position 11 has any significance or represents an artefactual degradation during isolation remains to be established.

There is an apparent contradiction between the published M_r reported for unreduced chondrocalcin (M_r 69000) and the theoretical M_r of the C-propeptide of type II collagen (M_r 105000) which remains to be determined. Further work is required to reconcile their difference. Previous work on the type I C-propeptide, however, has shown that, on SDS/polyacrylamide-gel electrophoresis, the unreduced trimer has a lower apparent M_r than would be expected from the apparent M_r values of the reduced chains [17].

The C-propeptide plays a key role in the initial assembly of the triple helix of the procollagen molecule [18, 19] and is then thought to be released extracellularly from procollagen as fibril formation occurs [20]. Since our recent studies indicate that it is intimately associated with mineral formation in calcifying cartilage matrix [10] an intriguing possibility is raised, namely that the C-propeptide may play a dual role, first in the assembly of type II collagen fibrils and then in the calcification of cartilage matrix which occurs subsequently. This possibility requires investigation.

We thank Ms. Elisa De Miguel and Mrs. Elida Rodriguez for helping with the experimental work, Mrs. Denyse Lavalée and Mrs. Farla Miller for secretarial assistance and Mr. Mark Lepik for the illustrations. This work was supported by the Shriners of North America and the Medical Research Council of Canada (to M.v.d.R. and A.R.P.) and by the National

Institutes of Health (to B.R.O. and L.C.R.). M.v.d.R. is a Chercheur-Boursier from the Fonds de la Recherche en Santé du Québec.

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Received 2 May 1986; accepted 29 May 1986