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Bovine type XII collagen: amino acid sequence of a 10 kDa pepsin fragment from periodontal ligament reveals a high degree of homology with the chicken α_1(XII) sequence

B. Dublet, E. Dixon, E. de Miguel and M. van der Rest

Genetics Unit, Shriners Hospital, 1529 Cedar Avenue, Montréal, Québec H3G 1A6 and Departments of Surgery and Biochemistry, McGill University, Montréal, Canada

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A 10 kDa collagenous peptide, derived from a 30 kDa disulfide bonded fragment, was purified from bovine periodontal ligament. Amino acid sequence analysis of tryptic peptides demonstrated a 92.8% homology with the chicken α_1(XII) cDNA derived sequence, demonstrating for the first time the presence of type XII collagen in a mammalian species and in an adult tissue.

Type XII collagen; Structure homology; Amino acid sequence; (Bovine periodontal ligament)

1. INTRODUCTION

Non-fibril forming collagens are thought to perform important functions in the extracellular matrix. Type IX collagen has been shown to be cross-linked to the type II fibril to probably perform a linker function between the fibril and other cartilage matrix constituents [1,2]. Since several collagen genes are coordinately expressed in different tissues, an analogue for type IX collagen in type I collagen containing extracellular matrices has been postulated by some investigators. The first demonstration of the existence of such an analogue came from the description of a cDNA clone (pMG377), isolated from a 17-day-old chick embryo tendon library, by Gordon et al. [3]. These authors designated the encoded polypeptide chain as the α_1 chain of type XII collagen. At the protein level, we have isolated from the same tissue pepsin derived fragments of α_1(XII) chain [4].

Correspondence address: B. Dublet, Genetics Unit, Shriners Hospital, 1529 Cedar Avenue, Montréal, Québec H3G 1A6, Canada

Prompted by the earlier description by Yamauchi et al. [5] of a 30 kDa disulfide bonded collagenous fragment, reducible into a 10 kDa subunit, in a pepsin extract of bovine periodontal ligament, we decided to investigate whether such a fragment could originate from a bovine type XII collagen. We report here the purification of a 10 kDa fragment from the bovine periodontal ligament and demonstrate by amino acid sequence analysis that it is highly homologous to the chicken α_1(XII) sequence reported by Gordon et al. [3]. Our data provide the first evidence for the presence of type XII collagen in a mammalian tissue and demonstrate for the studied region, a much higher sequence conservation between avian and mammalian α_1(XII) chains than between avian and mammalian α_1(IX) chains.

2. MATERIALS AND METHODS

2.1. *Tissue collection*

Jaws from 1.5–2-year-old cattle were obtained immediately after slaughtering at a local slaughterhouse and kept on ice. The primary incisors were extracted and the periodontal ligaments were removed with a wax-carving tool.

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2.2. *Collagen preparation*

The fresh tissue (27 g wet wt) was milled in a freezer mill. The powder was dissolved in approx. 10 vols of distilled water containing 10 mM EDTA, 5 mM *N*-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride and 10 mM *p*-amino-benzamidine. The homogenized tissue was centrifuged at 10000 × *g* and the precipitate was washed repeatedly (5 times) with the same solution. The residue was suspended in 400 ml of 0.5 M acetic acid and treated with pepsin (0.5 mg/ml, Sigma) for 24 h at 4°C. After centrifugation (12000 × *g*, 30 min) the supernatant was fractionated by differential salt precipitation by dialysis against increasing concentration of NaCl (0.7 M, 1.2 M and 2.0 M) in 0.5 M acetic acid.

2.3. *Two-dimensional gel electrophoresis*

The first dimension SDS-polyacrylamide slab gel electrophoresis was performed without reduction in 7 cm × 8 cm × 0.75 mm (L × W × D) gels in a mini PROTEAN II cell (Bio-Rad) according to Laemmli [6] using a 15% acrylamide concentration. Sliced lanes of the first dimension were laid down in a 1% agarose solution containing 125 mM Tris-HCl buffer, pH 6.8, and 10% of β-mercaptoethanol on a 15% slab gel and the electrophoresis was performed as for the first dimension. The gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 (Bio-Rad) in methanol/acetic acid/water (40:10:50) and destained with methanol/acetic acid/water (40:10:50).

2.4. *High-performance liquid chromatography (HPLC)*

Separations of the collagenous fragments by HPLC were done as described before [7] using either 9 mM trifluoroacetic acid or 10 mM heptafluorobutyric acid as ion pairing agent. The equipment used was from Beckman and consisted of a model 334 chromatograph, a model 160 UV monitor equipped with a zinc lamp and a CR 1B data system. The column was a C18 Vydac TP 201 (4.6 × 250 mm) (The Separation Group) and was protected with a guard column filled with pellicular C18 resin (Waters).

2.5. *Amino acid analysis*

Amino acid compositions of the fragments were established on a Dionex D-500 amino acid analyzer as described previously [8]. Hydrolysis was performed with 6 N HCl at 110°C for 20 h under vacuum in a Waters Picotag workstation.

2.6. *Reduction and alkylation of the fragments*

The fractions were dissolved in a 50 mM Tris-HCl, pH 8.0, buffer containing 5 M urea. After addition of 0.1 M β-mercaptoethanol and heating at 100°C for 3 min, the samples were cooled down to ambient temperature and iodoacetamide was added to a final 0.2 M concentration. After 30 min incubation, the samples were loaded as such on the HPLC.

2.7. *Trypsin digestion*

The purified fragments were dissolved in 0.2 M NH_4HCO_3 and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Millipore) in 1:30 enzyme/substrate ratio. HPLC peptide maps were obtained as described previously [7].

2.8. *Amino acid sequence analysis*

Amino acid sequences were determined by automated Edman degradation in an Applied Biosystem 470 A gas-phase sequence, using the trifluoroacetic acid conversion program. The phenylthiohydantoin derivatives of the amino acids were identified by HPLC in an Applied Biosystem 120 A on-line analyzer.

3. RESULTS

The 2.0 M NaCl precipitate contained two major disulfide bonded fragments, one yielding a 19 kDa fragment upon reduction, the other yielding a 10 kDa fragment (fig.1). These two fragments were purified by HPLC using the same two step strategy used previously for the purifica-

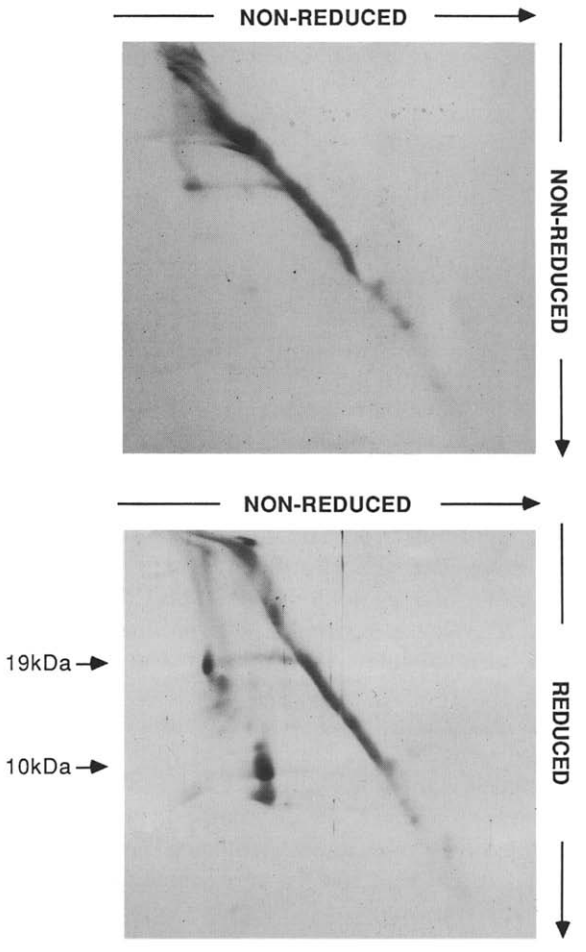

Fig.1. Two-dimensional gel electrophoresis of the 2.0 M NaCl precipitate. First dimension: no reduction; second dimension: no reduction (top), with reduction (bottom). The 10 and 19 kDa fragments are indicated on the left of the figure. The molecular masses are based on a standard of collagen α_1(I) CB peptide (not shown).

tion of the chicken fragments [4], i.e. a separation of the unreduced sample followed by an identical run of the fractions containing the peptides of interest after reduction and alkylation. The results of these separations were essentially identical to the results of the chicken preparation [4] and are therefore not shown. The 10 kDa and the 19 kDa fragments were digested with trypsin and the resulting peptides separated by HPLC (fig.2). These peptide maps indicate that the two fragments are completely different and are therefore either different domains of the same molecule or are derived from different molecules.

The tryptic peptides of the 10 kDa fragment were further purified by a second HPLC, using heptafluorobutyric acid as ion pairing agent. The amino acid compositions and amino acid sequences of these purified peptides were established. These sequences could be precisely aligned by homology with the chicken cDNA derived sequence of α_1(XII) (fig.3). With one exception, all the tryptic fragments sequences could be identified by homology with the chicken sequence. The sequence that did not align was: Asn-Gln-Pro-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Ser-Ala-.

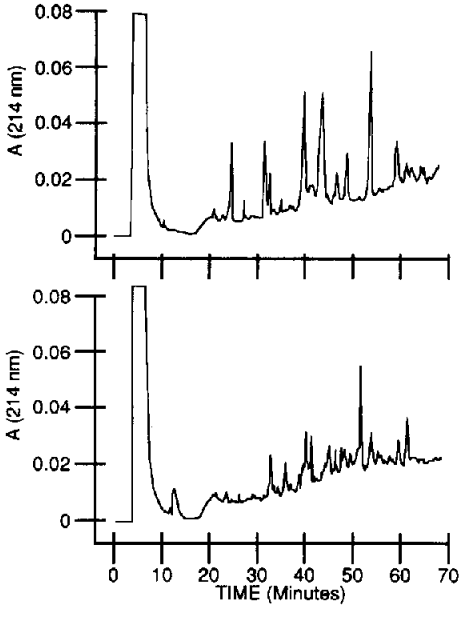

Fig.2. HPLC peptide maps of the tryptic peptides of the 10 (top) and 19 kDa (bottom) fragments. The separations were done in the presence of 9 mM trifluoroacetic acid with an aqueous gradient of acetonitrile (0–32%) over 90 min.

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CHICKEN SEQUENCE:        Arg-Gly-Glu-Pro-Gly-Pro-Gly-
BOVINE SEQUENCE:            Gly-Glu-Hyp-Gly-Pro-Gly-

Gly-Arg-Pro-Gly-Phe-Pro-Gly-Pro-Pro-Gly-Phe-Gln-Gly-
Gly-Arg-Hyp-Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Met-Gln-Gly-

Pro-Pro-Gly-Glu-Arg-Gly-Met-Pro-Gly-Glu-Lys-Gly-Glu-
Pro-Gln-Gly-Glu-Arg Gly-Leu-Hyp-Gly-Glu- X -Gly-Glu-

Arg-Gly-Thr-Gly-Ser-Gln-Gly-Pro-Arg-Gly-Leu-Pro-Gly-
Arg                                 Gly-Leu-Hyp-Gly-

Pro-Pro-Gly-Pro-Gln-Gly-Glu-Ser-Arg-Thr-Gly-Pro-Pro-
Pro-Hyp-Gly-Pro-Gln-Gly-Glu-Ser-Arg Thr-Gly-Pro-Hyp-

Gly-Ser-Thr-Gly-Ser-Arg-Gly-Pro-Pro-Gly-Pro-Pro-Gly-
Gly-Ser-Thr-Gly-Ser-Arg Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-

Arg-Pro-Gly-Asp-Ala-Gly-Ile-Arg-Gly-Pro-Pro-Gly-Thr-
Arg-Hyp-Gly-Asp-Ser-Gly-Ile-Arg
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Fig.3. Comparison of the chicken cDNA derived sequence of the α_1(XII) chain and the bovine tryptic peptide sequences. The five residues differing between the two species are underlined.

Since the cDNA pMG377 does not extend to the amino-terminal end of the COL 1 domain of α_1(XII) and since the peptide which could not be aligned with the pMG377 sequence marks a transition between a non-collagenous domain and a collagenous domain, we surmise that this peptide represents the amino-terminal end of the COL 1 domain of bovine α_1(XII). For the 70 residues which could be aligned with the chicken sequence, only 5 residues differ from the chicken sequence, yielding a 92.8% sequence homology between the two species.

4. DISCUSSION

The very high homology (92.8%) between the sequence of the 10 kDa collagenous fragment from the bovine periodontal ligament and the chicken α_1(XII) sequence, as encoded in the cDNA pMG377, indicates that this fragment is derived from bovine type XII collagen. So far, type XII collagen had only been described in chick embryonic tissues, particularly in tendons and in skin. Our data constitute the first demonstration of the presence of type XII collagen in an adult tissue and in a mammalian species.

We have decided to investigate the periodontal ligament because of the earlier report by Yamauchi et al. [5] of a disulfide bonded small molecular mass collagenous fragment in a pepsin extract of

this tissue. Our data confirm the presence of such a fragment and establish that it is derived from type XII collagen. Similar fragments have also been isolated from fetal bovine skin (unpublished).

Bovine type XII collagen presents, for the studied triple helical region, a very high homology with its chicken counterpart. In contrast, the homology between rat and chicken α_1(IX) chains at identically positioned residues is only 68% [Kimura, T., Stevens, J.W., Goldring, M.B., Ninomiya, Y. and Olsen, B.R., J. Biol. Chem., submitted]. We have very recently demonstrated that chicken type XII collagen is a homotrimer $[\alpha_1(XII)]_3$ [Sugrue, S.P., Gordon, M., Seyer, J., Dublet, B., Van der Rest, M. and Olsen, B.R., submitted], while type IX collagen is a heterotrimer of three different α chains [9]. The differences in sequence conservation between avian and mammalian molecules may be a reflection of this stoichiometry. Assuming indeed that most amino acid substitutions in the collagen triple helix are not neutral, a mutation in one allele of the gene of a homotrimeric molecule results in 87.5% mutant molecules while a mutation in one allele of the gene of a chain of heterotrimeric molecules results in only 50% mutant molecules, resulting in a weaker evolutionary pressure for the conservation of the primary structure.

Our data do not definitely prove that bovine type XII collagen is a homotrimer and actually the 2D gel shown in fig.1 might suggest that another chain is present. However all the 10 kDa reduced fragments were in the same fractions of the second HPLC purification step. Since the tryptic peptides obtained from these fractions appear to be all derived from the α_1(XII) chain, it is very likely that the bovine type XII collagen is also a homotrimer and that the size heterogeneity observed in fig.1 is due to the pepsin digestion.

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