ISOLATION AND CHARACTERIZATION OF COLLAGEN A AND B CHAINS FROM CHICK EMBRYOS

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1. Introduction

From human fetal membranes two new collagen chains, called A and B chains, have been isolated which are genetically different to the α-chains of types I, II, III and IV collagen [1]. These collagen chains were also found in human skin and in vessel walls [2], in skeletal bovine muscle [3] and in bone and cartilage [4]; in vitro A and B chains are produced by cultures of smooth muscle cells [5]. From most tissues and cell cultures twice as much B chains as A chains were obtained which is consistent with the existence of a collagen molecule of the subunit composition AB_2 (1.6), similar to type I collagen $(\alpha 1(I))_2\alpha 2$.

However, another study suggests that A and B chains are subunits of two different triple-helical molecules A_3 and B_3 [4].

The similarity of A,B-collagen to basement membrane collagen in terms of amino acid composition [1,4,6] and its localization in the endomysium [3] suggest the possibility that A,B-collagen may be a constituent of the muscle basal lamina. To answer this question, we attempted to localize this collagen type in chick muscle cell cultures immunohistologically using specific antibodies to chick A,B-collagen.

The existence of A,B-collagen-like molecules in non-mammalian species was suggested recently by two in vitro studies.

In suspension cultures of freshly-isolated chick tendon fibroblasts [8] and in cultures of embryonic chick neural retina cells [9] two chains were observed which migrated in the positions corresponding to human A and B chains. However, no conclusive data were available as to whether a collagen which is structurally and genetically related to human A,B-collagen exists in avian species.

Here we describe the preparation of collagen chains from whole chick embryos by limited digestion with pepsin, which are homologous to human A,B-collagen in terms of chromatographic and electrophoretic behaviour, amino acid composition and the finger-print pattern of cyanogen bromide-derived peptides.

The preparation of antibodies against this collagen has allowed its localization and identification in cultures of chick and quail breast muscle cells (J. Sasse, H. v/d M., K. v/d M., in preparation.)

2. Materials and methods

2.1. *Preparation of A,B-collagen*

One hundred and fifty day 14 chick embryos were killed by decapitation, washed and drained, and homogenized in a waring blender in 1 l 0.5 M acetic acid, titrated to pH 2.0 with HCl. The homogenate was digested with pepsin (swine stomach mucosa pepsin, 2500 Anson U/mg, Boehringer, Mannheim; 1 g/1000 g wet tissue) at 4°C for 24 h [10] and clarified by centrifugation for 6 min at 50 000 × *g*. From the supernate types I, II, III and IV collagen were precipitated by adding NaCl to 4% [2] and removed by centrifugation as above. The supernate, containing A,B-collagen, was titrated to pH 8.0 with 1 M NaOH, and dialyzed into tap water. The precipitate which formed overnight was collected by centrifugation as above, dissolved in 0.5 M acetic acid and clarified by centrifugation at 50 000 × *g* for 1 h. All operations were carried out at 0–4°C.

2.2. *DEAE-cellulose chromatography [11]*

A,B-collagen was dialyzed against 0.05 M Tris–HCl, 0.02 M NaCl, 2 M urea pH 8.6 [6] and applied to a DEAE-cellulose column (DE 52, Whatman, 1.5 × 10 cm) equilibrated with the same buffer at 12°C. The collagens were eluted from the column with a linear salt gradient from 0–0.3 M NaCl over 600 ml total vol.

2.3. *Carboxymethyl (CM)-cellulose chromatography*

This was done as in [12].

2.4. *Agarose-gel chromatography*

A and B chains were purified by molecular sieve chromatography on 4% agarose (A15m, Bio-Rad; column size 1.5 × 130 cm) in 1 M $CaCl_2$ as in [13].

2.5. *Analytical methods*

Discontinuous slab gel electrophoresis was performed in 0.1% SDS/0.1 M Tris–glycine buffer (pH 8.6) according to [14]. Whole collagen chains were separated on gradient gels of 5–10% acrylamide, while CNBr-derived peptides were separated on a gradient of 10–18% acrylamide. For amino acid analysis, samples were hydrolyzed under N_2 at 110°C in 6 N HCl for 24 h, and analyzed on a Durrum D500 amino acid analyzer.

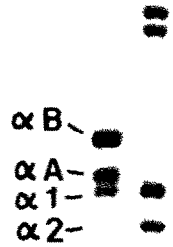
Cyanogen bromide cleavage was performed in 70% formic acid at 30°C for 4 h according to [15].

3. Results

About 5–10% of the collagen which can be solubilized by pepsin from day 14 chick embryos is A,B-collagen. Precipitation of collagen types I–IV with 4% sodium chloride at acidic pH [2,4] leaves A,B-collagen as the main collagenous constituent in the supernate. It precipitates by dialysis against tap water or 0.02 M Na_2HPO_4 buffer, and the precipitate, when dissolved in acetic acid and clarified by centrifugation, consists of > 95% pure A,B-collagen.

For further purification A,B-collagen was chromatographed on DEAE-cellulose under non-denaturing conditions (not shown).

A,B-collagen eluted in a single peak 100–150 ml after application of the salt gradient, while residuals of type I collagen eluted with the buffer front. The



a b c d e

Fig.1. SDS–gel electrophoresis on 6% acrylamide slab gels, stained with Coomassie blue. (a) Mixture of type I and A,B-collagen extracted from chick embryos with pepsin (4% NaCl supernate); (b) type I collagen; (c) A,B-collagen purified by DEAE-cellulose chromatography; (d) A chains; (e) B chains separated by CM-cellulose chromatography and purified by agarose gel filtration.

A,B-collagen thus purified revealed only two bands, the A and the B chains, on SDS–gel electrophoresis, both migrating slower than α1(I) (fig.1).

Separation of A and B chains was achieved by chromatography on CM-cellulose under conditions used for separating α1(I) and α2 chains (fig.2). While

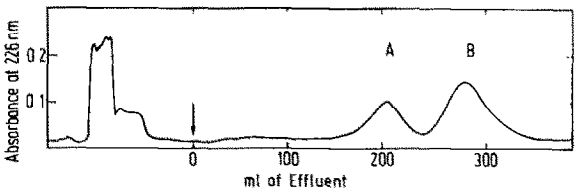


Fig.2. Separation of A and B chains by CM-cellulose chromatography. A,B-collagen was denatured and applied to CM-cellulose (CM52, 1.5 × 12 cm) at 42°C, equilibrated in 0.04 M sodium acetate, 6 M urea (pH 4.8). Elution was achieved by a linear gradient between 0–0.12 M NaCl over 600 ml.

A and B chains from human sources do not separate well under those conditions [6], the A chain eluted in the position of α1(I), and the B chain eluted between the position of β_{12} and α2 (fig.3).

In three separate preparations the chain ratio for A and B chains eluting from CM-cellulose was (2.0 ± 0.2) :1.

Further purification of A and B chains and estimation of the molecular weight was achieved by molecular sieve chromatography on 4% agarose (fig.4). While the A chain eluted in the position of α1(I), the B chain eluted slightly earlier which is consistent with its migration on SDS–gel electrophoresis (fig.1d,e).

For identification, purified A and B chains were subjected to amino acid analysis and cyanogen bromide cleavage. The typical features of the amino acid composition which characterize the isolated chains as A and B chains are the low alanine and the high leucine and hydroxylysine content (table 1). The B chain contains almost twice as much hydroxylysine as the A chain, but less alanine. One-third of the amino acids are glycine.

The relatively high methionine content of the A chain is reflected by the rather complex peptide pattern on SDS–gel electrophoresis produced by cyanogen bromide cleavage at methionyl residues (fig.5). The B chain gives rise to less peptides, the two

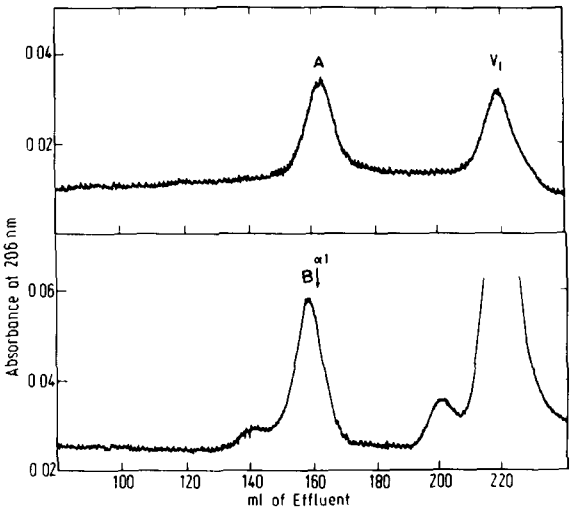


Fig.3. Molecular sieve chromatography of A and B chains on 4% agarose (A15m) in 1 M $CaCl_2$ (pH 7.5). V_i included volume.

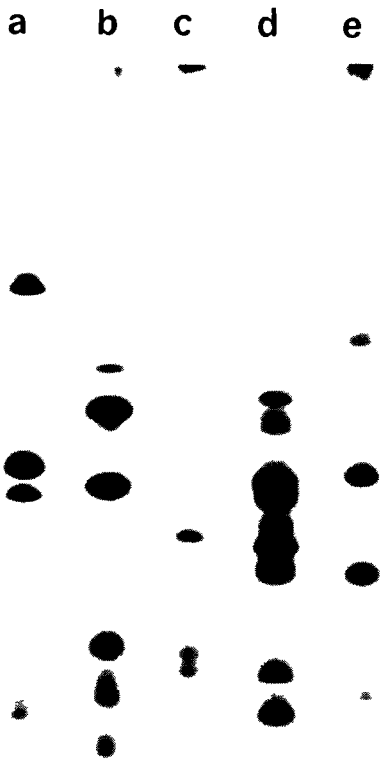


Fig.4. Cyanogen bromide peptides of chick α1(I) (a), α1(II) (b), α1(III) (c), A chains (d) and B chains (e), separated by SDS–gels electrophoresis on slab gels [14] using a linear gradient between 10–18% acrylamide. Stained with Coomassie blue.

prominent ones showing the same R_F values as 2 CNBr peptides of the A chain. The peptide patterns of both chains differ significantly from the CNBr pattern of α1(I), α1(II) and α1(III).

4. Discussion

The amino acid composition and electrophoretic behaviour of the isolated chick A and B chains clearly suggest homology to mammalian A and B chains [6].

Table 1
Amino acid composition of chick A and B collagen chains recovered after CM-cellulose and agarose chromatography, compared to the composition of human A and B chains (taken from [4]) (residues/1000 amino acids ± SD)

	Chick A chain	Human placenta A chain [4]	Chick B chain	Human placenta B chain [4]
3-Hyp	1.8 ± 0.6[a]	3	5 ± 1.3	5
4-Hyp	87 ± 15.4	106	81 ± 9.3	110
Asp	47 ± 2.4	50	46 ± 9.3	50
Thr	28 ± 1.3	29	21 ± 1.5	22
Ser	40 ± 5.1	34	29 ± 4.1	22
Glu	93 ± 3.9	89	105 ± 4.1	95
Pro	103 ± 2.6	107	124 ± 4.5	135
Gly	330 ± 18	331	324 ± 6	335
Ala	76 ± 5.9	54	48 ± 4.1	39
Val	21 ± 1.4	27	15 ± 1.5	17
Cys	0			
Met	11.7 ± 1.1	11	8.4 ± 0.9	9
Ile	13 ± 1.7	15	18 ± 0.9	17
Leu	31 ± 2.1	37	39 ± 1.8	36
Tyr	3.8 ± 0.5	2	2.3 ± 0.6	4
Phe	11.3 ± 0.9	11	10 ± 0.7	12
Hyl	18 ± 0.9	23	39 ± 3.1	36
His	6.2 ± 1.3	10	5.8 ± 0.8	6
Lys	16 ± 1.2	13	15 ± 0.7	14
Arg	49 ± 2.1	48	40 ± 1.8	40

[a] The values represent averages from 6 different analyses of 3 separate preparations

However, chick and human A chains differ to some extent in the degree of hydroxylation of prolyl- and lysyl residues, as well as in the content of alanine, proline, leucine and serine. The cyanogen peptide pattern of chick and human B chains [1,6] are homologous; the peptide pattern of the chick A chain differs from that of the human A chain reported in [1], however it is in agreement with that observed by Bentz et al. (personal commun.).

In the light of the presented data it appears likely that the two new collagen chains produced by embryonic chick tendon fibroblasts [8] or retina cells [9] actually represent A and B chains as was suggested by the authors. In both reports A and B chains were consistently found in ratios of 1:2 which is compatible with the stochiometry reported in our study. This ratio suggests the existence of a single molecule with the chain composition AB_2; however, no conclusive data are available to confirm this hypothesis in the chick system.

Immunofluorescence studies performed with antibodies specific to chick A,B-collagen indicated that this collagen originates in the chick embryo mainly from skeletal muscle, smooth muscle and heart muscle (H. v/d M. K. v/d M., in preparation) which is consistent with the localization of A,B-collagen in the endomysium [3]. It is also distributed rather ubiquitously in most fibrous tissues, but it could not be detected in cartilage or bone which contrasts to the report in [4]. Cell culture experiments suggest that skeletal myoblasts synthesize A,B-collagen also in vitro, where it constitutes in part the extracellular collagen fibers and perhaps the basement membranes of myotubes (J. Sasse, H. v/d M., in preparation).

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References

[1] Burgeson, R. E., El Adli, F. A., Kaitila, I. I. and Hollister, D. W. (1976) Proc. Natl. Acad. Sci. USA 73, 2579–2583.
[2] Chung, E., Rhodes, R. K. and Miller, E. J. (1976) Biochem. Biophys. Res. Commun. 71, 1167–1173.
[3] Duance, V. C., Restall, D. J., Beard, H., Bourne, F. J. and Bailey, A. J. (1977) FEBS Lett. 79, 248–252.
[4] Rhodes, R. K. and Miller, E. J. (1978) Biochemistry 17, 3442–3448.
[5] Mayne, R., Vail, M. S. and Miller, E. J. (1978) Biochemistry 17, 446–452.
[6] Bentz, H., Bächinger, H. P., Glanville, R. and Kühn, K. (1979) Eur. J. Biochem. in press.
[7] Von der Mark, H., von der Mark, K. and Gay, S. (1976) Dev. Biol. 48, 237–249.
[8] Jimenez, S. A., Yankowski, R. and Bashey, R. I. (1978) Biochem. Biophys. Res. Commun. 81, 1298–1306.
[9] Linsenmayer, T. F. and Little, C. D. (1978) Proc. Natl. Acad. Sci. USA 75, 3225–3239.
[10] Trelstad, R. and Lawley, K. R. (1977) Biochem. Biophys. Res. Commun. 76, 376–383.
[11] Smith, B. D., Byers, P. H. and Martin, G. R. (1972) Proc. Natl. Acad. Sci. USA 69, 3260–3262.
[12] Von der Mark, K. and Von der Mark, H. (1977) J. Cell Biol. 73, 736–747.
[13] Piez, K. A. (1968) Anal. Biochem. 26, 305–312.
[14] Laemmli, U. K. (1970) Nature 227, 680–685.
[15] Epstein, E. H., Scott, R. R., Miller, E. J. and Piez, K. A. (1971) J. Biol. Chem. 246, 1718–1724.