IDENTIFICATION OF A NEW BASEMENT MEMBRANE COLLAGEN BY THE AID OF A LARGE FRAGMENT RESISTANT TO BACTERIAL COLLAGENASE

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1. Introduction

Type IV collagen has been identified as the major component of basement membranes found in a variety of tissues [1]. Recent studies [2–6] have indicated that basement membrane collagen is not a single protein but rather a group of related proteins, similar to the collagen types I, II and III of interstitial connective tissue. A transplantable mouse tumor [7] has been used as a convenient source to purify and characterize a soluble form of basement membrane collagen. It consisted of disulfide-bonded polypeptide chains (mol. wt $16–18 \times 10^4$) and resembled a procollagen-like protein [8]. Pepsin degraded this material to several triple helical fragments giving rise to 5 distinct polypeptide chains (mol. wt $27–72 \times 10^3$). The data indicated that these fragments originate from two different type IV collagens [4]. A pepsin digest prepared from the insoluble tumor collagen contained the same fragments as found with the soluble collagen and some additional components [4] suggesting an even more complex nature of basement membrane collagen. Here we describe the isolation from the tumor of a large collagenous fragment Col 1(IV) which resisted digestion with pepsin and collagenase. The chemical and immunological properties of Col 1(IV) show that it is derived from a hitherto unrecognized additional species of type IV collagen.

2. Experimental

The basement membrane matrix was prepared from a tumor (EHS sarcoma) maintained by subcutanous passages in lathyritic mice [7]. An acetic acid extract of this matrix was used to purify a soluble form of basement membrane collagen [8]. Pepsin treatment at 15°C of the insoluble residue remaining after acetic acid extraction solubilized all of the residual collagen. From this digest a mixture of disulfide-bonded, collagenous fragments (P3) could be separated from cysteine-free fragments (P1,P2) on agarose [4,6]. These collagenous proteins were dissolved in 0.05 M Tris–HCl (pH 7.4), 0.2 M NaCl, 0.002 M $CaCl_2$ (10 mg/ml) and treated with bacterial collagenase (0.1 mg/ml; CLSPA, Worthington) for 16 h at 37°C. Purification of peptide Col 1(IV) from this digest was carried out on a Sephadex G-50 column (2.6×140 cm) equilibrated in 0.2 M ammonium bicarbonate (pH 8.5); on an agarose A5m column (3.6×125 cm) equilibrated in 1 M $CaCl_2$, 0.05 M Tris–HCl (pH 7.4) and on a column (1.5×10 cm) of CM-cellulose which was equilibrated in 0.01 M sodium acetate (pH 4.0), 4 M urea and eluted with a linear NaCl gradient (0–0.2 M). Purified peptides were analyzed by sodium dodecylsulfate disc electrophoresis using type I collagen or CNBr peptides of type I collagen for calibration [9].

The amino acid composition was determined after hydrolysis with 6 M HCl under N_2 (24 h, 110°C) on a Durrum D-500 analyzer. Reduction and *S*-aminoethylation of peptides in 8 M urea followed the method in [10]. Circular dichroism spectra were measured in a Cary 61 spectropolarimeter equipped with thermostated quartz cells. The analytical ultracentrifugation measurements were performed at 20°C with a Spinco

Model E ultracentrifuge (Beckman Instruments) equipped with a scanner. Sedimentation velocity runs were carried out at 56×10^3 rev./min in single sector cells and equilibrium runs at 7×10^3 rev./min in double sector cells. The A_{280} was measured and a partial specific volume of 0.70 ml/g was assumed to calculate molecular weights.

For radioimmunoassays peptide Col 1(IV) was labeled with ^{125}I. All reactions (binding by antibody and inhibition by non-labeled peptides) were carried out in the presence of 0.04% Tween 20 [11]. Rabbit antisera which show distinct binding of the peptide were obtained by immunization with soluble basement membrane collagen or with fragment P3 [8].

3. Results

Previous studies have shown that pepsin digestion of insoluble basement membrane collagen from the tumor produces a mixture of disulfide-bonded fragments (P3) and two fragments (P1,P2) which lack disulfides [4,6]. Further treatment of P3 with bacterial collagenase produced in 15–20% yield a large collagenous fragment Col 1(IV) which could be separated from smaller peptides on Sephadex G-50 (fig.1). No

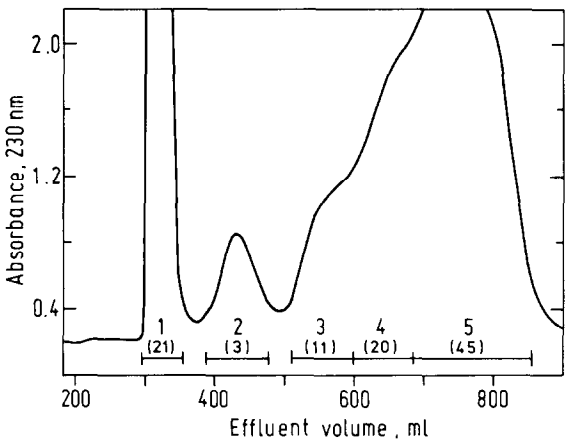

Fig.1. Separation of a collagenase digest (150 mg) of the basement membrane collagen fragment P3 on Sephadex G-50. The column (2.6 × 140 cm) was equilibrated in 0.2 M ammonium bicarbonate (pH 8.5). The relative amount (in %) of peptide material in pools 1–5 is given in brackets. Total recovery from the column was 92%. Pool 1 was used for further purification of peptide Col 1(IV).

or only negligible amounts of Col 1(IV) were found in collagenase digests prepared from the soluble basement membrane collagen or from the pepsin fragments P1 and P2. Peptide Col 1(IV) was further purified on agarose A5 and finally eluted from CM cellulose as a single peak. Amino acid analysis (table 1) showed about one-third of glycine, high levels of 4-hydroxyproline and hydroxylysine and a relatively low content of alanine and arginine which is characteristic for basement membrane collagen [1]. Peptide Col 1(IV) differed from other collagens or collagenous fragments obtained from the tumor [4,8] by an extraordinarily high cysteine content (45–50 residues/1000) and distinctly lower levels of 3-hydroxyproline, serine and phenylalanine.

Examination of Col 1(IV) in sodium dodecyl sulphate electrophoresis showed mainly a single broad band having a slower mobility than collagen γ components (fig.2). The peptide was homogeneous by sedimentation velocity analysis in 0.05 M phosphate buffer, pH 7.0 ($s_{20,w} = 7.1$) and showed in phosphate buffer as well as in 6 M guanidine · HCl a mol. wt 20×10^4 when determined by sedimentation equilib-

Table 1
Amino acid composition of peptide Col 1(IV) and its reduced chains (B–D)

	Col 1(IV)	B	C	D
		(residues/1000)		
3-Hyp	< 2	< 2	< 2	< 2
4-Hyp	90	95	100	98
Asp	68	78	69	67
Thr	25	29	26	29
Ser	19	30	16	14
Glu	85	81	83	83
Pro	62	58	64	67
Gly	323	288	310	313
Ala	41	40	37	36
Cys	45	48[a]	46[a]	46[a]
Val	28	34	32	27
Met	4	4	2	4
Ile	23	24	23	27
Leu	51	47	42	45
Tyr	13	14	15	14
Phe	12	13	13	12
His	15	19	15	16
Hyl	39	44	47	45
Lys	14	14	14	16
Arg	43	40	46	41

[a] As aminoethyl cysteine

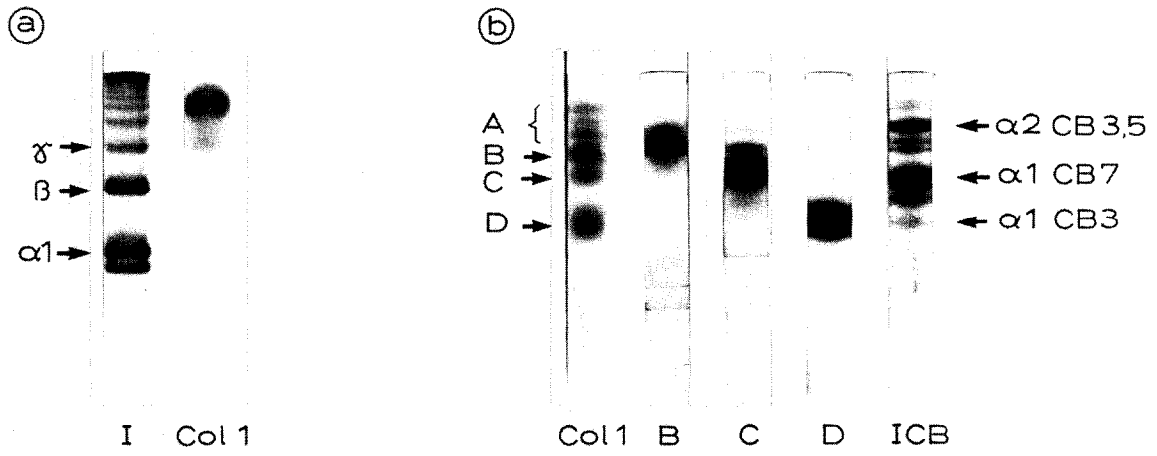

Fig.2. Sodium dodecyl sulphate disc electrophoresis of nonreduced ((a) 3.5% gels) and reduced ((b) 7.5% gels) peptide Col 1(IV). A–D denote peptides obtained from reduced Col 1(IV) after chromatography on agarose A 1.5 m. Patterns of bovine type I collagen (I) and of cyanogen bromide peptides of type I collagen (ICB) are included for reference.

rium runs. Reduction and alkylation of Col 1(IV) produced several polypeptide bands as judged by electrophoresis. These bands could be partially resolved into 4 peaks on agarose A1.5 (not shown). Three major constituents (peaks B,C,D) could be isolated in ~90% electrophoretic homogeneity (fig.2) and had apparent molecular weights as based on electrophoretic mobility of 39, 28 and 14×10^3, respectively. Amino acid analysis of these chain fragments showed a close resemblence to the non-reduced peptide Col 1(IV) (table 1). Additionally, 3 larger minor polypeptide bands could be identified.

Studies on the conformation of peptide Col 1(IV) at 15°C by circular dichroism showed a spectrum characteristic for triple helical collagen, e.g., a large negative deflection around 200 nm and a smaller positive peak around 225 nm. Upon heating, the spectrum changed to that of randomly coiled polypeptide chains. The melting temperature of the helix was found to be ~70°C but thermal transition took place over a wide temperature range (55–80°C). The high stability of the helix apparently explains the resistance of Col 1(IV) towards collagenase digestion. However, after reduction of disulfide bonds of Col 1(IV) in 8 M urea the peptide could be completely degraded by collagenase as judged from chromatography on Sephadex G-50.

A radioimmunoassay was established for peptide Col 1(IV) and used to study the relation between various collagenous fragments of the tumor by inhibition tests (fig.3). The non-reduced peptide Col 1(IV)

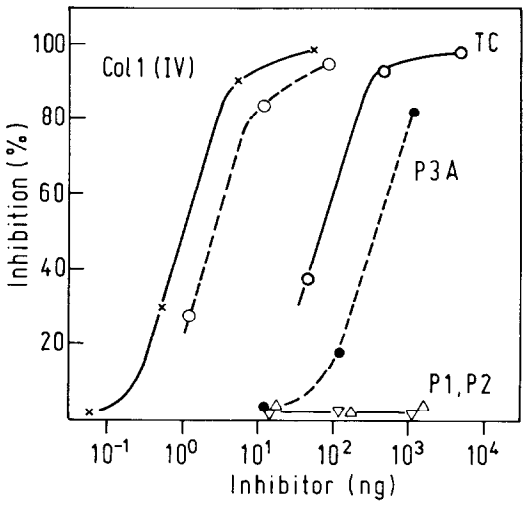

Fig.3. Identification of distinct antigenic determinants on peptide Col 1(IV) by radioimmunoassay. The test system was ^{125}I-labelled peptide Col 1(IV) and antibody against tumor collagen. Inhibitors of the reaction were non-reduced Col 1(IV) (×——×), reduced Col 1(IV) (○——○), acid-soluble tumor collagen (TC, ○——○) and the pepsin fragments P1 (△——△), P2 (▽——▽) and P3A (●——●) obtained from TC.

as well as the reduced chain components were effective inhibitors of the reaction. Soluble tumor collagen and the major chain constituent of P3 (P3A) were only inhibitory when used at 60–300-fold higher concentrations. No activity was found for the fragments P1 and P2.

4. Discussion

A new triple helical fragment Col 1(IV) could be prepared from a pepsin digest of insoluble basement membrane collagen produced by a mouse tumor. The acid-soluble form of the tumor collagen contains, as shown by radioimmunoassay, only 1–2% of this new component even though this low contamination is sufficient to elicit antibodies which bind to Col 1(IV). The most unusual property of Col 1(IV) is its resistance towards bacterial collagenase presumably due to a high stability of the triple helix (T_m 70°C). A large number of interchain disulfide bonds apparently provides the basis for this stability resembling previous observations on the precursor-specific triple helical segment of type III procollagen [12,13] which has T_m 53°C and is also resistant against collagenase. Other collagens including the acid-soluble tumor collagen [8] show T_m 35–40°C and are readily degraded by collagenase.

The major pepsin fragments of the soluble tumor collagen (P1, P2, P3A) differ from Col 1(IV) in size and their amino acid composition [4,6] and as shown here in their antigenic properties. In [4] evidence was provided indicating that the soluble tumor collagen consists of at least two different protein species. From the present data it is obvious that Col 1(IV) represents a third type of tumor collagen even though it may be only a fragment of the original protein. Reduction and denaturation of Col 1(IV) produced several polypeptide chains all smaller in size than one would expect for chains comprising a triple helix with mol. wt 20×10^4. Since pepsin and collagenase have been used to solubilize and to purify Col 1(IV) these proteases may have cleaved several peptide bonds along the constituent chains which in the non-reduced Col 1(IV) are still held together by the triple helical conformation and disulfide bridges.

Previous studies have shown that the tumor basement membrane contains no or only negligible amounts of interstitial collagens and that antibodies produced against tumor collagen react in immunofluorescence tests exclusively with the tumor matrix and with authentic basement membranes [4,8,14]. It is therefore likely that the collagen comprising Col 1(IV) is a variant of basement membrane collagen which may occur also in normal tissues. In fact, a fragment similar to Col 1(IV) has been recently obtained from human placenta (J. R., R. T., unpublished) a tissue known to be rich in basement membranes. Thus, digestion of authentic basement membranes by pepsin and bacterial collagenase may be a useful approach to identify this new type of collagen.

Acknowledgements

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