Isolation from bovine elastic tissues of collagen type VI and characterization of its form *in vivo*

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Foetal-bovine nuchal ligament and aorta, together with adult-bovine aorta and pregnant uterus, were extracted under dissociative conditions in the absence and in the presence of a reducing agent. A collagenous glycoprotein of M_r 140000 [designated component 140K(VI)], identified in these extracts as the major periodate/Schiff-positive component, was shown to be related to collagen type VI. Digestion of non-reduced extracts with pepsin yielded periodate/Schiff-positive peptides that, on the basis of their electrophoretic mobilities, amino acid analyses and peptide 'maps', were identical with type VI collagen fragments prepared by standard procedures. It is concluded that collagen type VI occurs *in vivo* as molecule comprising three chains of M_r 140000 in which the helical domains account for about one-third of each polypeptide. Biosynthetic experiments with nuchal-ligament fibroblasts in culture demonstrated that a bacterial-collagenase-sensitive [^3H]fucose-labelled glycoprotein, M_r 140000, was immunoprecipitated from culture medium by a specific antibody to the pepsin-derived form of collagen type VI. This result suggests that the collagenous polypeptides [140K(VI) components] represent the biosynthetic precursors of type VI collagen that do not undergo processing to smaller species before deposition in the extracellular matrix. Analyses of 5M-guanidinium chloride extracts of tissues with markedly different elastin contents and at different stages of development suggested that there was no relationship between collagen type VI and elastic-fibre microfibrils, a conclusion supported by the observation that the immunoprecipitated glycoprotein, M_r 140000, was distinct from the glycoprotein MFPI, M_r 150000, believed to be a constituent of these microfibrils [Sear, Grant & Jackson (1981) *Biochem. J.* **194**, 587–598].

Extraction of collagen from a variety of tissues by pepsin digestion together with rigorous fractionation techniques have demonstrated that collagen is not a single species but a family of related glycoproteins (for reviews see Bornstein & Sage, 1980; Miller & Gay, 1982; Weiss & Ayad, 1982). In the case of the major interstitial collagens (types I, II and III) the pepsin-derived forms are not significantly different from those observed *in vivo* (Miller, 1976). However, for many other collagens, notably basement-membrane collagen (type IV) (Schuppan *et al.*, 1980) and the disulphide-bonded cartilage collagen type IX (Ninomiya & Olsen, 1984; van der Rest *et al.*, 1985), non-collagenous interruptions along the main helix lead to consider-

able fragmentation on proteolysis, with consequent difficulty in determining the structure of the intact molecules. Similar problems have arisen in the case of an unusual high- M_r disulphide-bonded collagenous aggregate that was first isolated from pepsin digests of aortic intima (Chung *et al.*, 1976), but has since been isolated from a wide variety of tissues, including placenta (Furuto & Miller, 1980, 1981; Jander *et al.*, 1981, 1983; Odermatt *et al.*, 1983), liver (Rojkind *et al.*, 1979), skin (Laurain *et al.*, 1980), kidney (Risteli *et al.*, 1980), skeletal muscle (Sanes & Cheney, 1982), uterus (Abedin *et al.*, 1982) and nuchal ligament (Chambers *et al.*, 1984). This collagen has been termed 'intima collagen', 'short-chain collagen' and 'high-molecular-weight aggregate', but it is now designated collagen type VI (Furthmayr *et al.*, 1983; Jander *et*

Abbreviation used: SDS, sodium dodecyl sulphate.

et al., 1983). Detailed physicochemical studies have shown that the pepsin-derived aggregates are stabilized by intra- and inter-molecular disulphide bonds. Reduction of these bonds gives rise to several peptides that consist of a collagenous segment (M_r approx. 38000) attached at both ends to non-collagenous sequences, the lengths of which vary according to the extent and conditions of pepsin digestion, and results in chains of M_r in the range 40000–70000 (Jander *et al.*, 1981, 1983; Odermatt *et al.*, 1983). The molecular configuration of these chains is still controversial.

Extraction of extracellular-matrix components by using strong dissociative solvents with or without a disulphide-bond reducing agent, but in the presence of proteinase inhibitors, has resulted in the isolation of a collagenous glycoprotein from nuchal ligament and aorta (Sear *et al.*, 1981a; Gibson & Cleary, 1982; Knight *et al.*, 1984). This glycoprotein is highly disulphide-bonded, but on reduction gives rise to a component of M_r 140000 or 105000 estimated by non-collagenous and collagenous standards respectively. Evidence has been presented that the glycoprotein from nuchal ligament is related to the pepsin-derived form of type VI collagen (Knight *et al.*, 1984). We have now examined this relationship further and present additional proof that this glycoprotein of M_r 140000 extractable from nuchal ligament, aorta and uterus is a constituent polypeptide of collagen type VI *in vivo*. A preliminary account of this work has been reported (Ayad *et al.*, 1984).

Experimental

Materials

Pepsin (EC 3.4.23.1) from pig stomach mucosa (1:60:000), *Staphylococcus aureus* V8 proteinase and the protein standards used in gel electrophoresis were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). [^3H]Acetic anhydride (10Ci/mmol) and L-[5- ^3H]fucose (24Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.).

Foetal and adult bovine tissues were obtained from the abattoir within 1 h of death, and the foetal age was determined by the crown-rump length (Bogart, 1959). Adult pregnant uterus (150 days gestation) was examined either as a whole, or the myometrium and endometrium were examined separately. Different segments of the adult aorta were also analysed separately.

Extraction of intact collagen type VI

The original procedure used to extract nuchal ligament with dissociative solvents (Knight *et al.*, 1984) was modified as described by Ayad *et al.* (1984). Two fractions, designated G and GD, were

obtained by extracting each foetal- or adult-bovine elastic tissue with 5M-guanidinium chloride/50mM-Tris/HCl buffer, pH 8.0, and 5M-guanidinium chloride/100mM-Tris/HCl buffer, pH 8.3, containing 50mM-dithiothreitol respectively. Proteinase inhibitors (Knight *et al.*, 1984) were used throughout the extraction procedure. The extracts were extensively dialysed against water, and the resulting precipitates (Gp and GDp) were separated from the water-soluble (Gs and GDs) fractions by centrifugation. All fractions were stored at -20°C in the presence of proteinase inhibitors.

Preparation of the pepsin-derived form of collagen type VI

Tissues were extracted sequentially with 50mM-NaCl/50mM-Tris/HCl buffer, pH 7.4, and with 0.6M-KCl/50mM-Tris/HCl buffer, pH 7.4 (Ayad *et al.*, 1984), and the residues were digested with pepsin in 0.5M-acetic acid (enzyme/substrate ratio 1:100, w/w) at 4°C for 48 h. The solubilized collagens were fractionated to give collagen types I, III, IV, V and VI, and the type VI collagen was purified further (Abedin *et al.*, 1982; Chambers *et al.*, 1984).

DEAE-cellulose chromatography

The Gp fractions were stirred in 6M-urea/50mM-Tris/HCl buffer, pH 8.3, containing 1mM-N-ethylmaleimide, 10mM-6-aminohexanoic acid and 0.2mM-phenylmethanesulphonyl fluoride (6M-urea buffer) at 4°C for 24 h, and the solubilized material (80–100 mg of protein) was applied to a DEAE-cellulose column (1.6 cm $\times$ 14 cm) equilibrated with the 6M-urea buffer. The column was eluted at a rate of 55 ml/h with the 6M-urea buffer until the absorbance at 280 nm reached baseline after elution of the non-retarded material. A linear gradient of 0–0.3M-NaCl in the 6M-urea buffer was then applied over a total volume of 300 ml. Fractions (5 ml) were pooled as appropriate, dialysed against water and freeze-dried.

The GDs fractions were dialysed against 2M-urea/50mM-Tris/HCl buffer, pH 8.3, containing 1mM-N-ethylmaleimide, 10mM-6-aminohexanoic acid and 0.2mM-phenylmethanesulphonyl fluoride (2M-urea buffer), and approx. 100–150 mg of protein was applied to a DEAE-cellulose column (2.5 cm $\times$ 10 cm) equilibrated with the 2M-urea buffer. The column was eluted at the rate of 85 ml/h with the 2M-urea buffer until the pre-gradient peak was eluted and the absorbance at 280 nm reached baseline. A linear gradient of 0–0.5M-NaCl in 2M-urea buffer was then applied over a total volume of 300 ml. Fractions (5 ml) were pooled, dialysed against water and freeze-dried.

Affinity chromatography

The GDs fraction was purified by affinity

chromatography on concanavalin A-agarose and wheat-germ lectin-agarose as described in the *Affinity Chromatography Manual* (Pharmacia Fine Chemicals).

Pepsin digestion of Gp and GDs fractions

Gp and GDs fractions and the pooled samples obtained therefrom by DEAE-cellulose chromatography were treated with pepsin (enzyme/substrate ratio 1:100 or 1:10, w/w) for various time intervals (0–24 h) at 4°C in 0.5 M-acetic acid. Pepsin-derived soluble material from the uterus Gp fraction was fractionated by differential salt precipitation and by precipitation against phosphate-buffered saline (0.12 M-NaCl/10 mM-Na₂HPO₄/3 mM-KH₂PO₄), pH 7.2 (Abedin *et al.*, 1982). The uterus Gp fraction was also incubated in 5 M-guanidinium chloride/100 mM-Tris/HCl buffer, pH 8.3, containing 50 mM-dithiothreitol in the presence of proteinase inhibitors for 24 h at 4°C and, after equilibration into 0.5 M-acetic acid, digested with pepsin.

SDS/polyacrylamide-gel electrophoresis

Samples were analysed by the method of Laemmli (1970). Glycoproteins were detected with the periodate/Schiff reagent (Fairbanks *et al.*, 1971), and all proteins were detected with Coomassie Brilliant Blue.

Preparative SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide (6.5 or 8%, w/v) separating gels (15 cm × 15 cm × 0.3 cm) and 3% stacking gels (1 cm × 15 cm × 0.3 cm) without sample wells were prepared by the method of Laemmli (1970). Band detection (see below) was considerably improved when a stacking gel was used. In order to remove impurities, the gels were pre-electrophoresed for 4 h with two changes of electrode buffer. Samples (10–15 mg of protein/ml of sample buffer) were applied and electrophoresis continued at 10 mA/gel. After electrophoresis the gels were immersed in a freshly prepared solution of 4 M-sodium acetate for 10–20 min and the protein bands of interest were detected and excised (Higgins & Dahmus, 1979). The excised bands were eluted with 8 M-urea/0.5 M-Tris/HCl buffer, pH 8.3, containing 50 mM-2-mercaptoethanol (two 10 ml portions, each for 2–3 days), and the combined extracts were dialysed against 0.03% (w/v) SDS and then freeze-dried. The freeze-dried material was redissolved in water (0.5–1 ml) and the protein was precipitated by the addition of 10 vol. of pre-cooled (–30°C) acidified acetone (1 ml of 1 M-HCl/40 ml of acetone) (Bressan *et al.*, 1983). The protein precipitate was collected by centrifugation after 24 h at –30°C, dissolved in water and freeze-dried. Samples were (a) hydrolysed in constant-boiling HCl at 110°C for

20 h *in vacuo* and analysed on an LKB 4400 amino acid analyser and (b) analysed by SDS/polyacrylamide-gel electrophoresis to assess their purity.

Peptide 'mapping'

Proteins were radiolabelled with [³H]acetic anhydride (Gisslow & McBride, 1975) and electrophoresed in the first dimension on SDS/8% (w/v) polyacrylamide disc gels (Laemmli, 1970). The separated components were then digested within the gel with either CNBr (Barsh *et al.*, 1981) or *Staphylococcus aureus* V8 proteinase (Gibson *et al.*, 1983), and the resulting CNBr-cleavage or V8 proteinase-digest peptides were separated in the second dimension on SDS/polyacrylamide slab gels, 12% and 15% (w/v) respectively. The gels were then processed for fluorography (Laskey & Mills, 1975).

Immunoprecipitation of fibroblast culture medium with anti-(type VI collagen) antibodies

Foetal-calf nuchal-ligament fibroblasts were cultured until confluent and subsequently labelled with [³H]fucose (20 µCi/ml) in the presence of ascorbate (50 µg/ml) in serum-free medium for 24 h as described by Sear *et al.* (1981b). Proteinase inhibitors were then added to the medium. Antibodies against type VI collagen (pepsin-derived form) were raised in guinea pigs and purified by affinity chromatography (Evans *et al.*, 1983).

Fibroblast culture medium was incubated with the purified antibody followed by a second rabbit anti-(guinea pig IgG) antibody (Sear *et al.*, 1981b), and the immunoprecipitate was analysed by SDS/polyacrylamide-gel electrophoresis and fluorography as described above.

Results

Extraction of the intact form of collagen type VI

Nuchal ligament and aorta at various stages of foetal development and adult aorta and uterus were each extracted with guanidinium chloride in the absence and in the presence of a reducing agent to yield extracts G and GD respectively. Dialysis of the G and GD extracts against water resulted in the formation of precipitates Gp and GDp, which were separated from the water-soluble fractions Gs and GDs by centrifugation. A typical SDS/polyacrylamide-gel-electrophoretic analysis of the various fractions obtained from foetal aorta is shown in Fig. 1. Each fraction contained a complex spectrum of polypeptides, but the major glycoprotein, *M_r* 140 000, previously shown to be related to pepsin-extracted type VI collagen (Knight *et al.*, 1984), was observed only in the Gp and GDs fractions. In both fractions the glycoprotein ap-

Fig. 1. SDS/polyacrylamide-gel electrophoresis of dissociative extracts from foetal bovine aorta

Foetal bovine aorta was sequentially extracted with 5M-guanidinium chloride in the absence and in the presence of 50mM-dithiothreitol to yield extracts designated G and GD respectively as described in the text. Dialysis of the G and GD extracts against water produced insoluble (Gp and GDp) and soluble (Gs and GDs) fractions, which were analysed by SDS/6.5%-(w/v)-polyacrylamide-gel electrophoresis under non-reducing (tracks 1–4) and reducing (tracks 5–8) conditions. Tracks 1 and 5, Gs; tracks 2 and 6, Gp; tracks 3 and 7, GDs; tracks 4 and 8, GDp. The gels were stained with Coomassie Blue. The migration positions of standard type I collagen α1- and α2-chains are indicated together with component 140K(VI), which represents the major glycoprotein in the Gp and GDs fractions.

peared to be present as a large- M_r disulphide-bonded aggregate that did not enter the gel without reduction. However, subsequent isolation by ion-exchange chromatography and concentration of this glycoprotein from the GDs fractions revealed that the re-aggregation occurring after dialysis was incomplete (see Fig. 2b). This glycoprotein [referred to below as 140K(VI) component] was also found in foetal nuchal ligament, in both the myometrial and endometrial layers of the adult uterus and in both the upper and lower regions of the adult aorta.

Purification of 140K(VI) component from Gp and GDs fractions

Chromatographic analyses of the Gp and GDs fractions on DEAE-cellulose are shown in Figs. 2(a) and 2(b) respectively. The elution profiles of the Gp fraction were identical for all three tissues, but those of the GDs fraction differed considerably according to tissue source. However, in all cases, collagen type I was observed in the pre-gradient

peak, whereas the 140K(VI) component was eluted throughout the gradient (see inset gels). The 140K(VI) component from the Gp fraction was still present as a large- M_r aggregate before reduction (Fig. 2a inset), whereas that from the GDs fraction was present partly in a dissociated form, as shown by the diffuse band of approx. M_r 140000 (Fig. 2b). Differences between the GDs fractions of the three tissues were due mainly to the amount of material that was strongly bound to the DEAE-cellulose.

When the GDs fractions were chromatographed on concanavalin A-agarose, type I collagen was not retained, whereas the 140K(VI) component was bound together with other minor components and could be eluted with methyl α-D-mannoside (see Fig. 2b inset). When the concanavalin A-bound material was subsequently chromatographed on wheat-germ lectin-agarose, virtually all was retained and no further purification was apparent (result not shown). Preferential binding of the 140K(VI) component to concanavalin A-

Fig. 2. Chromatography of Gp and GDs fractions on DEAE-cellulose

(a) The Gp fractions from nuchal ligament (L), aorta (A) and uterus (U) were applied to a DEAE-cellulose column (1.6 cm $\times$ 14 cm) in 6M-urea/50 mM-Tris/HCl buffer, pH 8.3, containing proteinase inhibitors. The column was eluted until the absorbance at 280 nm reached baseline. A linear NaCl gradient (0–0.3 M) was then applied (arrow) over a total volume of 300 ml, and fractions 1 to 4 were pooled as shown. Inset shows the SDS/6.5%-(w/v)-polyacrylamide-gel electrophoresis of eluted fractions 1–4 from nuchal ligament analysed under reducing conditions (tracks 1–4 respectively) and fraction 3 analysed also in the unreduced state (track 5). (b) The GDs fractions of nuchal ligament, aorta and uterus were applied to a DEAE-cellulose column (2.5 cm $\times$ 10 cm) in 2M-urea/50 mM-Tris/HCl buffer, pH 8.3, containing proteinase inhibitors and eluted until the absorbance at 280 nm reached baseline. A linear NaCl gradient (0–0.5 M) was then applied (arrow) over a total volume of 300 ml, and fractions 1–4 were pooled as shown. Inset shows SDS/6.5%-(w/v)-polyacrylamide-gel electrophoresis of fractions 1–4 from uterus analysed under reducing conditions (tracks 1–4 respectively) and fraction 2 analysed also in the unreduced state (track 5). The concanavalin A-agarose-bound peak from a uterus GDs fraction analysed in the reduced state (see the text) is shown in track 6. Gels in (a) and (b) were stained with Coomassie Blue. The migration positions of non-collagenous proteins of known M_r are indicated.

agarose and wheat-germ lectin-agarose is consistent with the presence of mannose and glucosamine respectively (Knight *et al.*, 1984) and the presence of glucosamine in the pepsin-derived form of collagen type VI (Jander *et al.*, 1981, 1983).

Preparative SDS/polyacrylamide-gel electrophoresis and amino acid analysis of the 140K(VI) component

In order to prepare pure 140K(VI) component for amino acid analysis, polypeptides [particularly the $\alpha 1(I)$ chain of type I collagen] with electrophoretic mobilities close to that of the 140K(VI) component were first removed by either DEAE-cellulose or concanavalin A-agarose chromatography (see Figs. 2a and 2b) before preparative SDS/polyacrylamide-gel electrophoresis. Amino acid analyses of the 140K(VI) component prepared from the Gp and GDs fractions of nuchal ligament and uterus are shown in Table 1, and were similar to each other and to those reported previously for this component from nuchal ligament and aorta (Gibson & Cleary, 1982; Knight *et al.*, 1984). However, the contents of half-cystine and of methionine were often lower than in previous analyses or, in some cases, absent, and this was a

frequent observation for proteins eluted from polyacrylamide gels.

Relationship between 140K(VI) component and pepsin-derived form of collagen type VI

The effect of pepsin on the aggregated non-reduced form of the 140K(VI) component was monitored by staining the gels for periodate/Schiff-positive peptides after electrophoresis of the Gp fraction digested with pepsin for 24h at an enzyme/substrate ratio 1:100 (w/w) (Fig. 3). The 140K(VI) component of all three tissues was digested and three major components were observed (approx. M_r 35000, 45000 and 50000), as assessed by collagenous standards, which migrated in similar positions to those of the pepsin-derived peptides obtained by digestion of whole uterine tissue. Identical results were obtained after pepsin digestion of the DEAE-cellulose-bound peaks from the Gp fraction (Fig. 3). These peptide profiles were unchanged when a higher enzyme/substrate ratio of 1:10 (w/w) was used, and all subsequent digestions were carried out with a ratio of 1:100 (w/w). When the control and pepsin-digested Gp fractions were analysed in the non-

Table 1. *Amino acid analyses of the intact 140K(VI) component and pepsin-derived fragments of type VI collagen*
The 140K(VI) component was partially purified from the guanidinium chloride (Gp) or guanidinium chloride + dithiothreitol (GDs) fractions of nuchal ligament (L) and uterus (U) by ionby ion-exchange or affinity chromatography before isolation by preparative SDS/polyacrylamide-gel electrophoresis. Three major glycopeptides of M_r 35000, 45000 and 50000 produced by pepsin digestion of the uterus Gp fraction were also isolated by preparative SDS/polyacrylamide-gel electrophoresis.

Amino acid composition (residues/1000 residues)									
Amino acid	140K(VI) component				$10^{-3} \times M_r$...	Pepsin-derived fragments from uterus Gp fraction			
	GP		GDs			50	45	35	
	L	U	L	U					
Hyp	21	13	25	20		62	58	57	
Asp	108	90	83	84		77	90	71	
Thr	48	47	52	45		23	27	28	
Ser	63	77	129	87		40	41	59	
Glu	123	125	84	118		113	112	91	
Pro	48	50	67	54		96	57	93	
Gly	166	136	211	177		290	281	284	
Ala	77	72	77	70		52	57	61	
Cys	—	10	—	8		6	8	—	
Val	65	57	45	56		20	29	27	
Met	2	12	2	17		12	14	—	
Ile	42	37	25	39		24	23	26	
Leu	75	75	47	61		32	34	39	
Tyr	12	20	18	15		13	13	17	
Phe	35	31	26	30		21	18	21	
Hyl	10	9	10	8		40	46	22	
Lys	42	56	36	51		25	28	52	
His	14	15	29	21		4	4	10	
Arg	49	67	34	38		50	60	42

Fig. 3. Relationship between the 140K(VI) component and collagen type VI (pepsin-derived)

Gp fractions from aorta, nuchal ligament and uterus were analysed by SDS/8%-(w/v)-polyacrylamide-gel electrophoresis under reducing conditions before (tracks 1, 3 and 8) and after (tracks 2, 4, 6 and 7) digestion with pepsin for 24h at an enzyme/substrate ratio 1:100 (w/w) (see the text). Gels were stained with the periodate/Schiff reagent (Fairbanks *et al.*, 1971). Tracks 1 and 2, aorta; tracks 3 and 4, nuchal ligament; tracks 6, 7 and 8, uterus; track 5, collagen type VI (pepsin-derived) from whole uterus; track 9, fraction 3 from DEAE-cellulose chromatography of ligament Gp (see Fig. 2a) after pepsin treatment. M_r values are based on collagenous standards.

reduced state, the periodate/Schiff-positive material remained aggregated and did not penetrate the gel (results not shown). Analysis of the pepsin digests at various time intervals during the 24h incubation indicated the immediate loss of the 140K(VI) component and the appearance of components with M_r values between 140 000 and 50 000. Some of these intermediate components persisted in certain digests after 24h and longer incubation periods (Fig. 3).

When the periodate/Schiff-stained gels (Fig. 3) were overstained with Coomassie Blue, no peptides were revealed migrating near the three major periodate/Schiff-positive components of M_r 35 000–50 000, which were therefore eluted in a pure form after preparative gel electrophoresis. Typical amino acid analyses are shown in Table 1 for the pepsin-resistant peptides of the Gp fraction from uterus, and similar analyses were found for aorta and nuchal ligament. The analyses of the three peptides were similar to each other and characteristic of the pepsin-derived form of collagen type VI (Jander *et al.*, 1983). These results were substantiated by peptide 'mapping' with CNBr and V8 proteinase, which indicated that the three major peptides derived from the Gp fraction were identical with those of the type VI collagen peptides derived from pepsin digestion of whole tissue (Figs. 4a and 4b).

Pepsin digestion of either the GDs fraction or reduced Gp fraction resulted in the digestion of the 140K(VI) component to very small peptides, most of which migrated with the dye front on gel electrophoresis (results not shown).

Immunoprecipitation of newly synthesized type VI collagen

When antibodies to the pepsin-derived form of type VI collagen were used to precipitate newly synthesized macromolecules from ligament fibroblast culture medium, two fucosylated components of approx. M_r 140 000 and 240 000 were observed (Fig. 5). The major component, of M_r 140 000, co-migrated with the 140K(VI) tissue-extracted form, but was clearly distinguished from the fucosylated glycoprotein previously designated MFPI (Sear *et al.*, 1978, 1981b), which was not precipitated by the antibody (Fig. 5). The minor component, of M_r 240 000, was non-collagenous, as judged by its insensitivity to purified bacterial collagenase (result not shown).

Discussion

Earlier studies in this laboratory indicated a relationship between a collagenous glycoprotein, of M_r 140 000, extracted from nuchal ligament by guanidinium chloride, and collagen type VI,

Fig. 4. Peptide 'mapping' of ^3H -labelled pepsin-derived peptides from uterus Gp fraction and of collagen type VI (pepsin-derived)

Samples containing the pepsin-derived peptides from uterus Gp fraction (1) and collagen type VI (pepsin-derived) from whole uterus (2) were labelled with [^3H]acetic anhydride and subjected to two-dimensional peptide 'mapping' with either (a) CNBr cleavage or (b) *S. aureus* V8 proteinase digestion as described in the text. Peptides were detected by fluorography, and the arrows indicate the direction of migration of peptides of M_r 50000, 45000 and 35000 in the first dimension (see Fig. 3, tracks 5 and 7).

prepared from this tissue by pepsin digestion (Knight *et al.*, 1984). This relationship has now been further investigated in three tissues, nuchal ligament, aorta and uterus, which are known sources of the pepsin-derived type VI collagen (Chung *et al.*, 1976; Abedin *et al.*, 1982; Chambers *et al.*, 1984; Knight *et al.*, 1984) but differ markedly

in their elastin contents. The collagenous glycoprotein [now designated 140K(VI) component] was observed in all three tissues, had a similar amino acid composition to that observed previously (Gibson & Cleary, 1982; Knight *et al.*, 1984) and was extracted in an aggregated form by guanidinium chloride in the absence of a reducing agent. Pepsin digestion of the non-reduced aggregate resulted in the appearance of three major peptides, which had similar electrophoretic mobilities, amino acid compositions and CNBr-cleavage and V8 proteinase-digest peptide profiles to those of the corresponding peptides of type VI collagen prepared by pepsin digestion of the whole tissues. Moreover, the pepsin-derived peptides of the uterus Gp fraction had solubility properties identical with those previously described for the pepsin-derived form of uterus type VI collagen (Abedin *et al.*, 1982). It is therefore concluded that the 140K(VI) component represents a reduced component of the intact form of type VI collagen *in vivo*.

The relationship between the apparently single 140K(VI) component and the disparate pepsin-derived peptides is difficult to evaluate. Amino acid analyses (Table 1) indicate that about one-third of the 140K(VI) component is collagenous, and therefore the pepsin-resistant fragments of M_r 35000, 45000 and 50000 cannot be part of the same polypeptide chain. The CNBr-cleavage and V8 proteinase-digest peptide profiles (Figs. 4a and 4b) also indicate that at least two (and possibly all three) of the fragments are genetically distinct. It therefore appears that the intact form of type VI collagen consists of three chains of identical molecular mass (M_r 140000), at least two of which are genetically distinct. Immunoprecipitation of a major fucosylated component, of M_r 140000, from cell-culture medium supports this conclusion, and also suggests that type VI collagen is not processed to a smaller species before deposition in the extracellular matrix. This observation is in agreement with other similar studies on the origin of type VI collagen (Hessle & Engvall, 1984; von der Mark *et al.*, 1984; Heller-Harrison & Carter, 1984). In contrast, the biosynthetic and immunoblotting studies by Trueb & Bornstein (1984) suggest that the newly synthesized chains have a much larger molecular mass and are processed to components of M_r 180000 and 190000 in the tissue. We have observed components of similar molecular mass in extracts of the various tissues, but these bands were usually less pronounced and less periodate/Schiff-positive than the 140K(VI) component (see Figs. 2a and 2b inset gels) and were not found in our immunoprecipitation studies (Fig. 5).

The assembly and function of type VI collagen *in vivo* have not been established, although rotary-

Fig. 5. Comparison of [^3H]fucose-labelled glycoproteins synthesized and secreted by bovine nuchal-ligament fibroblasts in culture

Foetal-calf nuchal-ligament fibroblasts were labelled with [^3H]fucose in the presence of ascorbate, and the culture medium was incubated subsequently with a specific anti-[collagen type VI (pepsin-derived)] antibody as described in the text. The immunoprecipitate (track 1) and medium containing glycoprotein MFPI (Sear *et al.*, 1978, 1981*b*) from which component 140K(VI) had been precipitated (track 2) were analysed by SDS/6.5%-(w/v)-polyacrylamide-gel electrophoresis under reducing conditions. Samples in tracks 1 and 2 were also combined and analysed in track 3. Polypeptides were detected by fluorography. The mobility of the tissue-extracted 140K(VI) component is indicated.

shadowing studies suggest a microfibrillar origin (Furthmayr *et al.*, 1983). Microfibrillar structures are ubiquitous constituents of connective tissues, providing a link between the major extracellular macromolecules (collagen, proteoglycans, elastin), basal lamina and cells (Kraus, 1983; Cleary & Gibson, 1983). Early work on the microfibrils associated with elastic fibres in nuchal ligament concluded that these structural elements comprised disulphide-bonded non-collagenous glycoproteins (Ross & Bornstein, 1969). However, the original observation by Ross & Bornstein (1969) that these elastin-associated microfibrils could be extracted by highly dissociative solvents only in the presence of a reducing agent has since been refuted (Prosser *et al.*, 1984). In the present study a

reducing agent was also not a prerequisite for the extraction of intact type VI collagen, as a large proportion was extracted by guanidinium chloride alone. However, extraction of the three tissues with different elastin contents at different stages of development indicated that type VI collagen (a) did not predominate during the early gestational phase when elastic microfibrils are prevalent (Greenlee *et al.*, 1966), (b) was not preferentially extracted from the aortic arch (where the elastin concentration is highest), and (c) was more pronounced in the less elastic uterine tissue. These observations suggest that there is no relationship between type VI collagen and the elastin-associated microfibrils, a conclusion supported by immunofluorescent staining of this collagen in several tissues (Jander *et al.*, 1981; Cleary & Gibson, 1983; von der Mark *et al.*, 1984).

The glycoprotein described here as 140K(VI) component was previously designated tissue MFPI (Knight *et al.*, 1984; Ayad *et al.*, 1984) because of its similarity to a major glycoprotein, MFPI (M_r 150000), synthesized and secreted by cultured nuchal-ligament fibroblasts and reported to be a constituent of elastic microfibrils (Sear *et al.*, 1978, 1981*a,b*). However, antibodies to type VI collagen (pepsin-derived form) did not precipitate glycoprotein MFPI but precipitated only the 140K(VI) component (Fig. 5). It is therefore concluded that these two glycoproteins are immunologically distinct. This observation is confirmed by other studies demonstrating that collagen type VI and glycoprotein MFPI differ in their respective solubilities, enzymic susceptibilities and the effect of ascorbate on their synthesis (S. Ayad, C. A. Chambers, L. Berry, C. A. Shuttleworth & M. E. Grant, unpublished work). We therefore propose that type VI collagen and glycoprotein MFPI are constituents of different microfibrillar systems.

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References

- Abedin, M. Z., Ayad, S. & Weiss, J. B. (1982) *Biosci. Rep.* **2**, 493–502
- Ayad, S., Shuttleworth, C. A. & Grant, M. E. (1984) *Biochem. Soc. Trans.* **12**, 1052–1053
- Barsh, G. S., Peterson, K. E. & Byers, P. H. (1981) *Collagen Relat. Res.* **1**, 543–548
- Bogart, R. (1959) *Improvement of Livestock*, p. 316, Macmillan, New York
- Bornstein, P. & Sage, H. (1980) *Annu. Rev. Biochem.* **49**, 957–1003
- Bressan, G. M., Castellani, I., Colombatti, A. & Volpin, D. (1983) *J. Biol. Chem.* **258**, 13262–13267
- Chambers, C. A., Shuttleworth, C. A., Ayad, S. & Grant, M. E. (1984) *Biochem. J.* **220**, 385–394

- Chung, E., Rhodes, R. K. & Miller, E. J. (1976) *Biochem. Biophys. Res. Commun.* **71**, 1167–1174
- Cleary, E. G. & Gibson, M. A. (1983) *Int. Rev. Connect. Tissue Res.* **10**, 97–209
- Evans, H. B., Ayad, S., Abedin, M. Z., Hopkins, S., Morgan, K., Walton, K. W., Weiss, J. B. & Holt, P. J. L. (1983) *Ann. Rheum. Dis.* **42**, 575–581
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Furthmayr, H., Wiedemann, H., Timpl, R., Odermatt, E. & Engel, J. (1983) *Biochem. J.* **211**, 303–311
- Furuto, D. K. & Miller, E. J. (1980) *J. Biol. Chem.* **255**, 290–295
- Furuto, D. K. & Miller, E. J. (1981) *Biochemistry* **20**, 1635–1640
- Gibson, M. A. & Cleary, E. G. (1982) *Biochem. Biophys. Res. Commun.* **105**, 1288–1295
- Gibson, G. J., Kielty, C. M., Garner, C., Schor, S. L. & Grant, M. E. (1983) *Biochem. J.* **211**, 417–426
- Gisslow, M. T. & McBride, B. C. (1975) *Anal. Biochem.* **68**, 70–78
- Greenlee, T. K., Ross, R. & Hartman, J. L. (1966) *J. Cell Biol.* **30**, 59–71
- Heller-Harrison, R. A. & Carter, W. G. (1984) *J. Biol. Chem.* **259**, 6858–6864
- Hessle, H. & Engvall, E. (1984) *J. Biol. Chem.* **259**, 3955–3961
- Higgins, R. C. & Dahmus, M. E. (1979) *Anal. Biochem.* **93**, 256–260
- Jander, R., Rauterberg, J., Voss, B. & von Bassewitz, D. B. (1981) *Eur. J. Biochem.* **114**, 17–25
- Jander, R., Rauterberg, J. & Glanville, R. W. (1983) *Eur. J. Biochem.* **133**, 39–46
- Knight, K. R., Ayad, S., Shuttleworth, C. A. & Grant, M. E. (1984) *Biochem. J.* **220**, 395–403
- Krauh, J. M. (1983) *Connect. Tissue Res.* **11**, 153–167
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- Laurain, G., Delvincourt, T. & Szymanowicz, A. G. (1980) *FEBS Lett.* **120**, 44–48
- Miller, E. J. (1976) *Mol. Cell. Biochem.* **13**, 165–192
- Miller, E. J. & Gay, S. (1982) *Methods Enzymol.* **82**, 3–32
- Ninomiya, Y. & Olsen, B. R. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3014–3018
- Odermatt, E., Risteli, J., Van Delden, V. & Timpl, R. (1983) *Biochem. J.* **211**, 295–302
- Prosser, I. W., Gibson, M. A. & Cleary, E. G. (1984) *Aust. J. Exp. Biol. Med. Sci.* **62**, 485–505
- Risteli, J., Bachinger, H. P., Engel, J., Furthmayr, H. & Timpl, R. (1980) *Eur. J. Biochem.* **108**, 239–250
- Rojkind, M., Giambrone, M.-A. & Biempica, L. (1979) *Gastroenterology* **76**, 710–719
- Ross, R. & Bornstein, P. (1969) *J. Cell Biol.* **40**, 366–381
- Sanes, J. R. & Cheney, J. M. (1982) *J. Cell Biol.* **93**, 442–451
- Schuppan, D., Timpl, R. & Glanville, R. W. (1980) *FEBS Lett.* **115**, 297–300
- Sear, C. H. J., Kewley, M. A., Jones, C. J. P., Grant, M. E. & Jackson, D. S. (1978) *Biochem. J.* **170**, 715–718
- Sear, C. H. J., Jones, C. J. P., Knight, K. R. & Grant, M. E. (1981a) *Connect. Tissue Res.* **8**, 167–170
- Sear, C. H. J., Grant, M. E. & Jackson, D. S. (1981b) *Biochem. J.* **194**, 587–598
- Trueb, B. & Bornstein, P. (1984) *J. Biol. Chem.* **259**, 8597–8604
- van der Rest, M., Mayne, R., Ninomiya, Y., Seidoh, N. G., Chretien, M. & Olsen, B. R. (1985) *J. Biol. Chem.* **260**, 220–225
- von der Mark, H., Aumailley, M., Wick, G., Fleischmajer, R. & Timpl, R. (1984) *Eur. J. Biochem.* **142**, 493–502
- Weiss, J. B. & Ayad, S. (1982) in *Collagen in Health and Disease* (Weiss, J. B. & Jayson, M. I. V., eds.), pp. 1–17, Churchill-Livingstone, London