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A collagenous glycoprotein found in dissociative extracts of foetal bovine nuchal ligament

Evidence for a relationship with type VI collagen

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A collagenous glycoprotein (M_r 140000) was isolated from dissociative extracts of foetal bovine nuchal ligament and purified by a combination of ion-exchange and gel-filtration chromatography. This glycoprotein (designated MFPI) exists as a large-M_r disulphide-bonded aggregate in the absence of a reducing agent. The purified glycoprotein was shown to contain about 6% (w/w) carbohydrate, mostly as galactose, glucose and mannose. Amino acid analysis showed the presence of hydroxyproline and hydroxylysine, indicative of its collagenous nature. The collagenous nature of this glycoprotein was further investigated by enzyme digestion. Pepsin digestion produced three major fragments, which were identical with peptides of type VI collagen. Bacterial-collagenase digestion of the unreduced glycoprotein also produced several discrete peptides. However, reduction of the glycoprotein before bacterial-collagenase digestion resulted in the degradation of these discrete peptides. Glycoprotein MFPI extracted in dissociative conditions appears to be a larger-M_r form of type VI collagen, believed to originate from microfibrillar components in the intact tissue.

Much of our current understanding of elastic-fibre formation is derived from studies on the developing bovine nuchal ligament. A well-defined sequence of morphological changes has been described in which the deposition of amorphous elastin in the extracellular matrix is preceded by the appearance of microfibrillar structures aligned in the direction of the developing elastic fibre. Accordingly it has been proposed that the microfibrils may function as a scaffold for the deposition of soluble elastin. The nature of the microfibrils is ill-defined, but they are generally believed to comprise one or more glycoproteins (for review see Cleary & Gibson, 1983).

Early studies by Ross & Bornstein (1969) demonstrated that highly dissociative conditions in combination with a reducing agent were particularly effective in solubilizing the microfibrillar components of elastic fibres. Most subsequent studies of the glycoproteins in elastic tissues have adopted this approach, and it has been possible to identify in extracts of foetal and mature bovine nuchal ligaments discrete glycoproteins whose M_r values have been determined to be 34000 (Serafini-Fracassini *et al.*, 1981), 35000 (Kawaguchi, 1982), 140000 (Gibson & Cleary, 1982), 150000 and 300000 (Sear *et al.*, 1978, 1981*a*,*b*).

Considerable interest has centred on the two glycoproteins designated MFPI (M_r 150000) and MFPII (M_r 300000), which were identified in ligament extracts and could be immunoprecipitated from culture medium of bovine nuchal-ligament fibroblasts by using an antiserum raised against a microfibrillar protein extract (Sear *et al.*, 1978, 1981*a*,*b*). These two species are synthesized by a number of different cell types, including human skin fibroblasts (Sear *et al.*, 1977), foetal bovine tendon fibroblasts (Taylor *et al.*, 1982), dental-pulp fibroblasts (Shuttleworth *et al.*, 1982) and periodontal-ligament fibroblasts (J. W. Smalley, C. A. Shuttleworth & M. E. Grant, unpublished work). The native glycoprotein MFPI recovered from cell-culture fluid is partially collagenous (Sear *et al.*, 1981*a*,*b*; Taylor *et al.*, 1982), and a protein

Abbreviation used: SDS, sodium dodecyl sulphate.

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identified by Gibson & Cleary (1982) in extracts of foetal-calf aorta and nuchal ligament exhibits similar properties. The definitive establishment of a relationship between this collagenous glycoprotein and elastic-fibre microfibrils will require the production of totally specific antisera. The preparation of the purified glycoprotein from intact tissue is a first stage in this process, and in the present paper we report on the isolation, purification and partial characterization of the glycoprotein MFPI from foetal bovine nuchal ligament. We also present evidence that this glycoprotein is a larger-M_r form of the unique highly disulphide-bonded collagenous aggregate known variously as 'high-molecular-weight' (HMW) aggregate (Furuto & Miller, 1980, 1981; Laurain *et al.*, 1980; Abedin *et al.*, 1982) or as short-chain or intima collagen (Jander *et al.*, 1981, 1983), but more recently classified as type VI collagen (Odermatt *et al.*, 1983; Furthmayr *et al.*, 1983; Jander *et al.*, 1983).

Experimental

Materials

Bacterial collagenase (EC 3.4.24.3) (form III) was obtained from Advance Biofactures Corporation (Lynbrook, NY, U.S.A.). Pepsin (EC 3.4.23.1) from pig stomach mucosa (1:60000) was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), as were the protein standards ovalbumin, bovine serum albumin, phosphorylase *a*, β-galactosidase and myosin used in gel electrophoresis.

Foetal calves were obtained from Manchester Abattoir within 1 h of maternal death. Nuchal ligaments were dissected from calves of 100–230 days foetal age as determined by crown-rump length (Bogart, 1959), and sequentially extracted as shown in Scheme 1.

Purification of the glycoproteins from the crude extract

Portions of the dialysed freeze-dried G extract or D extract (200mg) were suspended in 20ml of 6M-urea buffered with 25mM-Tris/HCl buffer, pH7.5, and containing 2mM-*N*-ethylmaleimide and 10mM-6-amino-n-hexanoic acid. Dithiothreitol was added to 50mM concentration, and incubation was continued at pH8.0 for 2h at 37°C with occasional stirring. The reaction mixture was cooled to room temperature, iodoacetamide added to 100mM and the reaction continued for a further 30min at 20°C. The reduced and *S*-carboxymethylated extract was clarified by centrifugation at 3000*g* for 20min, then dialysed overnight at 4°C against two changes of 20vol. of urea/Tris/HCl solution. This material was applied to a column (14.0cm × 1.8cm) of DEAE-cellulose equilibrated at 4°C in urea/Tris/HCl solution and washed on with a further 3 column vol. of starting buffer. The

Nuchal ligament (31.4g wet wt.)

1. Milled in liquid N_2
2. Extracted with 250ml (×3) of chloroform/methanol (3:1, v/v)
3. Extracted with 300ml (×2) of buffered saline

→ Supernatant: saline extract (0.38g dry wt.)

4. Extracted with 300ml (×2) of 0.5M-acetic acid

→ Supernatant: acid extract (0.11g dry wt.)

5. Extracted 300ml (×2) of 6M-guanidinium chloride

→ Supernatant: G extract (1.79g dry wt.)

6. Extracted with 300ml (×2) of 6M-guanidinium chloride containing 50mM-dithiothreitol

→ Supernatant: D extract (0.54g dry wt.)

Residue (1.30g dry wt.)

Scheme 1. *Schematic flow diagram of the extraction procedure applied to foetal bovine nuchal ligament*
Step 2 was carried out at 4°C for 4h. All subsequent steps were carried out at 4°C for 24h in the presence of the proteinase inhibitors 6-amino-n-hexanoic acid (0.1M), *N*-ethylmaleimide (2mM), phenylmethanesulphonyl fluoride (0.5mM), EDTA (2mM) and NaN_3 (0.1%, w/v). Each extract and the final residue were dialysed exhaustively against distilled water at 4°C, freeze-dried and stored desiccated at 4°C. The total yields of each fraction for a typical experiment are shown in parenthesis.

bound material was eluted with a linear gradient of 0–0.3M-NaCl in the urea/Tris/HCl solution. The column was washed with at least 3 column vol. of the 0.3M-NaCl/urea/Tris/HCl solution and finally with 3 column vol. of 2M-NaCl/urea/Tris/HCl solution. The flow rate was 20ml/h, and 5ml fractions were collected.

The major large-M_r glycoprotein, which was usually eluted early in the linear gradient, was concentrated in the cold on a 50ml Amicon ultrafilter with a PM 10 membrane to approx. 5ml final volume. This sample was applied to a column (48cm × 3.5cm) of Sepharose CL-4B and eluted with 25mM-Tris/HCl buffer, pH7.5, containing 6M-urea, 0.5M-NaCl and the same inhibitors as for the previous column. This column was run at room temperature at 16.3ml/h, and 5ml fractions were collected. Suitable fractions were dialysed in the cold-room against running tap water and then finally against distilled water at 4°C, and freeze-dried.

Fractions requiring further purification (20mg) were redissolved in 3ml of 62.5mM-Tris/HCl buffer, pH6.8, containing 2M-urea, 2% (w/v) SDS, 2mM-phenylmethanesulphonyl fluoride and 10% (v/v) glycerol. A small amount of insoluble material was removed by centrifugation and the supernatant was applied to a column (80cm × 1.8cm) of Bio-Gel A-15m and eluted at room temperature at 3.5ml/h, with 1.55ml fractions being collected. The eluent used was 0.1% (w/v) SDS buffered at pH8.0 with 50mM-NH_4HCO_3 and containing 1mM-dithiothreitol, 20mM-6-amino-n-hexanoic acid, 0.02% (w/v) NaN_3 and 0.5mM-NaCl. Fractions were analysed by their absorbance at 280nm and dialysed at 4°C against distilled water containing approx. 2g of anion-exchange resin AG 1-X2 (Bio-Rad Laboratories) to bind any diffusible SDS.

Pepsin digestion

Samples containing glycoproteins of interest were incubated with pepsin at an enzyme/substrate ratio of 1:100 (w/w) at 4°C for 4h and 24h in 0.5M-acetic acid, pH2.8. The incubation mixture was freeze-dried and analysed by SDS/polyacrylamide-gel electrophoresis.

Nuchal ligament that had only been extracted with 50mM-Tris/HCl buffer, pH7.4, containing 50mM-NaCl was also digested with pepsin for 24h as described above, and the solubilized collagens were fractionated according to established methods (Abedin *et al.*, 1982; Chambers *et al.*, 1984). A collagen fraction purifying in a manner identical with, and having similar properties to, HMW aggregate or type VI collagen (see the introduction) was obtained.

Bacterial collagenase digestion

Appropriate fractions, either reduced or unreduced, were incubated with highly purified bacterial collagenase at an enzyme/substrate ratio of 1:10 (w/w) in 50mM-Tris/HCl buffer, pH7.4, containing 5mM-$CaCl_2$, 2mM-*N*-ethylmaleimide and 1mM-phenylmethanesulphonyl fluoride at 37°C for 4h or 24h. The incubation mixture was centrifuged (40000*g* for 1h), and the bacterial collagenase-insoluble and -soluble fractions were analysed by SDS/polyacrylamide-gel electrophoresis. Control samples of bovine serum albumin and a mixture of collagen types I and III prepared from whole nuchal ligament (Chambers *et al.*, 1984) were used to confirm the specificity of the collagenase.

SDS/polyacrylamide-gel electrophoresis

The procedures described by Sear *et al.* (1981*a*) were used. The gels were scanned and the M_r values of the separated polypeptides estimated by using standards of known M_r.

Amino acid analysis

Protein samples (1mg) were hydrolysed for 24h at 110°C in 2ml of redistilled 6M-HCl in tubes initially flushed with N_2 and then evacuated. Amino acids were determined on a JEOL model JLC6AH analyser.

Carbohydrate analysis

The trimethylsilyl derivatives were prepared by the method of Clamp *et al.* (1973) and analysed by g.l.c. in a Pye model 24 series 104 chromatograph.

Results

Extraction of ligament glycoproteins

When milled and delipidated tissue was extracted according to the procedures outlined in Scheme 1 it was found that the bulk of the material (over 60% by wt.) was solubilized by 6M-guanidinium chloride. Initial extractions with 50mM-NaCl buffered to pH7.4 extracted mainly serum proteins, and two glycoproteins of M_r 65000 and 35000. The dilute-acetic acid extract accounted for less than 4% of the total weight of extracted material and contained mainly type I collagen and two glycoproteins of M_r 68000 and 65000.

Analyses of the G extract obtained with 6M-guanidinium chloride revealed a complex spectrum of polypeptides, the major Coomassie Blue-staining species present migrating in positions corresponding to M_r approx. 250000, 180000, 140000, 68000, 65000 and 45000 as judged by the mobilities of non-collagenous proteins of known M_r. When a duplicate gel was stained with periodic acid/Schiff reagent the major large-M_r glycopro-

tein present in the extract corresponded to the protein band of M_r 140000. This glycoprotein was found to be similar to a major fucosylated glycoprotein designated MFPI, synthesized by foetal bovine ligament fibroblasts (Sear *et al.*, 1978, 1981*a*). Accordingly, this tissue-derived glycoprotein is referred to below as glycoprotein MFPI. Two other glycoproteins (M_r 68000 and 65000) were also observed.

When the residue after 6M-guanidinium chloride extraction was treated with 6M-guanidinium chloride in the presence of a reducing agent, 50mM-dithiothreitol, approx. 12–15% (w/w) of the original tissue was solubilized. This D extract was similar to the 6M-guanidinium chloride extract in terms of polypeptide composition and amino acid analysis (results not shown). MFPI was the predominant glycoprotein in this fraction, but quantitatively more of this species was recovered in the G extract, which was therefore used in subsequent purification procedures.

Purification of the glycoprotein MFPI

The G extract was not readily soluble in all non-denaturing solutions tested, even after reduction and carboxymethylation. All subsequent purification procedures were therefore conducted under highly dissociative conditions, and the initial purification was achieved at 4°C on DEAE-cellulose in 25mM-Tris/HCl buffer, pH7.5, containing 6M-urea and in the presence of proteinase inhibitors (Fig. 1). Trace amounts of collagen type I in the extract did not bind to the column and were eluted in fraction 1 (Fig. 1). The glycoprotein MFPI was eluted early in the linear salt gradient (fraction 3) with the glycoprotein of M_r 65000 and several other polypeptides, and was partially separated from the glycoprotein of M_r 68000 and other larger-M_r glycoproteins in fraction 4. Fraction 6, representing the material eluted in the 2M-NaCl wash, contained very little protein, but appeared to be predominantly proteoglycan, as all the uronic acid in the extract was recovered in this fraction.

The material recovered in fraction 3 generally constituted approx. 30% by weight of the total material applied to the column. To obtain maximum yields of glycoprotein MFPI it was best to avoid precipitation of the polypeptides in fraction 3 because they could not always be readily redissolved. Samples were therefore concentrated by ultrafiltration before being applied to a preparative column of Sepharose CL-4B run in 25mM-Tris/HCl buffer, pH7.5, containing 6M-urea and 0.5M-NaCl (Fig. 2). This procedure yielded one major included peak with a shoulder on the leading edge. The large peak at the total volume of the

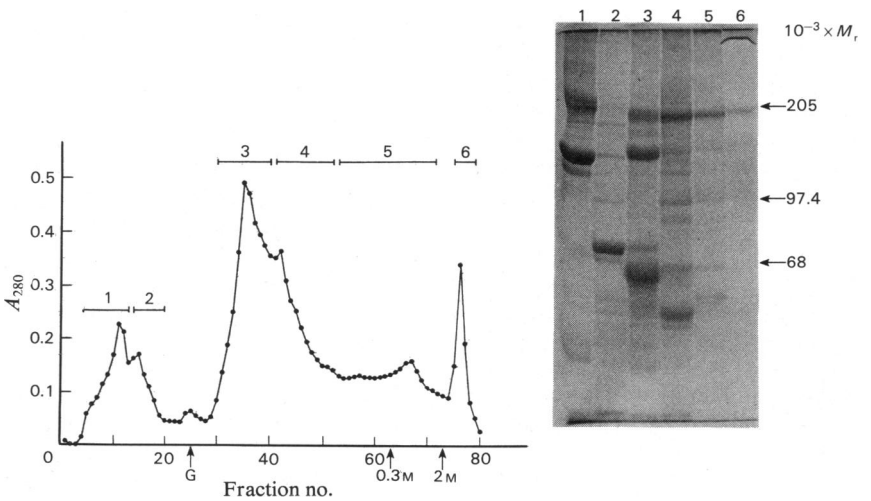

Fig. 1. *Chromatography of G extract on DEAE-cellulose*

G extract (200mg) isolated as shown in Scheme 1 was reduced and alkylated (see the Experimental section) and applied to a DEAE-cellulose column (14.0cm × 1.8cm) in 6M-urea/25mM-Tris/HCl, pH7.5, containing 2mM-*N*-ethylmaleimide and 10mM-6-amino-n-hexanoic acid. The column was eluted at 4°C with urea/Tris/HCl buffer, pH7.5 (20ml/h), until a constant baseline was established, and then with a linear NaCl gradient (0–0.3M) in the urea/Tris/HCl buffer over a total volume of 200ml. The column was finally washed with 0.3M- and 2M-NaCl in urea/Tris/HCl buffer. G, start of linear gradient; 0.3M and 2M indicate start of NaCl washes. Tubes were pooled as shown to give fractions 1–6. Inset shows the SDS/polyacrylamide-gel electrophoresis [6.5% (w/v) gels] of these fractions. The gels were stained with Coomassie Blue. M_r values of non-collagenous proteins are indicated.

column can be attributed to the absorbance of proteinase inhibitors included in the sample. Glycoprotein MFPI was present as the major polypeptide in elution tubes numbered 39–45, which were pooled to yield fraction I (Figs. 2*a* and 2*b*). Polypeptides with apparent M_r values in the range 60000–70000 were eluted in fraction II (Figs. 2*a* and 2*c*).

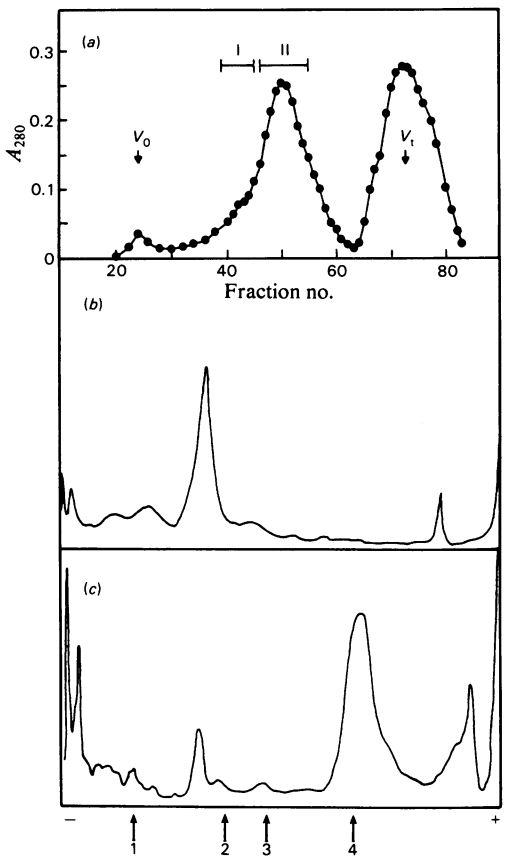

Fig. 2. *Chromatography on Sepharose CL-4B*
(*a*) Fraction 3 from the DEAE-cellulose column (Fig. 1) was concentrated by ultrafiltration, applied to a Sepharose CL-4B column (48cm × 3.5cm) and eluted at room temperature (16.3ml/h) with 6M-urea/25mM-Tris/HCl, pH7.5, containing 0.5M-NaCl, 2mM-*N*-ethylmaleimide and 10mM-6-amino-n-hexanoic acid. Tubes 39–45 and 47–55 were pooled to give fractions I and II respectively. Fractions I and II were analysed on 6.5% (w/v) SDS/polyacrylamide gels [direction of migration cathode (–) to anode (+)], stained with Coomassie Blue and scanned in a Beckman model 25 spectrophotometer at 560nm. (*b*) and (*c*) show the densitometric traces of fractions I and II respectively. Non-collagenous standards of (1) myosin (M_r 205000), (2) β-galactosidase (M_r 116000), (3) phosphorylase *a* (M_r 97400), and (4) bovine serum albumin (M_r 68000) are indicated.

The fraction containing primarily glycoprotein MFPI was further purified on a Bio-Gel A-15m column by dissolving the dialysed freeze-dried fraction I (Fig. 2) in Tris/HCl/urea buffer containing 2.0% SDS (Fig. 3). The glycoprotein was recovered in a highly purified form (fraction A, Fig. 3) and represented the only detectable component on SDS/polyacrylamide-gel electrophoretograms stained with Coomassie Blue. The final yield represented approx. 0.1–0.3% of the original dry tissue weight. Fraction B and the V_t peak (Fig. 3) appeared consistently where the sample was dissolved in the buffer system described in the Experimental section, but neither fraction appeared to contain proteinaceous material.

Chemical composition of glycoprotein MFPI

The amino acid analysis of purified glycoprotein MFPI recovered from the Bio-Gel A-15m column is shown in Table 1, where it is compared with those of a 'microfibrillar protein fraction' and other glycoproteins purified from bovine nuchal ligament. The presence of hydroxyproline and hydroxylysine residues in significant amounts is important to note, for the collagenous glycoprotein MFPI detectable in cell cultures (Sear *et al.*, 1981*a*; Taylor *et al.*, 1982) contains similar proportions of these hydroxylated amino acids. Also noteworthy is the presence of half-cystine residues, which appear to participate in intermolecular cross-link formation, for glycoprotein MFPI exists as disulphide-bonded aggregates that are excluded from 6.5% SDS/polyacrylamide gels when analysed in the absence of a reducing agent (Fig. 4*a*). The carbohydrate content of glycoprotein MFPI was found to be 6.1% (w/w), and g.l.c. analysis (average of two determinations) indicated the following composition expressed as percentage of the carbohydrate total weight: galactose (33.0%); glucose (24.3%); mannose (25.0%); *N*-acetylglucosamine (12.9%); fucose (4.5%); sialic acid (trace).

Susceptibility of glycoprotein MFPI to enzymic digestion

The collagenous nature of glycoprotein MFPI was investigated by the classical methods of pepsin digestion and bacterial-collagenase digestion. In these experiments it was found possible to use as substrate a G extract that had been dialysed exhaustively against water. This procedure yielded an insoluble precipitate (Gp) that was found to contain all the glycoprotein MFPI, but the other glycoproteins (M_r 68000 and 65000) remained water-soluble. Thus it was possible to follow the fate of glycoprotein MFPI by monitoring polyacrylamide-gel electrophoretograms for periodic

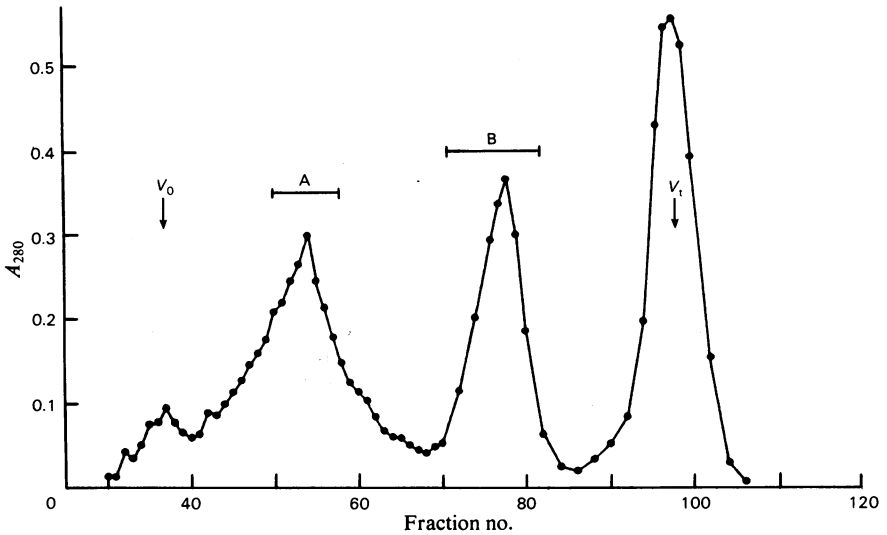

Fig. 3. *Chromatography on Bio-Gel A-15m*

Fraction I (20mg) from the Sepharose CL-4B column (Fig. 2) was dissolved in 62.5mM-Tris/HCl buffer, pH6.8, containing 2M-urea, 2% (w/v) SDS, 2mM-phenylmethanesulphonyl fluoride and 10% (v/v) glycerol (3ml) and applied to a Bio-Gel A-15m column (80.0cm × 1.8cm). The column was eluted at room temperature with 50mM-NH_4HCO_3 buffer, pH8.0, containing 0.5M-NaCl, 0.1% (w/v) SDS, 0.02% (w/v) NaN_3, 1mM-dithiothreitol and 20mM-6-amino-n-hexanoic acid at the rate of 3.5ml/h. Tubes 50–58 were pooled to give fraction A, which contained glycoprotein MFPI as the only polypeptide observed on SDS/polyacrylamide-gel electrophoresis. Tubes 71–82 were pooled to yield fraction B.

Table 1. *Amino acid analyses of ligament glycoproteins extracted under dissociative conditions*

	Composition (residues/1000 residues)				
	Glycoprotein MFPI*	Ligament glycoprotein	Glycoprotein A	Structural glyco-protein	Microfibrillar protein preparation
M_r ...	140000	140000	35000	34000	
Reference ...		Gibson & Cleary (1982)	Kawaguchi (1982)	Serafini-Fracassini *et al.* (1981)	Ross & Bornstein (1969)
Amino acid					
Hyp	21	22	12	0	
Asp	98	98	121	119	114
Thr	50	36	48	44	55
Ser	63	40	69	61	59
Glu	123	105	118	103	111
Pro	77	64	65	47	70
Gly	120	194	106	112	120
Ala	76	67	66	72	59
½Cys	17†	16	51†	27	80
Val	55	65	49	56	54
Met	10	10	11	14	16
Ile	34	38	37	33	45
Leu	83	62	76	84	57
Tyr	19	18	39	43	30
Phe	31	34	46	48	32
Hyl	7	23	1		
Lys	45	35	31	47	37
His	18	13	14	18	14
Arg	54	59	37	55	45

* Analyses represent the averages for nine determinations.
† Measured as *S*-carboxymethylcysteine.

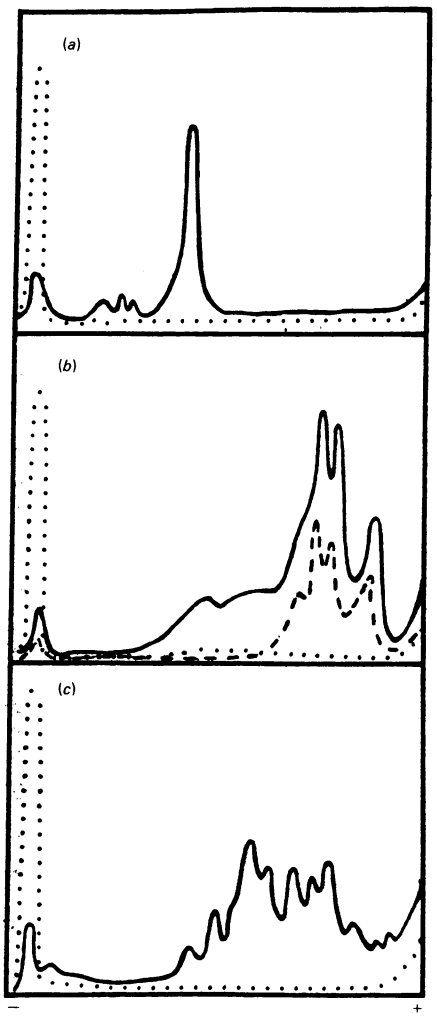

Fig. 4. *Susceptibility of glycoprotein MFPI to pepsin and bacterial collagenase*

G extract was dialysed against water, and the insoluble precipitate (Gp) containing the glycoprotein MFPI, but not the glycoproteins of M_r 65000 and 68000 (see the text), was analysed on SDS/6.5% (w/v) polyacrylamide gels before (*a*) and after digestion with either pepsin (*b*) or bacterial collagenase (*c*). Electrophoresis was carried out in the absence (·····) and presence (——) of 50mM-dithiothreitol. The reduced form of type VI collagen prepared from pepsin digests of nuchal ligament by established procedures (see the text) was also analysed (– – – –). The gels were stained with the periodic acid/Schiff reagent (Fairbanks *et al.*, 1971) and scanned on a Beckman model 25 spectrophotometer at 580nm. (*a*) Gp; (*b*) Gp incubated in 0.5M-acetic acid (pH2.8) at 4°C for 24h with pepsin at an enzyme/substrate ratio of 1:100 (w/w); (*c*) Gp incubated in 50mM-Tris/HCl buffer, pH7.4, containing 5mM-$CaCl_2$, 2mM-*N*-ethylmaleimide and 1mM-phenylmethanesulphonyl fluoride at 37°C for 4h with bacterial collagenase (enzyme/substrate ratio 1:10, w/w). Only the bacterial-collagenase-insoluble fraction is shown.

acid/Schiff-positive peptides after digestion of the Gp fraction (Fig. 4).

Following pepsin digestion for either 4h or 24h all the periodic acid/Schiff-positive material remained at the top of an SDS/polyacrylamide gel if electrophoresis was conducted without prior reduction. However, when these digests were reduced and then electrophoresed, three major periodic acid/Schiff-positive components were obtained, which migrated in positions identical with those observed for the reduced peptides of type VI collagen (Fig. 4*b*). When gels were overstained with Coomassie Blue, α-chains of collagen types I and III were detected (results not shown), indicating that collagenous species in the Gp fraction assume a native pepsin-resistant helical conformation when the guanidinium chloride is removed by dialysis.

After digestion of the Gp fraction with bacterial collagenase all the periodic acid/Schiff-positive material remained in the insoluble fraction (see the Experimental section). The gel-electrophoretic analysis of the insoluble fraction obtained after bacterial-collagenase digestion for 4h is shown in Fig. 4(*c*). In the unreduced form only large-M_r aggregates were observed, whereas reduction of the sample before electrophoresis resulted in the appearance of ten to twelve periodic acid/Schiff-positive components.

Discussion

Glycoproteins closely associated with the fibrous elements of connective tissues have become recognized as important structural and biological determinatants of tissue function (for reviews see Anderson, 1976; Kleinman *et al.*, 1981; Aplin & Hughes, 1982). However, their presence within the insoluble extracellular matrix, usually in relatively minor amounts, poses considerable difficulties of extraction and isolation, especially in the native form. Consequently many groups have resorted to the use of highly dissociative conditions to release both glycoproteins and proteoglycans from collagenous and elastic tissues.

In the present study the glycoprotein MFPI was detected in both the G extract and the D extract (Scheme 1) of foetal bovine nuchal ligament. Also present in these extracts were glycoproteins of apparent M_r 68000 and 65000, but subsequent studies indicated that they could be separated from glycoprotein MFPI by dialysis against water. No evidence was obtained for a glycoprotein of M_r

34000–35000 that has been described in dissociative extracts of nuchal ligament (Serafini-Fracassini *et al.*, 1981; Kawaguchi, 1982), although a glycoprotein of this size was detected in the initial NaCl/Tris/HCl extract (results not shown). The marked insolubility of glycoprotein MFPI required that purification procedures were conducted under dissociative conditions, and by a combination of ion-exchange and gel-filtration chromatography it was possible to isolate a fraction that was judged by SDS/polyacrylamide-gel electrophoresis to contain glycoprotein MFPI free of contaminating proteins. Amino acid analyses of this glycoprotein (Table 1) are virtually identical with that of the collagenous glycoprotein (CL-glycoprotein) extracted from nuchal ligament and aorta by Gibson & Cleary (1982). Both species have similar electrophoretic mobilities, but they appear to exhibit different chromatographic behaviour in that glycoprotein MFPI consistently bound to DEAE-cellulose (Fig. 1), whereas CL-glycoprotein did not (Gibson & Cleary, 1982).

It is noteworthy that collagenous glycoproteins, whose M_r values have been reported in the range 130000–160000, have been isolated from lung parenchyma (Lafuma *et al.*, 1982), skeletal muscle (Marton & Arnason, 1982), and corneal stroma (Alper, 1983), as well as from cultured lung (Carter, 1982) and tendon (Taylor *et al.*, 1982) fibroblasts. The accuracy of the M_r values quoted for these hybrid molecules is difficult to assess, for the measurements have generally been based on non-collagenous protein standards. However, glycoprotein MFPI isolated from tissue extracts migrates only slightly more slowly than the α1(I) collagen chains and has M_r 105000 based on collagen standards (see Fig. 1). Earlier studies on the radiolabelled glycoprotein MFPI synthesized by cultured ligament fibroblasts demonstrated that the newly synthesized component migrated on electrophoresis in SDS/polyacrylamide gels just ahead of pro-α2(I) chains (Sear *et al.*, 1981*a*) with an M_r of 150000 based on standard non-collagenous proteins. Preliminary studies indicate that the extracted tissue glycoprotein MFPI does not co-migrate with newly synthesized glycoprotein MFPI on gel electrophoresis (S. Ayad, C. A. Shuttleworth & M. E. Grant, unpublished work), and further work will be required to establish if any processing of this molecule occurs before deposition in the extracellular matrix.

The properties of glycoprotein MFPI synthesized by ligament fibroblasts established that this collagenous component was distinct from the well-characterized collagen types I to V (Sear *et al.*, 1981*a*,*b*). However, the occurrence of a short-chain collagen, now designated type VI collagen (Odermatt *et al.*, 1983; Furthmayr *et al.*, 1983; Jander *et al.*, 1983), in peptide digests of a variety of tissues known to contain microfibrillar glycoproteins raised the possibility that glycoprotein MFPI might represent the type VI collagen parent molecule *in situ*. Gibson & Cleary (1982) had also noted that the hydroxyproline, hydroxylysine and cystine contents of CL-glycoprotein suggested the possibility of a relationship to the short-chain collagens from aortic intima and placenta. In studies conducted in this laboratory on the collagens present in peptic digests of foetal bovine nuchal ligament it had been noted that a minor fraction with some of the properties of type VI collagen could be obtained (Chambers *et al.*, 1984). When the fate of glycoprotein MFPI treated with pepsin was monitored by SDS/polyacrylamide-gel electrophoresis, the pattern of polypeptides obtained was identical with that of a type VI collagen preparation obtained by standard pepsin digestion of intact tissue followed by appropriate salt fractionation procedures (Fig. 4*b*). Both pepsin-derived fractions consisted of disulphide-bonded aggregates, which on reduction gave peptides of identical mobility and similar staining intensities (Fig. 4).

Studies on the susceptibility of glycoprotein MFPI to digestion with bacterial collagenase provided further evidence for a relationship with type VI collagen. The unreduced aggregated form of glycoprotein MFPI was cleaved by collagenase at a limited number of sites, resulting in a modified aggregate that on reduction yielded a complex mixture of discrete peptides similar to those observed in collagenase digests of newly synthesized glycoprotein MFPI (Sear *et al.*, 1981*a*). However, when glycoprotein MFPI was reduced before incubation with collagenase, the glycoprotein was completely degraded. This behaviour is totally analogous to that of type VI collagen (Furuto & Miller, 1981; Abedin *et al.*, 1982). The extensive disulphide-bonding of glycoprotein MFPI and type VI collagen is apparently responsible for this stability against proteolysis, but the ability to extract glycoprotein MFPI with 6M-guanidinium chloride in the absence of a reducing agent suggests that intermolecular disulphide bonding is less important than intramolecular or interchain disulphide linkages. A similar conclusion has been reached by Furthmayr *et al.* (1983) in their electron-microscopical studies on the structure of type VI collagen, which indicate that the pepsin-derived species might originate from a microfibrillar component.

Evidence is accumulating that glycoprotein MFPI is present in all tissues from which type VI collagen can be isolated with pepsin (S. Ayad, C. A. Shuttleworth & M. E. Grant, unpublished work), and the results described in the present paper provide evidence that the pepsin-derived

microfibrillar type VI collagen is indeed derived from glycoprotein MFPI. Further studies will be necessary to define the true organization of this collagenous glycoprotein *in vivo* and to establish a definitive relationship between glycoprotein MFPI and the microfibrillar glycoprotein that plays a key role in elastogenesis (Cleary & Gibson, 1983).

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