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Type VI collagen is composed of a 200 kd subunit and two 140 kd subunits

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We have isolated type VI collagen, a transformation-sensitive glycoprotein of the extracellular matrix, in an intact, disulfide-bonded form. The protein contains a 200 kd subunit and two different 140 kd subunits in a stoichiometric ratio. Based on the amount of hydroxyproline and hydroxylysine, the sensitivity to bacterial collagenase and the cross-reactivity with antibodies to pepsin-extracted type VI collagen, we have identified the 200 kd subunit as the α3(VI) chain and the two 140 kd subunits as the α1(VI) and α2(VI) chains. The α3(VI) chain is synthesized by cells in culture as a precursor of 260 kd, while no precursor form of the other two chains could be detected.

Key words: collagen type VI/extracellular matrix/beaded filaments/procollagen/transformation-sensitive

Introduction

Type VI collagen is a widely distributed glycoprotein of the extracellular matrix produced by fibroblasts and other mesenchymal cells (see Engel *et al.*, 1985, for a review). Considerable interest has focused on this protein because its biosynthesis is almost completely blocked after transformation of fibroblasts by viruses (Carter, 1982a; Trüeb *et al.*, 1985). Type VI collagen is a hybrid molecule consisting of one collagenous and two globular domains. The detailed structure of the intact molecule, however, has not been elucidated due largely to its insolubility in physiological buffers and to the difficulty of isolating it in pure form. The collagenous domain is readily extracted from tissues with pepsin and has consequently been characterized extensively. It is composed of three different polypeptide chains α1(VI)-pepsin, α2(VI)-pepsin and α3(VI)-pepsin which are disulfide-linked to form large aggregates. Several laboratories have prepared antisera against these pepsin-extracted fragments in order to identify the intact subunits of type VI collagen in guanidinium extracts of tissues or in fibroblast cultures by the immunoblotting technique (Jander *et al.*, 1984; Trüeb and Bornstein, 1984; Von der Mark *et al.*, 1984). Depending on the antisera used, polypeptides of 140 kd (Jander *et al.*, 1984; Von der Mark *et al.*, 1984; Heller-Harrison and Carter, 1984; Hessle and Engvall, 1984; Ayad *et al.*, 1985; Gibson and Cleary, 1985), 180/190 kd /Trüeb and Bornstein, 1984) or 240 kd (Trüeb and Bronstein, 1984; Hessle and Engvall, 1984) have been reported to represent the full-size subunits of type VI collagen. To account for such diversity in molecular mass, one would have to assume that the three subunits differ considerably in size and/or that the subunits are synthesized in the form of precursors which might be processed incompletely before deposition in the matrix. We sought therefore to purify intact type VI collagen in amounts sufficient for direct biochemical, rather than indirect immunological analyses. Here we identify the intact α1(VI) and α2(VI) chains as two different polypeptides of 140 kd and the intact α3(VI) chain as a third polypeptide of 200 kd.

Results

A guanidinium extract from bovine uterus was fractionated into four peaks when chromatographed on Sephacryl S-500 in the presence of SDS (Figure 1). The first peak did not appear to contain any protein as there were no Coomassie Blue stained bands evident on a polyacrylamide gel (Figure 2). The second peak contained a proteinaceous aggregate that did not enter the polyacrylamide gel in the absence of reducing agents, but gave rise to two bands of 200 and 140 kd after reduction. Based on the intensity of the stain (and considering the difference in molecular mass) the two bands were obtained in a stoichiometric ratio of 1.05:2 (n = 4). The third and fourth peak contained polypeptides which did not change their electrophoretic migration behaviour on a gel by reduction. When transferred to nitrocellulose and incubated with an antiserum against pepsin-extracted type VI collagen, only the polypeptides from the second peak showed a positive reaction. Affinity-purified antibodies against α1(VI)-pepsin bound specifically to the 140 kd band (Figure 3). Antibodies against α2(VI)-pepsin also reacted with the 140 kd region, however, the two staining patterns differed slightly from each other. Antibodies against α3(VI)-pepsin bound specifically to the 200 kd band. Thus, the 140 kd band must contain the intact parent polypeptides of α1(VI)-pepsin as well as α2(VI)-pepsin, while the 200 kd band must represent the parent polypeptide of α3(VI)-pepsin. This conclusion is supported by experiments with antibodies against the intact components (Figure 4). Antibodies prepared against the 200 kd band reacted

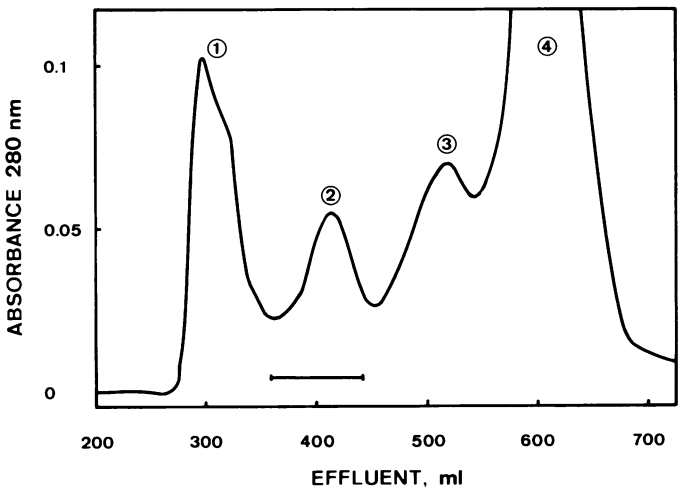

Fig. 1. Molecular sieve chromatography of an extract from bovine uterus. Protein (100 mg in 10 ml) was dissolved in a buffer containing 0.2% SDS and chromatographed on a 3.3 × 70 cm column of Sephacryl S-500. The pool containing type VI collagen is indicated by a bar.

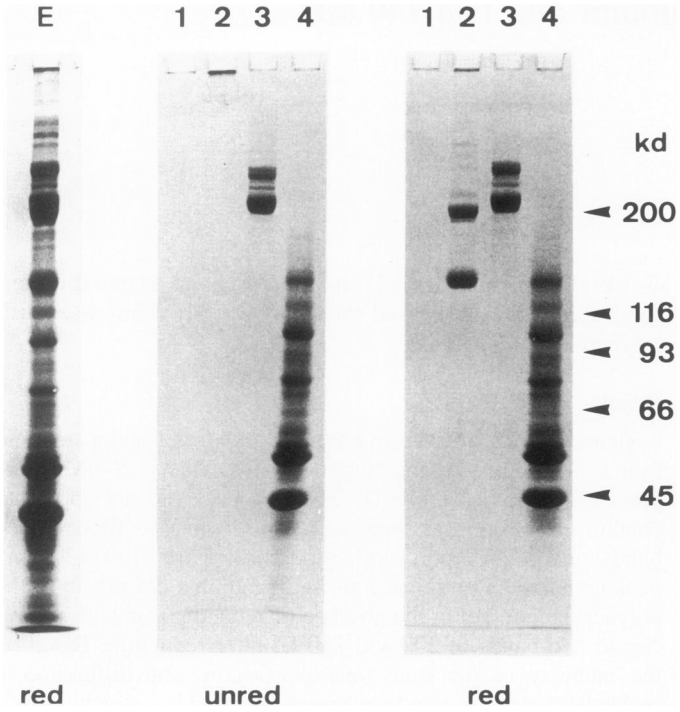

Fig. 2. SDS–polyacrylamide gel electrophoresis of proteins pooled from Figure 1. Samples were run in the absence (**unred**) or presence (**red**) of 3% 2-mercaptoethanol on a 5–10% gradient polyacrylamide gel. Lane numbers refer to the different peaks of Figure 1, E shows the crude extract prior to gel filtration. The migration of globular protein standards is indicated in the right margin.

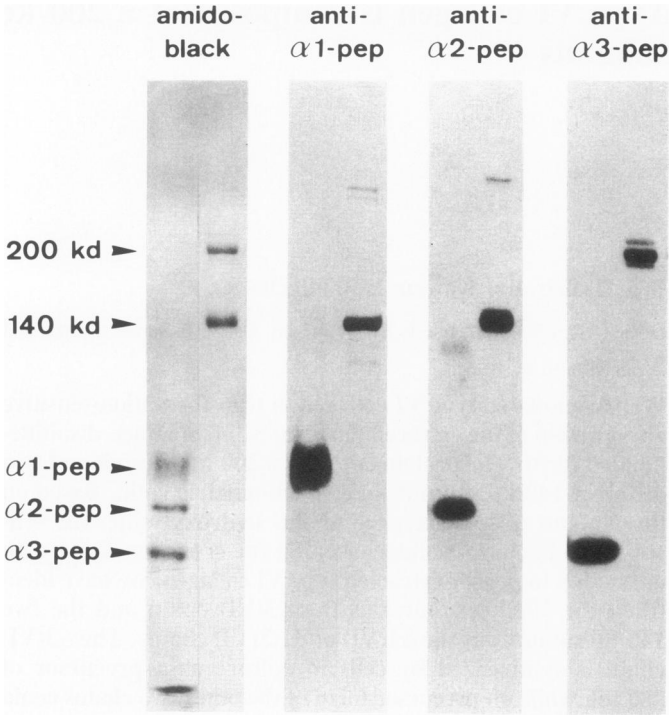

Fig. 3. Immunoblot with antibodies against pepsin-extracted type VI collagen. Pepsin-extracted type VI collagen, form B (left lanes) and the polypeptides from peak 2, Figure 1 (right lanes) were resolved on 5–10% gradient polyacrylamide gels in the presence of 2-mercaptoethanol, transferred to nitrocellulose and either stained with amido black or incubated with affinity-purified antibodies against the individual chains of type VI collagen as indicated. Bound antibodies were visualized by ^{125}I-labeled protein A and autoradiography.

specifically with α3(VI)-pepsin, while antibodies prepared against the 140 kd band bound to both α1(VI)-pepsin and α2(VI)-pepsin (and to the dimeric component of 100 kd).

After reduction and alkylation the 200 kd subunit could be separated from the 140 kd polypeptides on Sephacryl S-400 (Figure 5). Amino acid analyses revealed small amounts of hydroxyproline and hydroxylysine and relatively large amounts of glycine in the 200 kd and the 140 kd components (Table I). Assuming that a globular protein contains an average of 80 residues of glycine/1000 amino acids, we estimate that not more than 25–35% of the full length of the polypeptides consist of collagen-specific sequences (Gly-X-Y). In fact, hydroxyproline and hydroxylysine are 3- to 4-fold lower in the 200 kd and the 140 kd components when compared with the pepsin-extracted chains of type VI collagen (Jander *et al.*, 1983).

The existence of collagenous sequences is also indicated by digestion experiments with bacterial collagenase (Figure 6). From the 200 kd polypeptide a piece of 40 kd was removed after incubation with the enzyme. The collagenous domain of α3(VI) (M_r 38 kd) is therefore likely to be located close to one end of the 200 kd polypeptide. The 140 kd band was split by the action of collagenase into three fragments of 65, 55 and 35 kd. Since the total mass of the three fragments exceeds 140 kd, they must originate from two different 140 kd polypeptides. Additional digestion experiments with pepsin were then performed, these only being successful when SDS was exhaustively removed from the unreduced type VI collagen of peak 2, Figure 1. Three peptides were liberated from the protein by pepsin and these peptides migrated on a polyacrylamide gel with mobilities identical to those of a standard of pepsin-extracted type VI collagen (Figure 6C).

Affinity-purified antibodies against the 140 kd band were used for immunoprecipitation of type VI collagen from the cell culture medium of fibroblasts grown in the presence of ascorbic acid and [^{35}S]methionine. The precipitated material did not enter a polyacrylamide gel in the absence of reducing agents, but was resolved into two bands of 260 and 140 kd after reduction (Figure 7). Both bands were susceptible to digestion with bacterial collagenase. Similar polypeptides of 260 and 140 kd were obtained when the cell layers were subjected to sequential extraction with detergent and urea as described by Carter (1982b). Although this material may contain contaminating proteins in addition to type VI collagen, its amino acid composition closely resembled that of the purified type VI collagen subunits (Table I). An antiserum was prepared against the 260 kd band excised from the polyacrylamide gel. On immunoblots it reacted with the 200 kd band as well as with α3(VI)-pepsin, but not with the 140 kd band or α1(VI)-pepsin and α2(VI)-pepsin (Figure 4, right-hand side). Thus, the 260 kd band must represent a precursor of α3(VI). Accordingly, the 140 kd band must contain α1(VI) and α2(VI) which do not appear to be synthesized in a precursor form.

Discussion

Isolation of type VI collagen in an intact and native form has been attempted by several laboratories, but the final products were not sufficiently pure to determine the exact subunit composition of this protein (Carter, 1982b; Jander *et al.*, 1984; Von der Mark *et al.*, 1984; Gibson and Cleary, 1985). Even in the presence of 4 M guanidinium various contaminants tend to adhere tenaciously to type VI collagen (Gibson and Cleary, 1985). Efforts have therefore focused on investigating individual subunits

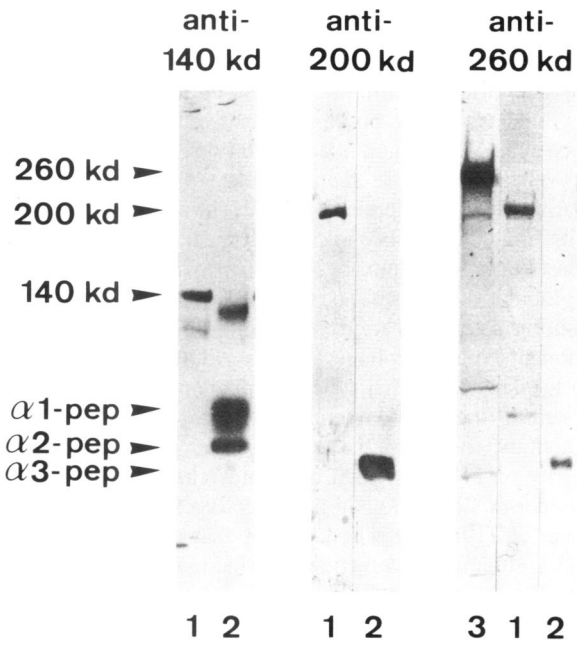

Fig. 4. Immunoblot with antisera against the intact subunits of type VI collagen. Intact type VI collagen (**lane 1**), pepsin-extracted type VI collagen, form B (**lane 2**) and an extract from fibroblast cultures (Carter, 1982b) (**lane 3**) were resolved on a 5–10% gradient polyacrylamide gel under reducing conditions, transferred to nitrocellulose and stained with different antisera (not affinity purified) against the 140 kd band, the 200 kd band or the 260 kd band as indicated.

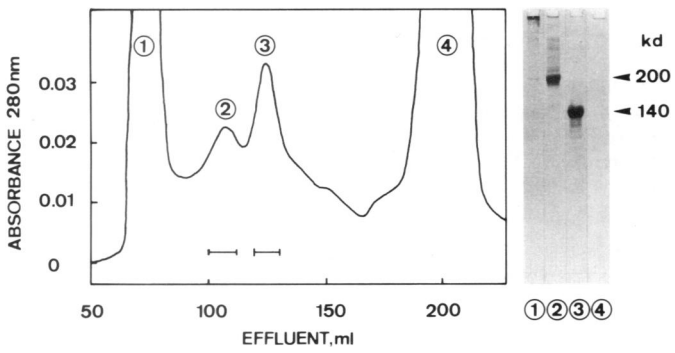

Fig. 5. Molecular sieve chromatography of the type VI collagen subunits. Reduced and alkylated type VI collagen (10 mg in 3 ml) was chromatographed on a 2.1 × 66 cm column of Sephacryl S-400 in a buffer containing 0.2% SDS. Pooled fractions of the peaks (numbered 1–4) were analysed on 5–10% gradient polyacrylamide gels (**lanes 1–4**). Peak 1 contains cross-linked material, peaks 2 and 3 contain the 200 kd and the 140 kd components (pooled as indicated) and peak 4 contains the reagents used for alkylation.

of the protein, such as the collagenous domain obtained by extraction of tissues with pepsin or the 140 kd chains isolated from guanidinium extracts after reduction and alkylation of disulfide bonds. Since at least two of the collagenous fragments proved to be immunologically related to the 140 kd chains, it was generally assumed that type VI collagen consists of three different 140 kd polypeptides which happen to migrate as one band on polyacrylamide gels (Jander *et al.*, 1984; Gibson and Cleary, 1985; Aumailley *et al.*, 1985).

Addition of SDS to all column buffers has enabled us to isolate intact, disulfide-bonded type VI collagen in a virtually pure form. The protein contained, in addition to the previously characterized 140 kd chains, a 200 kd polypeptide in a stoichiometric ratio of 2:1. Small amounts of hydroxyproline and hydroxylysine, sensitivity to bacterial collagenase and immunological cross-reactivity with antibodies against α3(VI)-pepsin clearly establish this polypeptide as the intact α3(VI) chain. Taken together with our

Table I. Amino acid composition of the subunits of type VI collagen and of its precursor produced by fibroblasts

	140 kd	200 kd	Precursor (140 + 260 kd)
Aspartic acid	117	107	98
Glutamic acid	119	117	110
Hydroxyproline	31	16	17
Serine	55	69	54
Threonine	38	43	46
Glycine	171	145	155
Alanine	70	85	71
Arginine	63	70	58
Proline	68	62	79
Valine	32	55	57
Methionine	9	7	12
Isoleucine	23	31	43
Leucine	67	83	70
Phenylalanine	35	35	36
Hydroxylysine	21	7	13
Lysine	32	33	40
Histidine	26	22	14
Tyrosine	23	13	27

Residues are given per 1000 amino acid residues. No corrections were made for incomplete hydrolysis or hydrolytic loss. Cysteine and tryptophan were not determined.

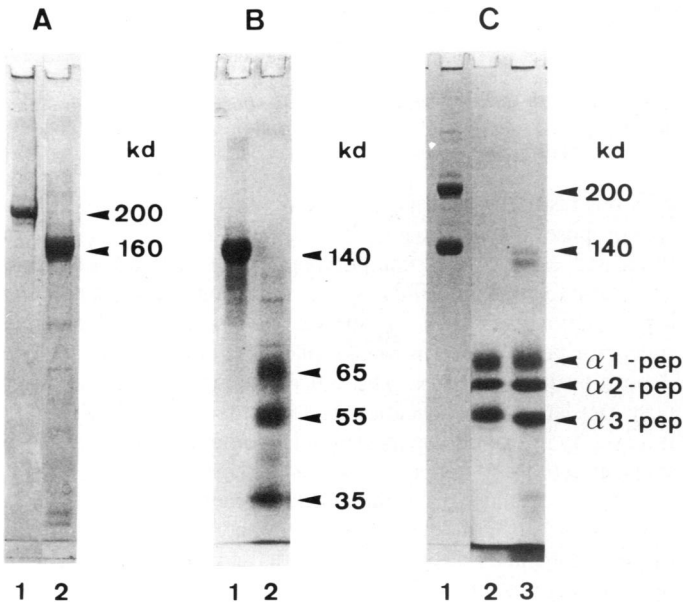

Fig. 6. Enzymatic digestion of type VI collagen. The purified 200 kd subunit (**A**) and the purified 140 kd subunits (**B**) were incubated for 2 h with bacterial collagenase and the digests were resolved on 5–10% gradient polyacrylamide gels (gel A contained a larger stacking gel than gel B). The starting material (1) and the collagenase-resistant fragments (2) are indicated by their molecular masses based on globular protein standards. Neither increasing the enzyme:substrate ratio nor prolonging the digestion times altered the results. Unreduced type VI collagen (**C**) was incubated for 24 h with pepsin and the digest was resolved on a 5–10% gradient polyacrylamide gel in the presence of 2-mercaptoethanol. 1 = starting material, 2 = digested material, 3 = standard of pepsin-extracted type VI collagen, form B.

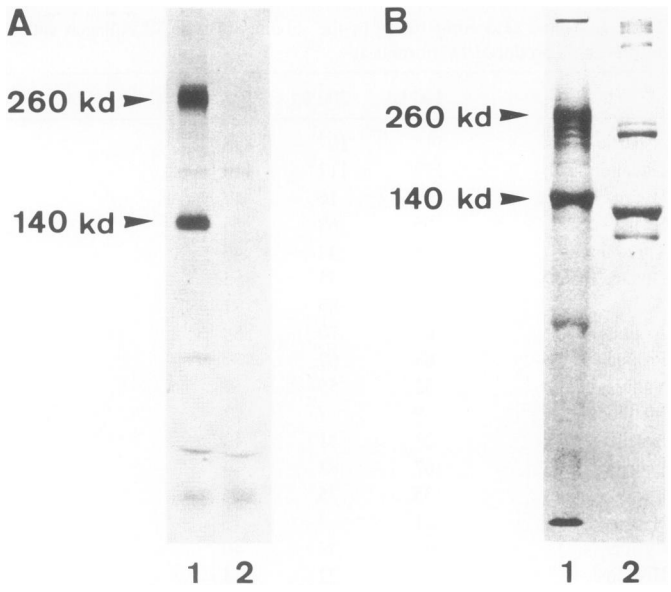

Fig. 7. Type VI collagen precursors produced by fibroblasts in culture. **A**: ^{35}S-methionine-labeled type VI collagen was precipitated from the culture media of WI 38 cells with antibodies against the 140 kd subunits. The precipitated material was resolved on a 6% polyacrylamide gel in the presence of 2-mercaptoethanol and visualized by fluorography (**lane 1**). A control obtained with unrelated antibodies is shown in **lane 2**. **B**: Cell layers of WI 38 cells were extracted with detergent and urea (Carter, 1982b). Undissolved material consisting mainly of type VI collagen was resolved on a 3–12% gradient polyacrylamide gel in the presence of 2-mercaptoethanol and visualized by staining with Coomassie Blue (**lane 1**). **Lane 2** shows a standard of type I collagen. The molecular mass of the major polypeptides based on globular protein standards is indicated in the left margins.

previous results (Trüeb and Bornstein, 1984; Trüeb *et al.*, 1985) we propose the following model: α1(VI) and α2(VI) are translated as two different polypeptides of 125 kd each and secreted into the extracellular space, following hydroxylation and glycosylation, as chains of 140 kd. No processing of putative precursor peptides seems to take place with these chains before deposition in the matrix. The α3(VI) subunit is secreted after post-translational modification as a precursor of 260 kd. There is as yet no information concerning its unmodified form because translation of this subunit *in vitro* has not been successful to date. In the extracellular space, the 260 kd precursor is converted to a polypeptide of 200 kd. This conversion, however, appears to be incomplete in tissues other than uterus and aorta or with species other than bovine (unpublished data). Most likely, the two 140 kd subunits and the 200 kd subunit form a single heterotrimeric molecule. So far, we have not succeeded in separating the two different 140 kd polypeptides from each other using extracts from bovine or human tissues. With other species, however, a separation of the two polypeptides in a ratio of 1:1 was readily achieved (unpublished data). Other investigators have not yet reported on the identification of the intact α3(VI) chain. Polypeptides of ~200 kd, however, have also been noted in type VI collagen preparations of other groups (Jander *et al.*, 1984; Gibson and Cleary, 1985) and these polypeptides could not be removed from the 140 kd subunits even under denaturing conditions. In addition, a polypeptide of 260 kd related to type VI collagen has been detected with the aid of monoclonal antibodies by Engvall *et al.* (1986) in the culture medium of fibroblasts. This 260 kd polypeptide was intimately associated with the 140 kd subunits and could not be separated by reduction without denaturation. Another glycoprotein of 250 kd has been found by Carter (1982a) in the extracellular matrix of cultured fibroblasts. This GP250, however, seems to be distinct from the α3(VI) precursor because it was not susceptible to digestion with bacterial collagenase.

With its hybrid structure of collagenous and globular domains and its curious way of processing one, but not the other two subunits, type VI collagen is unique among the known collagen types. What role these features may play remains purely speculative. The propeptides of α3(VI) could lend type VI collagen its limited solubility in physiological buffers, as noted in fibroblast culture medium, by shielding hydrophobic sequences of the globular domains. Once the propeptides are removed, the hydrophobic sequences are exposed and subsequently induce aggregation of type VI collagen via its globular domains. Bruns (1984) has suggested that type VI collagen represents a major component of 'beaded filaments', a structural component found throughout the connective tissue. These filaments exhibit a typical periodicity of 110 nm which correlates closely with the structural model of filamentous type VI collagen described by Furthmayr *et al.* (1983). It is conceivable that partial processing of the α3(VI) subunit may function as a fine-tuning tool to modulate aggregation of type VI collagen molecules, thereby regulating length and thickness of such beaded filaments. Different stages of processing, as noted with different tissues, would then lead to changes in the dynamic properties of the filaments. Before this hypothesis can be tested, however, the function of type VI collagen needs to be further elucidated.

Materials and methods

Purification of type VI collagen

Extracts from bovine uterus (Trüeb and Bornstein, 1984) were suspended in 100 mM NaCl, 0.2% SDS, 1 mM EDTA, 50 mM Tris–HCl pH 7.2 and denatured by heating at 80°C for 5 min. After centrifugation, the solubilized proteins were chromatographed on Sephacryl S-500 (Pharmacia, 3.3 × 70 cm, 17.4 ml/h) at room temperature. The effluent was monitored at 280 nm and analysed by polyacrylamide gel electrophoresis. Fractions containing type VI collagen were reduced with 100 mM 2-mercaptoethanol and alkylated with 4-vinylpyridine (Friedman *et al.*, 1970). Alkylated polypeptides were resolved on a column of Sephacryl S-400 as above (2.1 × 66 cm, 13.7 ml/h).

Gel electrophoresis

Proteins were analysed on SDS–polyacrylamide gels as described by Laemmli (1970). Gels were stained with Coomassie Blue or processed for fluorography (Bonner and Laskey, 1974).

Immunological procedures

Five different antisera against pepsin-extracted type VI collagen were raised in rabbits as previously described (Trüeb and Bornstein, 1984). The sera were purified on α1(VI)-pepsin, α2(VI)-pepsin or α3(VI)-pepsin which has been isolated according to Jander *et al.* (1983) and coupled to Affi-Gel 10 (Biorad). Antisera against the intact subunits of type VI collagen were prepared by excising individual bands from polyacrylamide gels and mixing them after equilibration with phosphate-buffered saline with an equal volume of Freund's adjuvant in a teflon potter. The mixture was injected s.c. into rabbits at 14-day intervals with blood being collected after the third injection. Antisera were tested by ELISA or immunoblotting (Trüeb and Bornstein, 1984).

Amino acid analysis

Samples were hydrolysed under vacuum in 6 N HCl (24 h, 110°C) and amino acid analysis was carried out by h.p.l.c. using pre-column derivatization with dabsyl chloride (Chang *et al.*, 1983) or on a Biotronic LC 5001 analyzer equipped with a ninhydrin detection system.

Enzymatic digestion

Polypeptides were suspended in 0.5 M NaCl, 2 mM $CaCl_2$, 2 mM *N*-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, 50 mM Tris/HCl pH 7.4 and digested with bacterial collagenase (Worthington, CLSPA 315 units/mg) at room temperature with an enzyme:substrate ratio of 1:100. The reaction was terminated by addition of SDS sample buffer containing 10 mM EDTA and heating at 95°C for 3 min. For digestion with pepsin, unreduced type VI collagen was dialysed against 0.1 M acetic acid, then against three changes of 8 M urea followed by five changes of 0.5 M acetic acid. Pepsin (Sigma, 0.5 mg/ml) was added and

digestion carried out at 4°C. The reaction was terminated after 24 h by lyophilization.

Cell culture

Human embryonic fibroblasts (WI 38 cells) were obtained from the American Type Culture Collection and propagated in the presence of 50 μg/ml ascorbic acid as described (Trüeb *et al.*, 1985). After metabolic labeling with [^{35}S]methionine (NEN, 100 μCi/ml) type VI collagen was precipitated from the conditioned media by the addition of affinity-purified antibodies against the 140 kd subunits (Zimmermann *et al.*, 1986) followed by agarose-immobilized goat anti-rabbit IgG antibodies (Sigma) essentially as described (Trüeb *et al.*, 1985).

Acknowledgements

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