Characterization of the Precursor Form of Type VI Collagen*

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Well characterized monospecific antisera against pepsin-extracted bovine type VI collagen were used to identify and characterize the intact form of type VI collagen. In immunoblotting experiments the antisera reacted with the pepsin-resistant fragments of the α1(VI) and α3(VI) chains, but not with the fragment of the α2(VI) chain. Extracts obtained from uterus and aorta with 6 M guanidine HCl contained two immunoreactive polypeptides of M, = 190,000 and 180,000 based on globular protein standards. Cleavage of extracts with pepsin generated the previously characterized pepsin-resistant fragments of α1(VI) and α3(VI), indicating that the higher molecular weight polypeptides represent the intact parent chains. α1(VI) and α3(VI). Digestion of extracts with bacterial collagenase released an M, = 100,000 noncollagenous fragment from the α1(VI) chain. Thus, intact type VI collagen in tissues contains a relatively short triple helical domain and at least one very large globular domain which is sensitive to pepsin but resistant to collagenase digestion. Immunoblotting revealed a polypeptide of M, = 240,000, which we suggest represents the pro-α1(VI) chain, in the culture medium of bovine fibroblasts. Bands intermediate in molecular weight between 240,000 and 190,000 were identified in cell layers. These findings establish type VI collagen as a protein with very large nontriple helical domains, a property that undoubtedly plays an important role in its function.

Pepsin digestion of tissues, under conditions that preserve the native conformation of the collagen triple helix, has been used in a number of studies to facilitate the extraction of collagens (1). The enzymatic treatment is effective because intermolecular cross-links in collagens tend to be located in regions that lack a triple helical structure and because other noncollagenous proteins that interact with collagens to form stable supramolecular complexes are generally susceptible to pepsin. In the case of the interstitial collagens (types I, II, and III) only minor changes are sustained during enzymatic extraction, but for other collagens it is now known that the pepsin-resistant forms differ considerably from the proteins as they exist in tissues. Thus pepsin extraction of type V collagen results in the loss of a large nontriple helical domain (2), whereas multiple cleavages, particularly in the α2(IV) chain, are sustained during extraction of type IV collagen (3).

In 1976 Chung et al. (4) reported the presence of a high molecular weight disulfide-bonded polypeptide fragment in pepsin digests of vascular tissues. This collagen was unusual in that it was soluble in high concentrations of NaCl at acidic pH and contained glycine and a relatively high content of cystine and hydroxylysine. Subsequently, similar or identical proteins were isolated from placenta, skin, uterus, liver, kidney, and skeletal muscle (5–14). Several terms for this collagen, including intima and short chain collagen, have been replaced by the now generally accepted designation, type VI collagen (13, 15).

The chemistry of type VI collagen, as obtained by pepsin digestion of bovine and human placenta, has been examined in recent studies (10, 13, 14). After reduction, chains with molecular weights in the range of 40,000–70,000 were characterized by a variety of electrophoretic and chromatographic techniques. Both Jander et al. (13) and Odermatt et al. (14) have identified two types of type VI collagen that differ in the degree to which they contain noncollagenous sequences, presumably a function of the extent of pepsin cleavage. Jander et al. (13) propose, on the basis of peptide mapping and other studies, that type VI collagen exists as a heterotrimer of three chains: α1(VI), α2(VI), and α3(VI).

Furthmayr et al. (15) have used the above chemical information, together with extensive electron microscopical examination of rotary shadowed and negatively stained type VI collagen preparations, to assemble a detailed structural model of the protein. According to this model, triple stranded monomers of type VI collagen (M, = 170,000) consist of a 105-nm triple helix terminating in a globular domain of M, = 30,000 at one end and one of M, = 40,000 at the other. Two monomers can align in an antiparallel fashion with a stagger of 30 nm to form a disulfide-linked dimer. The triple helices of such a dimer may be intertwined in a superhelix. Dimers can then associate laterally to form tetramers, and tetramers can aggregate end-to-end to form filamentous structures.

It is clear, however, that any realistic model of the structure of type VI collagen must take into consideration the role of pepsin-sensitive regions of the protein that exist in tissues. We have utilized specific antibodies to type VI collagen, as obtained by pepsin digestion of bovine aorta and uterus, to identify the intact form of type VI collagen in nonhydrolytic guanidine HCl extracts of these tissues and in fibroblast cultures. Extensive use was made of the protein-blotting techniques, in conjunction with enzymatic and chemical cleavage of the protein, to demonstrate that intact type VI collagen chains contain pepsin-sensitive regions that may be twice as long as the pepsin-resistant chains currently identified with type VI collagen.

EXPERIMENTAL PROCEDURES

Isolation of Collagens—Type VI collagen was isolated from bovine uterus and aorta following established procedures (11, 14). Digestion with pepsin (Sigma; 3085 units/mg) was performed for 60 h at 4 °C at an enzyme to substrate ratio of 1:100 (final concentration of pepsin, 1 mg/ml). The digest was fractionated by differential salt precipita-
tion, low ionic strength precipitation, and Cm-cellulose chromatography as previously described (11). 7 S collagen was obtained as a by-product of the type VI collagen preparation (11). The purification of types I and IV collagens from bovine kidney cortex has been described elsewhere (16). Type III collagen from calf skin was a gift from Dr. H. D. Fox (Western New England, West Springfield, MA). Type V collagen was prepared from the pepsin digests of bovine kidney cortex and bovine uterus by differential salt precipitation (17). The 0.7-1.2 M NaCl fraction, obtained at acidic pH, was redissolved in 2.5 M NaCl, 50 mM Tris-HCl, pH 7.4, and reprecipitated at neutral pH with 4.5 M NaCl. Type V collagen was further purified by chromatography on DE-52 cellulose (DR-52, Whatman; equilibrated in 0.9 M NaCl, 55 mM Tris-HCl, pH 7.5) and by low ionic strength precipitation at 20 mM NaCl, 20 mM Tris-HCl, pH 7.5.

Preparation of Guanidine HCl Extracts—Bovine aorta (100 g, wet weight) was homogenized and extracted for two periods of 24 h at 4 °C with 1000 ml of PBS containing the protease inhibitors EDTA (5 mM), N-ethylmaleimide (5 mM), and phenylmethylsulfon fluoride (1 mM). Insoluble material was collected by centrifugation and re-extracted twice with 500 ml of 1 M NaCl, 0.1 M Tris-HCl, pH 7.5, containing the above protease inhibitors. Residual material, containing type VI collagen, was finally extracted with 500 ml of 0 M guanidine HCl in Tris-HCl, pH 7.5, in the presence of protease inhibitors. The extracts were dialyzed extensively against 0.05 M acetic acid and lyophilized.

Bovine uterus was treated in a similar way except that extraction with 1 M NaCl was omitted in order to accelerate the procedure and thus diminish degradation.

Amino Acid Analysis—Samples were hydrolyzed under reduced pressure in 6 N HCl (24 h, 110 °C), and amino acid analysis was carried out on a Dionex D500 analyzer at AAA Laboratories, Mercer Island, WA.

Gel Electrophoresis—Protein was analyzed on SDS gradient polyacrylamide gels essentially as described by Laemmli (18). Gels were stained with Coomassie blue and destained with ethanol (19).

Chemical and Enzymatic Cleavage—Cleavage at cysteinyl residues with NTGCB was performed as previously described (16, 20). Digestion of the guanidine HCl extracts (5 mg/ml) with pepsin (1 mg/ml) was performed in 0.5 M acetic acid at 4 °C. The reaction was terminated after 24 h by lyophilization.

For digestion with collagenase, the guanidine HCl extracts (8 mg/ml) were denatured for 30 min at 56 °C in 0.2 M NaCl, 10 mM CaCl₂, 50 mM Tris-HCl, pH 7.4. Bacterial collagenase (Worthington; CLSPA, 0.15 units/mg) was added at a concentration of 30 units/ml and digestion carried out at 37 °C in the presence of 0.2 mM phenylmethylsulfon fluoride. The reaction was terminated by dilution with an equal volume of SDS sample buffer (containing 20 mM EDTA and 2% mercaptoethanol) and heating at 95 °C for 5 min. In parallel incubations, bovine serum albumin was not affected by these conditions.

Antiserum—Antibodies against type VI collagen were raised in female rabbits (New Zealand White) by subcutaneous injection of 0.5 mg of protein in PBS mixed with an equal volume of complete Freund’s adjuvant. Additional injections of 0.5 mg of protein in incomplete Freund’s adjuvant were given at 14-day intervals. Ten days after the fourth injection, the sera were collected. Anti-type VI collagen activity was tested by direct ELISA (21) and by indirect immunofluorescence (16). Aliquots of the antiserum were adsorbed on type IV and V native and denatured collagens immobilized on agarose. Absorbed and unabsorbed antiserum showed similar titers in ELISA and in immunofluorescence. Absorbed and unabsorbed antiserum showed similar titers in ELISA and in immunofluorescence.

Proteo Blots—Immunoblotting was performed essentially as described by Towbin et al. (22). Polypeptides were transferred overnight from SDS-polyacrylamide gels to nitrocellulose (Schleicher and Schuell; 0.45-µm pore size) in 192 mM glycine, 25 mM Tris, 20% methanol, pH 8.3, at 65 V. The nitrocellulose was stained with 0.1% Arcido Black (naphthol blue black) in 20% methanol, 10% acetic acid and destained with 20% methanol, 10% acetic acid. Positions of migration of standard proteins were marked by punching small holes into the nitrocellulose sheets. Residual binding sites for protein were saturated with 4% BSA in PBS (BSA/PBS) for 60 min at 37 °C. The sheets were then reacted for 4 h at room temperature with various antisera at a dilution of 1:50 to 1:200 in BSA/PBS. Unbound antibodies were removed with BSA/PBS (4 × 5 min), and the nitrocellulose was incubated for 60 min with [125I]-labeled protein A (immunoglobulin G) (ICN, Irvine, CA). After thorough washing with BSA/PBS (4 × 15 min), followed by PBS (3 × 10 min), the nitrocellulose was dried between filter paper and exposed to BB-5 x-ray film (Kodak).

Affinity Chromatography—Immunoglobulins were isolated from antisera VI(2) by ammonium sulfate precipitation (45% saturation) and affinity chromatography on protein A-agarose (Sigma) equilibrated in PBS. Retained IgG was eluted with 0.1 M glycine HCl, pH 2.8, and dialyzed immediately against 0.1 M HEPES, pH 8.0. Recovered IgG was coupled to Affi-Gel 10 (Bio-Rad) at a protein coupling concentration of approximately 1 mg/ml of gel according to the instructions of the manufacturer.

Conditioned media from various cell types were chromatographed on columns of immobilized antibodies (20 ml/ml of gel) in the presence of the protease inhibitors N-ethylmaleimide (5 mM) and phenylmethylsulfon fluoride (1 mM). The columns were rinsed with PBS containing protease inhibitors, and specifically absorbed protein was eluted with hot SDS sample buffer containing 2% mercaptoethanol.

RESULTS
Biochemical Characterization of Pepsin-extracted Type VI Collagen—Type VI collagen isolated from bovine aorta or uterus by pepsin extraction did not enter 3-10% gradient polyacrylamide gels in the absence of reducing agents. We, therefore, concluded that the protein forms disulfide-linked aggregates of molecular weight higher than 400,000. Upon reduction, these aggregates dissociated into three polypeptide chains with apparent molecular weights of 51,000, 40,000, and 32,000 based on collagenous protein standards, or 73,000, 60,000, and 49,000 based on globular protein standards (Fig. 1). Comparison with type VI collagen from bovine placenta (kindly provided by Dr. R. Jander, Münster, West Germany) established that the protein isolated in this study represents the shorter form of type VI collagen (form B, Ref. 14) presumably a consequence of extensive pepsin digestion. In accordance with a recent publication (13) the three polypeptide chains will be designated al(VI), a2(VI), and a3(VI), and the nature of the protein chain will be indicated by the suffix pepsin. While a2(VI)pepsin and a3(VI)pepsin were resolved as sharp bands, a1(VI)pepsin often migrated as a broad band or as loosely spaced doublet (Fig. 1, lanes 2 and 3). However, this chain was also resolved as a sharp band when a fresh preparation of type VI collagen was run on a 5-10% gradient polyacrylamide gel (see Fig. 3, lane 1). In addition to the three polypeptide chains, a faint band with a mobility similar to that of the a1(1) chain of type I collagen was sometimes noted. Since the intensity of this band increased with the age of the protein preparation, it may represent a dimer of two of the other chains, perhaps linked by lysyl-derived bonds.

The amino acid composition of type VI collagen clearly differed from the amino acid compositions of other collagen types. Most striking was the high content of cystine, tyrosine, and aspartic acid, the relatively high content of hydroxylysine, and the low content of hydroxyproline (Table 1). The glycine

The abbreviations used are: PBS, phosphate-buffered saline; pH 7.4; BSA, bovine serum albumin; ELISA, enzyme-linked immunosor-
Collagen—Antisera raised in rabbits against the two preparations of type VI collagen reacted with both antigens in an ELISA (Fig. 2). The antiserum against the protein from aorta showed a positive reaction up to a dilution of 1:4000; the antiserum against the protein from uterus was positive up to a dilution of 1:30,000. The latter antiserum exhibited the higher titer irrespective of whether the microtiter plates had been coated with type VI collagen from uterus or aorta. The difference in titers could well represent a difference in the immune response of the two animals used for immunization, rather than a difference in the immunogenicity of the two type VI collagen preparations. In the following text the antiserum raised against type VI collagen from aorta will be referred to as antiserum VI(1), and the antiserum raised against type VI collagen from uterus will be referred to as antiserum VI(2). Neither of the antiserum cross-reacted with types I, III, or IV collagens or with the 7 S fragment of type IV collagen. A moderate cross-reaction was observed with type V collagen prepared from bovine kidney cortex or from bovine uterus by differential salt precipitation. However, when this collagen was further purified by low ionic strength precipitation and DEAE-cellulose chromatography, virtually all of the cross-reacting material was removed (Fig. 2).

Indirect immunofluorescence on frozen sections of skeletal muscle, uterus, and aorta resulted in staining of interstitial structures with a tissue distribution similar to that of fibronectin (not shown). The antigenic determinants were, therefore, present and accessible, although the sections had not been pretreated with denaturing agents or with pepsin prior to incubation with antibodies.

Further characterization of the antiserum was performed by the protein-blotting technique. Antiserum VI(1) reacted with the α1(VI)pepsin and α3(VI)pepsin chains whereas antiserum VI(2) reacted exclusively with the α1(VI)pepsin chain (Fig.

Fig. 1. SDS-PAGE of type VI collagen and of the guanidine HCl extracts from uterus and aorta. Lyophilized protein (30 μg; lanes 4 and 5, 10 μg) was electrophoresed in the presence of mercaptoethanol on a 3–10% gradient polyacrylamide gel. Lane 1, type I collagen from bovine kidney cortex; lane 2, pepsin-extracted type VI collagen from bovine uterus; lane 3, pepsin-extracted type VI collagen from bovine aorta; lane 4, guanidine HCl extract from bovine uterus; lane 5, guanidine HCl extract from bovine aorta; lane 6, globular protein standard: plasma fibronectin (M, = 220,000), phosphorylase b (M, = 94,000), serum albumin (M, = 67,000) and ovalbumin (M, = 43,000). The gel was stained with Coomassie blue. α, β, and γ chains of type I collagen and α1(VI), α2(VI), and α3(VI) chains of pepsin-extracted type VI collagen are identified.

Table I

Amino acid composition of pepsin-extracted type VI collagen

| Residues per 1000 amino acid residues | Uterus | Aorta |
|--------------------------------------|-------|------|
| Hydroxyproline                       | 47    | 60   |
| Aspartic acid                        | 91    | 87   |
| Threonine                            | 30    | 22   |
| Serine                               | 52    | 35   |
| Glutamic acid                        | 102   | 107  |
| Proline                              | 89    | 95   |
| Glycine                              | 274   | 294  |
| Alanine                              | 47    | 46   |
| Half-cystine                         | 25    | 32   |
| Valine                               | 29    | 24   |
| Methionine                           | 9.2   | 5.7  |
| Isoleucine                           | 20    | 20   |
| Leucine                              | 37    | 28   |
| Tyrosine                             | 19    | 15   |
| Phenylalanine                        | 17    | 12   |
| Hydroxylysine                        | 39    | 44   |
| Lysine                               | 21    | 19   |
| Histidine                            | 4.1   | 2.8  |
| Arginine                             | 47    | 51   |

content (274–294 per 1000 amino acid residues) was considerably lower than one-third, indicating that regions lacking a stable triple helical conformation persist in the pepsin-resistant protein. There were slight but significant differences between the composition of the type VI collagen preparation from uterus and that from aorta. These differences might reflect slight differences in the extent of pepsin cleavage between the two protein preparations.

Characterization of Antisera to Pepsin-extracted Type VI Collagen

Fig. 2. Characterization of antisera to type VI collagen by direct ELISA. Antisera to pepsin-extracted type VI collagen from uterus (○, ○, antiserum VI(2)) and to pepsin-extracted type VI collagen from aorta (▲, ▲, antiserum VI(1)) were reacted in serial doubling dilution in microtiter plates coated with type VI collagen from aorta (○, ▲) or type V collagen from uterus (○, ▲). Coating of the plates with type VI collagen from uterus produced almost identical results. Incubation of the antisera in microtiter plates coated with types I, III, or IV collagens or with the 7 S fragment of type IV collagen resulted in background values (not indicated).
Neither serum reacted with the α2(VI)pepsin chain. Thus, the antigenic determinants of type VI collagen recognized by these two antisera may be limited to few sites. The α2(VI)pepsin chain apparently does not contain such a determinant. The α3(VI)pepsin chain appeared to be recognized by one rabbit (antisem VI(1)) but not by the other (antisem VI(2)). Both antisera, however, reacted equally well with type VI collagen from uterus or from aorta. It should be noted that only sequential determinants will be detected by the immunoblotting technique. It is, therefore, possible that conformation-dependent epitopes on the α2(VI) chain, and elsewhere in the molecule, contribute to the antibody population in these two antisera, but such antibodies would not detect type VI collagen chains after SDS-PAGE and transfer to nitrocellulose. When tested in the immunoblotting procedure, antisera to type VI collagen, absorbed with type V collagen, did not react with types I, III, IV, V, or VIII collagens, the 7 S fragment of type IV collagen, or with plasma fibronectin.

The Nature of Type VI Collagen Extracted from Uterus and Aorta—We used the above characterized antisera to search with type VI collagen from uterus or from aorta. It should be noted that these two antisera may be limited to few sites. The α3(VI)pepsin chain appeared to be recognized by one rabbit (antisem VI(1)) but not by the other (antisem VI(2)). Both antisera, however, reacted equally well in the immunoblotting technique. It is, therefore, possible that the a3(VI)pepsin chain appeared to be recognized by one rabbit (antisem VI(1)) but not by the other (antisem VI(2)). Both antisera, however, reacted equally well in the immunoblotting technique. It is, therefore, possible that the a3(VI)pepsin chain appeared to be recognized by one rabbit (antisem VI(1)) but not by the other (antisem VI(2)). Both antisera, however, reacted equally well by one rabbit (antisem VI(1)) but not by the other (antisem VI(2)). Both antisera, however, reacted equally well.

Immunoblots stained with antisem VI(2) showed only one band with an apparent molecular weight of 190,000 (Fig. 3, lanes 8 and 9). Since antisem VI(2) was shown to react only with the α1(VI)pepsin chain whereas antisem VI(1) reacted with both the α1(VI)pepsin and α3(VI)pepsin chains (Fig. 3, compare lanes 2 and 3 with lanes 6 and 7) we tentatively conclude that the 190,000 band represents the parent α1(VI) chain and the 180,000 band represents the parent α3(VI) chain. In support of this conclusion, neither of the two bands stained with antisera directed against types IV or V collagens and both bands were missing if the guanidine HCl extracts were electrophoresed in the absence of reducing agents (data not shown). The presumptive type VI collagen chains are, therefore, linked by disulfide bridges to form high molecular weight aggregates in tissues. No difference was observed between the extract obtained from uterus and the one obtained from aorta (Fig. 3).

In order to investigate further the nature of the two polypeptides recognized by anti-type VI collagen antibodies, the entire guanidine HCl extracts were digested with pepsin under conditions used to prepare type VI collagen. Immunoblots of such pepsin-treated extracts revealed that the bands of 190,000 and 180,000 daltons had disappeared, while two new bands with mobilities identical to those of the α1(VI)pepsin and α3(VI)pepsin chains appeared (Fig. 4, lanes 2 and 3). Again, antisem VI(2) stained only one band corresponding to the α1(VI)pepsin chain (Fig. 4, lanes 5 and 6).

These experiments indicate that intact type VI collagen contains large pepsin-sensitive domains in addition to the triple helical region preserved in pepsin-extracted type VI collagen. Our antisera, however, will react only with determinants located in, or close to, the triple helical region, since they were prepared against pepsin-extracted type VI collagen.

In the immunoblot might bind to the two polypeptides to different extents. In addition to this doublet, two faint poorly resolved bands in the molecular weight region of 60,000 were noted. The latter bands might represent degradation products of the two larger chains.

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**Fig. 3. Immunoblot of type VI collagen and of guanidine HCl extracts of uterus and aorta with anti-type VI collagen antisera.** Samples were resolved on 5–10% gradient polyacrylamide gels in the presence of mercaptoethanol and subsequently transferred to nitrocellulose. Lanes 2 and 6, pepsin-extracted type VI collagen from uterus; lanes 3 and 7, pepsin-extracted type VI collagen from aorta; lanes 4 and 8, guanidine HCl extracts of uterus; lanes 5 and 9, guanidine HCl extracts of aorta. The nitrocellulose was incubated with antiserum directed against types IV or V collagens. Our antisera, however, will react only with determinants located in, or close to, the triple helical region, since they were prepared against pepsin-extracted type VI collagen.

**Fig. 4. Immunoblot of the pepsin digestion products of a guanidine HCl extract from uterus.** The guanidine HCl extract was suspended in 0.5 M acetic acid and digested with pepsin. After 24 h, the reaction was terminated by lyophilization. Aliquots of the digest (120 µg) were resolved on 5–10% gradient polyacrylamide gels in the presence of mercaptoethanol and subsequently transferred to nitrocellulose. The nitrocellulose was incubated with antisem VI(1) (lanes 1–3) or antisem VI(2) (lanes 6–9). Bound antibodies were visualized by 125I-labeled protein A and autoradiography. Lane 1 depicts a standard of pepsin-extracted type VI collagen from uterus run on a 5–10% gradient polyacrylamide gel and stained with Coomassie blue. Lanes 1, 2–5, and 6–9 represent three different gels; the migration of corresponding polypeptides, therefore, differs slightly.
In an attempt to identify the large globular regions, the entire guanidine HCl extracts were heat-denatured and digested with bacterial collagenase under conditions that did not affect the migration of bovine serum albumin. Immunoblots of the digests stained with our antisera revealed the appearance of two new bands with apparent molecular weights of 125,000 and 100,000, based on globular protein standards (Fig. 5). In the case of the experiment described in Fig. 5 (lanes 5–7), digestion appeared to be incomplete. With increasing time of collagenase digestion there appeared to be a reversal of the upper to the lower band. Digestion of all the collagenous sequences in the $M_r = 125,000$ band might be prevented by partial renaturation of the denatured chains and/or by the existence of a highly compact disulfide-linked region at the transition of the collagenous to the globular region.

Both of the collagenase-produced bands stained with antiserum VI(2) which recognizes only the $\alpha 1$($VI$)pepsin chain (Fig. 5, lanes 5–8) suggesting that both are derived from the $\alpha 1$($VI$) chain. Staining of the protein blots with antisera VI(1) which recognizes both the $\alpha 1$($VI$)pepsin and $\alpha 3$($VI$)pepsin chains did not reveal any additional bands (Fig. 5, lanes 2 and 3). We interpret these findings to indicate that the major antigenic determinant that is recognized by antisera VI(1) in the $\alpha 3$($VI$) chain is located within the collagenous region of the chain and is thus susceptible to bacterial collagenase digestion. The major determinant of the $\alpha 1$($VI$) chain, however, appears to be located in a nontriple helical region that is preserved in the $\alpha 1$($VI$)pepsin chain at the transition of the collagenous to the globular region. We, therefore, conclude that the collagenase-resistant band of $M_r = 100,000$ represents one of the globular domains in the $\alpha 1$($VI$) chain occurring at one end of the triple helix of collagen type VI. It is likely, based on electron microscopic studies (15), that a globular domain also exists on the other end of the triple helix. However, we presume that this additional fragment, if preserved during collagenase digestion, was not visualized in the experiments described in Fig. 5 because the antigenic determinants in this domain are not recognized by antiserum VI(1).

In order to obtain additional information regarding the location of the antigenic determinants, the protein was cleaved at cysteinyl residues with NTCB. When the pepsin-extracted protein was treated and the products examined by SDS-PAGE (Fig. 6, lanes 1–3) only a small effect on migration was noted. Although the Coomassie-stained bands were less distinct, all three polypeptide chains were still visible. All three chains, however, exhibited slightly faster mobilities, the differences between treated and untreated chains representing about 5000 daltons. The disulfide bridges connecting the three polypeptides in pepsin-extracted type VI collagen, therefore, appear to be located within regions of about 50 amino acids at the ends of the chains.

An immunoblot of the NTCB cleavage products of pepsin-extracted type VI collagen, stained with antiserum VI(1), revealed only the band derived from the $\alpha 3$($VI$)pepsin chain (Fig. 6, lanes 5 and 6). The polypeptide derived from the $\alpha 1$($VI$)pepsin chain did not react with the antiserum even though the chain was visible by Coomassie blue staining, indicating that its antigenic determinant(s) had been removed by NTCB. The determinant(s) of the $\alpha 3$($VI$)pepsin chain as detected by antiserum VI(1) are, therefore, located in the collagenous region of the chain somewhere between the disulfide bridges, whereas the determinant(s) of the $\alpha 1$($VI$)pepsin chain are located approximately 50 amino acids from the ends of the chain. These experiments support the results of the collagenase digestion experiments (Fig. 5) which also demonstrated that the antigenic determinants in the $\alpha 3$($VI$) chain are located in a collagenase-resistant region.

Cleavage of the guanidine HCl extract from uterus with NTCB produced results that were compatible with the above findings. Staining of a protein blot with antiserum VI(1) showed the disappearance of the $M_r = 190,000$ ($\alpha 1$($VI$)) and $M_r = 180,000$ ($\alpha 3$($VI$)) bands with appearance of a band identical in migration to the NTCB cleavage product of the $\alpha 3$($VI$)pepsin chain (Fig. 6, lanes 8 and 9). In addition a band with $M_r = 61,000$ was stained. We presume that this chain is derived from the nontriple helical sequence of the $\alpha 1$($VI$) chain.

**Fig. 5.** Immunoblot of the collagenase digestion products of a guanidine HCl extract from uterus. The guanidine HCl extract was heat denatured in 0.2 M NaCl, 10 mM CaCl$_2$, 50 mM Tris-HCl, pH 7.4, and digested with bacterial collagenase for 0 (lanes 1 and 5), 3 (lanes 2 and 6), and 23 h (lanes 3 and 7). The reaction was terminated by dilution with an equal volume of SDS sample buffer containing 20 mM EDTA and 2% mercaptoethanol. Aliquots of the digests (40–80 µg) were resolved on 3%–18% gradient polyacrylamide gels and transferred to nitrocellulose. Bands were visualized with antibodies (lanes 1–4) or antiserum VI(2) (lanes 5–8). Bound antibodies were visualized by 125I-labeled protein A and autoradiography. Pepsin-extracted type VI collagen from uterus (lanes 4 and 8) was included as a standard. Lanes 1–4 and 5–8 represent two different gels from two different digestion experiments.

**Fig. 6.** SDS-PAGE of NTCB cleavage products of pepsin-extracted type VI collagen and of the guanidine HCl extract from uterus. Pepsin-extracted type VI collagen (lanes 1–6) and the guanidine HCl extract (lanes 7–9) were reduced with dithiothreitol and cleaved at cysteinyl residues with NTCB for 0 (lanes 1, 4, 7, 5 (lanes 2, 5, 8) and 23 h (lanes 3, 6, 9). The reaction was terminated by dilution with an equal volume of SDS sample buffer containing 2% mercaptoethanol. The cleavage products were resolved on 5–10% gradient polyacrylamide gels and stained with Coomassie blue (lanes 1–3). Similar gels were transferred to nitrocellulose, and the nitrocellulose was incubated with antisera VI(1) (lanes 4–9). Bound antibodies were visualized by 125I-labeled protein A and autoradiography.
chain and contains the antigenic determinant recognized by antiserum VI(1). This chain is smaller than either of the collagenase-resistant fragments recognized by the antiserum (Fig. 5, lanes 2 and 3) possibly because a cysteinyl residue is cleaved somewhere in the nontriple helical domain (roughly 60,000 daltons from the points of cleavage of collagenase and pepsin).

The Nature of Type VI Collagen Synthesized by Cells in Culture—Since intact type VI collagen from tissues is insoluble in neutral pH buffers and requires dissociation with 6 M guanidine HCl to bring it into solution, we thought it likely that cells in culture would synthesize a soluble precursor of this protein. To examine this question, media conditioned by fibroblasts were chromatographed on affinity columns containing IgG from antiserum VI(2), linked to agarose, and retained proteins were resolved on SDS-polyacrylamide gels. Immunoblots of these gels, stained with antiserum VI(2), revealed a sharp band with $M_r = 240,000$ based on globular standards (Fig. 7, lane 2). Conditioned media from other cell types including smooth muscle and endothelial cells did not contain such a polypeptide. Staining of the protein blots with antiserum VI(1) led to very weak staining of the $M_r = 240,000$ band, possibly as a consequence of the lower titer of this antiserum. When whole cell layers of fibroblasts were dissolved in SDS sample buffer and processed for immunoblotting, staining of two major bands with molecular weights of 215,000 and 200,000 was achieved with antiserum VI(1) (Fig. 7, lane 4). The occurrence of all these bands suggests a stepwise conversion of precursor chains of type VI collagen ($M_r = 240,000$) to the $\alpha_1$(VI) and $\alpha_3$(VI) chains found in tissues ($M_r = 190,000$ and 180,000). As with the culture media, the cell layers of smooth muscle and endothelial cells did not contain material reacting with either antiserum.

**DISCUSSION**

The use of pepsin digestion to extract collagens with large globular domains, or containing short nontriple helical sequences, is complicated by the substantial cleavage which such collagens undergo. However, hydrolytic procedures are frequently necessary because some collagens tend to be insoluble and may be stabilized in supramolecular complexes by extensive intermolecular cross-links. Type VI collagen was first identified as a pepsin-resistant fragment in vascular tissue (4) and, to date, all published reports have described the pepsin-extracted molecule. Such molecules, after reduction, have chains varying in molecular weight from 40,000–70,000 depending on the extent of pepsin digestion (13, 14); it is this characteristic that led to the use of the term "short chain" collagen (10). However, it has been apparent to many workers in the field that the properties of the intact protein may differ considerably from those currently attributed to the protein.

In the present study we have used well characterized monospecific antisera with the protein-immunoblotting technique to identify the intact form of type VI collagen as it exists in tissues prior to pepsin treatment. These antisera reacted exclusively with two polypeptide chains of $M_r = 190,000$ and 180,000 when guanidine HCl extracts of bovine uterus or aorta were examined after reduction (Fig. 3). We conclude that these two polypeptides represent the undegraded forms of the $\alpha_1$(VI) and $\alpha_3$(VI) chains of type VI collagen for the following reasons. 1) Both chains were sensitive to bacterial collagenase (Fig. 5). 2) After pepsin digestion of tissue extracts, the $\alpha_1$(VI) and $\alpha_3$(VI) chains were replaced by fragments with the same mobility as the $\alpha_1$(VI)pepsin and $\alpha_3$(VI)pepsin chains (Fig. 4). 3) Cleavage of tissue extracts with NTCB also led to loss of $\alpha_1$(VI) and $\alpha_3$(VI) chains and the appearance of a fragment with the same mobility on SDS-PAGE as the cleavage product of the $\alpha_3$(VI)pepsin chain (Fig. 6). 4) The $\alpha_1$(VI) and $\alpha_3$(VI) chains in tissue extracts form high molecular weight disulfide-linked aggregates as does pepsin-extracted type VI collagen. 5) The chains identified as $\alpha_1$(VI) and $\alpha_3$(VI) do not react with antisera against type IV or V collagens. 6) It seems unlikely that larger forms of type VI collagen exist in tissues because protease inhibitors were present throughout the extraction and there was no evidence on immunoblots for the presence of higher or lower molecular weight material. Furthermore, aorta and uterus yielded the same type VI collagen chains even though containing proteases might be expected to be different in the two tissues, and the extraction was carried out in slightly different ways.

Preliminary attempts to isolate the intact form of type VI collagen from guanidine HCl extracts of tissue have not been successful. The protein appears to be insoluble in neutral or acidic pH buffers, even in the presence of detergents such as Tween 20. In view of this insolubility, we looked for a soluble precursor to type VI collagen in cell culture material. We were able to identify an $M_r = 240,000$ chain in the culture medium of bovine fibroblasts that reacted with antiserum VI(2), an antiserum specific for the $\alpha_1$(VI) chain (Fig. 7). Furthermore, cell layers of these cultures contained at least two bands intermediate in size between $M_r = 240,000$ and 190,000 that reacted with antiserum VI(1). Thus, type VI collagen appears to be synthesized and secreted by fibroblasts in the form of a precursor to this chain.
higher molecular weight soluble procollagen which may undergo stepwise conversion to a more insoluble form in the extracellular space. The intermediate sized bands are unlikely to result from nonspecific proteolytic cleavage since these bands are discrete in size, and deposition occurred in the presence of fetal calf serum which contains protease inhibitors. The conditioned media of smooth muscle and endothelial cells lacked type VI procollagen and served as negative controls for these experiments. Experiments are currently in progress to determine whether the \( M_r = 240,000 \) band detected by antiserum VI(2) represents a mixture of pro-\( \alpha_1(\text{VI}) \) and pro-\( \alpha_3(\text{VI}) \) bands or whether a separate band can be detected by antisera specific for the \( \alpha_3(\text{VI}) \) chain.

The finding of an intact form of type VI collagen in guanidine HCl extracts of tissues raises the question of the relation of this protein to other collagenous proteins in tissues and in cell culture identified by other workers. Gibson and Cleary (23) have partially purified an \( M_r = 140,000 \) glycoprotein from guanidine HCl extracts of bovine aorta and nuchal ligament. The protein was sensitive to bacterial collagenase, relatively rich in cystine and tyrosine, and contained equal amounts of hydroxyproline and hydroxylysine, features that resemble type VI collagen. Jander et al.\(^3\) have now extended the studies of Gibson and Cleary (23) and have shown that the \( M_r = 140,000 \) glycoprotein is related to type VI collagen, based on similarity in electron microscopical appearance, immunological cross-reactivity, and the ability to generate the pepsin-resistant moiety of type VI collagen by pepsin digestion of the guanidine HCl-extracted glycoprotein.

There are also several points of similarity between type VI collagen, the \( M_r = 140,000 \) glycoprotein, and some collagenous proteins described in cell culture (24-26). Sear et al. (24) identified a glycoprotein of \( M_r = 150,000 \) (MFP) in cultures of bovine nuchal ligament fibroblasts. Their protein contained hydroxyproline and hydroxylysine and was cleaved by bacterial collagenase yielding relatively large collagenase-resistant fragments (\( M_r = 50,000 \) and 30,000). Carter (25, 26) has characterized a transformation-sensitive cell surface-associated protein, GP140, which is disulfide-bonded, contains equimolar amounts of hydroxyproline and hydroxylysine, and is sensitive to bacterial collagenase.

If it can be firmly established that these tissue and cell culture proteins are indeed derived from type VI collagen, some form of physiological processing must exist to account for the differences in molecular weight from the chains identified in this study. Alternatively, differences in carbohydrate content could conceivably account for the observed differences in migration of SDS-PAGE. A third possibility is that one of the three polypeptide chains in intact type VI collagen is actually shorter than the others. The shorter chain could be the \( \alpha_2(\text{VI}) \) chain which is not detected by our antisera. The latter possibility relates to the question of chain content. Thus far, only one chain has been found in tissue and cell culture proteins with \( M_r = 140,000 \), whereas type VI collagen contains at least two, and probably three, different chains. There is evidence that all three chains in type VI collagen exist in a single molecule (13), but this evidence is not conclusive. It seems possible that type VI collagen, as currently isolated, represents a mixture of two closely related proteins, one a heterotrimer of two different chains and the other a homotrimer.

The function of type VI collagen is not known. Sear et al. (24), based on earlier work (27), have suggested that the \( M_r = 150,000 \) glycoprotein in the culture medium of nuchal ligament fibroblasts is a component of elastic fiber microfibrils, but this relationship has been questioned (28). Our own work, together with published information, allows us to develop a preliminary model of the protein which may eventually provide a clue to its function. We have determined molecular weights of \( \approx 190,000 \) and 180,000 for the \( \alpha_1(\text{VI}) \) and \( \alpha_3(\text{VI}) \) chains, respectively, based on migration in SDS-PAGE compared with globular protein standards. These numbers are almost certainly overestimates since type VI collagen chains are hybrid molecules composed of collagenous and "noncollagenous" sequences, and collagenous chains obey a different molecular weight versus mobility relationship than do reduced globular proteins (29). Nevertheless, based on the size of pepsin-resistant type VI collagen chains and on the length of the rodlike segment as observed in the electron microscope (13-15), the mass of the triple helix can be no greater than 35,000-40,000 Da (per chain). The majority of the \( \alpha_1(\text{VI}) \) and \( \alpha_3(\text{VI}) \) chains is, therefore, composed of globular domains. Our collagenase digestion data reveal an \( \alpha_1(\text{VI}) \)-derived collagenase-resistant fragment with a molecular weight of about 100,000 (Fig. 5). Even though there must be some overlap in sequence between \( \alpha_1(\text{VI}) \) pepsin and the collagenase-resistant fragment (since antibodies prepared to pepsin-extracted type VI collagen were used to identify the latter) these findings point to a very large globular domain, possibly comprising one-half the length of the \( \alpha_1(\text{VI}) \) chain, at one end of the molecule.

We, therefore, envision type VI collagen as a dumbbell-shaped molecule with a rodlike segment separating two globular domains of different size. We do not know whether the very large globular domain is located NH\_2- COOH-terminally. This juxtaposition of triple helical and globular domains within a molecule brings to mind two other proteins, the \( \text{C}1\text{q} \) component of complement (30) and acetylcholinesterase (31), which share a similar molecular architecture. It is unclear whether a protein such as type VI collagen would be suitable to form filaments of the sort suggested by Puchmayr et al. (15). Possibly, the protein acts as a sort of biological adapter interacting both with fibrous structures and with the cell surface or with other globular proteins.

After the manuscript for this paper was completed we learned of the work of Hessle and Engvall (32). These workers were able to produce monoclonal antibodies to type VI collagen which was injected into mice as a homogenate of human fetal membranes. When such antibodies were linked to a solid support they were capable of selectively binding a metabolically labeled protein or proteins containing polypeptides of \( M_r = 240,000 \) and 140,000 from the culture medium of human lung fibroblasts.

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