Rodbard, D., & Chrambach, A. (1971) *Anal. Biochem. 40*, 95.
Seifter, S., Gallop, P. M., Michaels, S., & Meilmann, E. (1960) *J. Biol. Chem. 235*, 2613.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Biochem. 30*, 1190.
Tishler, P. V., & Epstein, C. J. (1968) *Anal. Biochem. 22*, 89.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Susceptibility of Type V Collagen to Neutral Proteases: Evidence That the Major Molecular Species Is a Thrombin-Sensitive Heteropolymer, $[\alpha 1(V)]_2\alpha 2(V)$†

Helene Sage, Pam Pritzl, and Paul Bornstein*

ABSTRACT: The susceptibility of human type V collagen to several neutral proteases was examined. Thrombin cleaved both the $\alpha 1(V)$ and $\alpha 2(V)$ chains of this protein at 34 °C, producing two pairs of fragments with apparent molecular weights of 95 000 and 10 000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Two-dimensional ^{125}I-labeled peptide mapping of the larger fragments demonstrated that the upper band [which comigrated with $\alpha 1(I)$] was derived from both the $\alpha 1(V)$ and $\alpha 2(V)$ chains, while the other component [which comigrated with $\alpha 2(I)$] was a product of $\alpha 1(V)$ alone. Cleavage of type V collagen, containing $\alpha 3(V)$ chains, with thrombin produced an analogous pattern with three high molecular weight bands. Chymotrypsin and trypsin cleaved type V collagen at 37 °C but not at lower temperatures. Digestion of type V collagen with elastase at 37 °C resulted in selective proteolysis of $\alpha 2(V)$, leaving $\alpha 1(V)$ essentially intact. Pepsin treatment of type V collagen from which $\alpha 2(V)$ had been removed by elastase treatment resulted in nearly complete degradation of $\alpha 1(V)$. These data support the hypothesis that a major fraction of native type V collagen is a heteropolymer with the chain composition $[\alpha 1(V)]_2\alpha 2(V)$. Cleavage of type V collagen by thrombin may have physiologic significance in that breakdown of pericellular matrix may be an important step in the response of a tissue to injury.

Collagens associated with basement membranes or with cell surfaces (types IV and V)[1] are not substrates for human skin collagenase under conditions which result in cleavage of interstitial collagens (types I, II, and III) (Woolley et al., 1978; Crouch & Bornstein, 1979; Sage et al., 1979; Sage & Bornstein, 1979; Liotta et al., 1979). These observations have prompted several investigations of the reactivities of other neutral proteases toward types IV and V collagens and consideration of the possible significance of such processes in inflammation, wound repair, and metastatic invasion. The concept that specific collagenases exist for certain collagen types has been reinforced by the finding of a granulocyte collagenase which exhibited preferential activity toward type I as compared to type III collagen (Horwitz et al., 1977) and by the isolation of a collagenase from a metastatic murine tumor which cleaved only type IV collagen (Liotta et al., 1979).

The collagen triple helix is unusually resistant to proteolytic attack. However, recent reports from several laboratories have shown that enzymes other than "classical" collagenases are capable of degrading native, triple-helical collagen molecules. Trypsin and neutrophil elastase cleaved type III collagen at or near the collagenase-sensitive site (Miller et al., 1976; Gadek et al., 1980). In addition, a neutral protease extracted from human leukocytes degraded native type IV collagen into several products of M_r <70 000 (Uitto et al., 1980), and an elastase purified from polymorphonuclear leukocyte granules digested type IV collagen at 37 °C into several fragments (Mainardi et al., 1980). Native type IV procollagen and collagen, but not types I, III, and V, were degraded by a neutral serine protease present in the secretory granules of rat mast cells (Sage et al., 1979; Crouch et al., 1980; Woodbury & Neurath, 1980). It should be noted, however, that the helix in type IV collagen is interrupted in several places by sequences that cannot form the triple helix (Shuppan et al., 1980) and that proteolysis may occur preferentially in these locations.

The collagen protein family is comprised of at least nine structurally distinct genetic chain types, three of which are found in type V collagen [for a review, see Bornstein & Sage (1980)]. This collagen type has been purified from a number of tissues which are enriched in basement membranes by solubilization with pepsin (Burgeson et al., 1976; Chung et al., 1976) and from cells in culture with which it appears to be preferentially associated (Sage et al., 1981b; Pöschl & von der Mark, 1980). However, although immunoferritin studies at the ultrastructural level have shown that types IV and V collagens are codistributed in kidney basement membranes (Roll et al., 1980), a distinction has been made between the localization of type IV collagen, which exists as an integral structural component of morphologically defined basement membranes, and that of type V collagen, which may be a ubiquitous pericellular collagen found in the extracellular matrix or exocytoskeleton (Gay et al., 1980).

Type V collagen consists of two or three α chains, depending upon the tissue of origin; the molecular organization of the

† From the Departments of Biochemistry and Medicine, University of Washington, Seattle, Washington 98195. *Received December 22, 1980.* This work was supported in part by National Institutes of Health Grants AM 11248 and HL 18645. H.S. is a recipient of a fellowship from R. J. Reynolds Industries, Inc.

[1] Collagen containing B and A chains and previously described as type AB has been referred to as type V collagen, and the chains as $\alpha 1(V)$ and $\alpha 2(V)$, respectively (Bornstein & Sage, 1980). Type V collagen from some tissues contains an additional chain, $\alpha 3(V)$, which was previously referred to as the C chain (Sage & Bornstein, 1979).

0006-2960/81/0420-3778$01.25/0

protein has been controversial. From denaturation–renaturation studies, Bentz et al. (1978) concluded that fetal membrane type V collagen was a heteropolymer of the formula $[\alpha 1(V)_2]\alpha 2$, while Rhodes & Miller (1978) proposed $[\alpha 1(V)]_3$ and $[\alpha 2(V)]_3$ on the basis of the biphasic melting curve of placental collagen. A variable distribution of the two chain types in different tissues has provided additional evidence for the existence of homopolymers (Rhodes & Miller, 1978; Deyl et al., 1979), and a recent report has demonstrated the synthesis of $[\alpha 1(V)]_3$ in vitro (Haralson et al., 1980). Studies have not been performed on the molecular organization of type V collagen containing $\alpha 3$ chains, as described by Brown et al. (1978) and Sage & Bornstein (1979).

The present study describes the sensitivity of type V collagen to several neutral proteases. Thrombin cleaved type V collagen at 34 °C, producing a limited number of specific fragments. At 37 °C, chymotrypsin and trypsin degraded both chains of type V collagen to one or two major fragments. In contrast, incubation with elastase at 37–39 °C resulted in the selective proteolysis of $\alpha 2(V)$. The remaining $\alpha 1(V)$ components were subsequently degraded by pepsin and were therefore non triple helical. These results provide evidence for the existence of native type V collagen as a heteropolymer composed of two different chain types, $\alpha 1(V)$ and $\alpha 2(V)$, in the same molecule.

Materials and Methods

Preparation of Substrates. Native types I and IV collagens were prepared from pepsin-treated human placenta by salt fractionation as previously described (Sage et al., 1979). Type V collagen containing $\alpha 1$ and $\alpha 2$ chains, prepared from human amnion and chorion by similar techniques, was a gift from Dr. D. Hörlein. Native type V collagen containing $\alpha 1$, $\alpha 2$, and $\alpha 3$ chains was purified from human placenta according to Sage & Bornstein (1979). Collagens were dissolved at a concentration of 1 mg/mL in 0.1 M acetic acid at 4 °C and were subsequently dialyzed against Tris–saline[2] buffer at the same temperature. Prior to incubation with enzyme, the samples were clarified in a microfuge at room temperature.

Enzymatic Digestions. α-Chymotrypsin (bovine pancreas, 3× crystallized, 65 units/mg, Worthington), trypsin (bovine pancreas, TPCK, 241 units/mg, Worthington), and elastase (porcine pancreas, 8.2 units/mg, Worthington) were dissolved at a concentration of 1 mg/mL in Tris–saline at 0 °C and were further diluted 1:10 with Tris–saline. Fresh solutions were prepared prior to each incubation. Human α-thrombin (factor IIa, 0.25 mg/mL, 2000 units/mg), in 0.2 M sodium phosphate buffer, pH 6.5, containing 1 μM DAPA to prevent conversion to the β form, was a gift from Drs. W. Kisiel and W. Canfield and was prepared according to Fenton et al. (1977). Approximately 95% of the enzyme was present as the α (active) form. It was completely inhibited by 5 mM diisopropyl fluorophosphate at pH 8.3 in 5 min. The preparation was demonstrated to be plasmin free by the lack of activity toward the synthetic substrate S-2251 even in the presence of soybean trypsin inhibitor. The enzyme, which was used at a concentration of 2.4 μg/mL (4.8 units/mL), was stored frozen in small aliquots and was thawed only once, immediately before use.

Aliquots corresponding to 25 μg of collagen in Tris–saline were transferred to 1-mL plastic microfuge tubes. A 2.5-μL sample of enzyme solution [corresponding to 0.25 μg of chymotrypsin, trypsin, and elastase, and to 0.06 μg (0.12 unit) of thrombin] was added, the mixtures were vortexed, and the reactions were incubated for various time intervals at temperatures ranging from 30 to 39 °C in a circulating water bath. The final enzyme to substrate ratio, by weight, was 1:100 for chymotrypsin, trypsin, and elastase, and 1:400 for thrombin. Control incubations were conducted at all temperatures in the absence of enzyme. For termination of the digestion, samples were diluted with an equal volume of 2× $NaDodSO_4$–polyacrylamide gel electrophoresis sample buffer containing 50 mM DTT (Laemmli, 1970) and were heated at 100 °C for 5 min. The digest, containing 25 μg of protein, was subsequently analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis on composite 4%/7.5%/12.5% slab gels, as previously described (Sage et al., 1979). Protein-containing bands were visualized by staining with Coomassie blue, and the extent of cleavage was quantitated with a Joyce-Loebl scanning densitometer.

In order to test whether the type V collagen which remained intact after elastase digestion had retained its native conformation, samples were treated with pepsin. Aliquots of solution containing 50 μg in 50 μL of type V collagen were incubated with elastase for different time intervals (10 min–1.5 h) at 37, 38, and 39 °C. The digest was cooled to either 4 or 20 °C and was subsequently diluted with an equal volume of 1 M acetic acid at the same temperature. A 25-μL aliquot was transferred to a separate microfuge tube, and 2.5 μL of a solution of pepsin A (porcine, 2× recrystallized, Worthington; freshly prepared at 1 mg/mL in H_2O at 4 °C) was added to this digest. The remaining 25 μL was used as a control. The samples were incubated at 4 or 20 °C for 21 h and were subsequently lyophilized directly. Control pepsin digestions were performed by using solutions of type V collagen which had been incubated at the temperature of the reaction but not exposed to elastase and by using collagen which had been denatured by heating at 40 °C for 10 min. In addition, type I collagen was incubated with elastase, followed by pepsin, under the same conditions as used for type V. The lyophilized samples were dissolved in 25 μL of sample buffer and were analyzed on 4%/7% $NaDodSO_4$–polyacrylamide slab gels.

Identification of Cleavage Products. The chain of origin of the major fragments produced by cleavage of type V collagen with trypsin, chymotrypsin, and thrombin was identified with a two-dimensional peptide mapping technique as described by Sage et al. (1981a). For these experiments, a 1:40 ratio by weight of thrombin to type V collagen was used (as shown in Figures 1 and 2), to maximize the extent of cleavage and to ensure that the fragments to be mapped were stable to higher concentrations of enzyme. Bands representing the cleavage products were cut from the gel, radioiodinated with the Bolton-Hunter reagent, and incubated with proteinase K (EM Biochemicals). The resulting peptides were resolved on cellulose-coated thin-layer plates by using high-voltage electrophoresis in the first dimension followed by chromatography in a butanol/pyridine/acetic acid/water buffer system. The patterns were visualized by fluorescence autoradiography.

Results

Susceptibility of Collagens to Thrombin. Type V collagen exhibited a unique sensitivity to thrombin. At an enzyme to substrate ratio of 1:40 or 1:400 by weight, approximately 90% of type V collagen was cleaved within 2 h at temperatures between 34 and 37 °C (Figure 1). The major fragments of type IV collagen (140K and 70K) were also partially susceptible to degradation by this enzyme (Figure 1). In contrast, type I collagen was not cleaved at 37 (Figure 2) or 38 °C, and

[2] Abbreviations used: DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; Tris–saline, 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5; DAPA, diethylaminonaphthalenesulfonylaminopropionate.

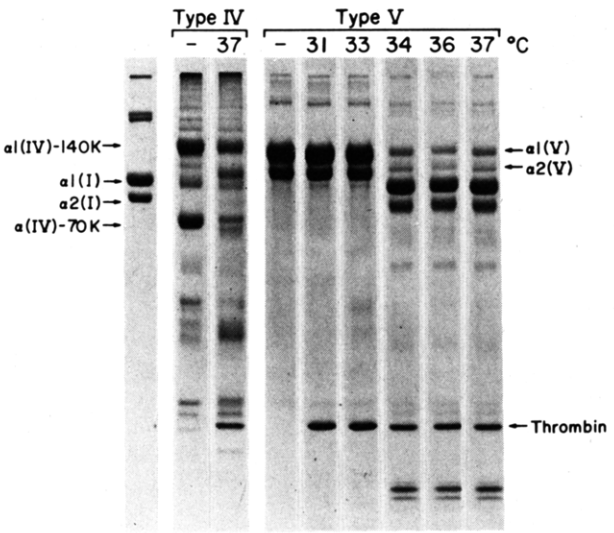

FIGURE 1: Incubation of types IV and V collagen with thrombin at different temperatures. Solutions of native collagen were incubated with thrombin at a 1:40 enzyme to substrate weight ratio for 2 h at various temperatures. Control digestions (indicated by –) received no enzyme and were incubated at 37 °C for 2 h. The reaction mixture was diluted with an equal volume of sample buffer containing 50 mM DTT, heated at 100 °C for 5 min, and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis on a composite 4%/7.5%/12.5% slab gel. A 25-μg sample of collagen was loaded per slot, and protein was visualized by staining with Coomassie blue. Lane on the far left contains type I collagen standard. The mobilities of several collagen chains and of thrombin are indicated.

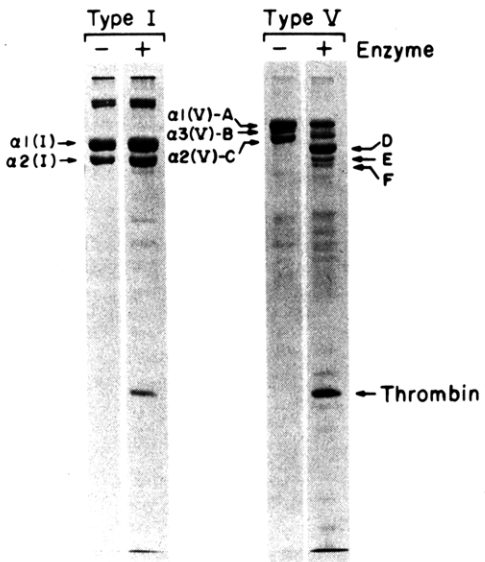

FIGURE 2: Incubation of types I and V collagen with thrombin at 37 °C. The type V collagen used for this cleavage was isolated from placenta and contained α1, α2, and α3 chains. Solutions of native collagens were incubated with thrombin at a 1:40 enzyme to substrate weight ratio at 37 °C for 2 h. Control incubations were conducted at 37 °C in the absence of enzyme. The digests were diluted with an equal volume of sample buffer and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis on a composite 4%/7.5%/12.5% slab gel. Each sample contained 25 μg of collagen, and visualization was by staining with Coomassie blue. The mobilities of types I and V collagen chains and of thrombin are indicated. Bands which were cut out of the gel and mapped as shown in Figure 3 have been identified by letters A–F.

less than 10% cleavage occurred at 39 °C (data not shown).

The cleavage products of type V collagen appeared as doublets on $NaDodSO_4$–polyacrylamide gel electrophoresis, one set of which comigrated with α1(I) and α2(I) chains and the other migrated as components of approximately 10 000

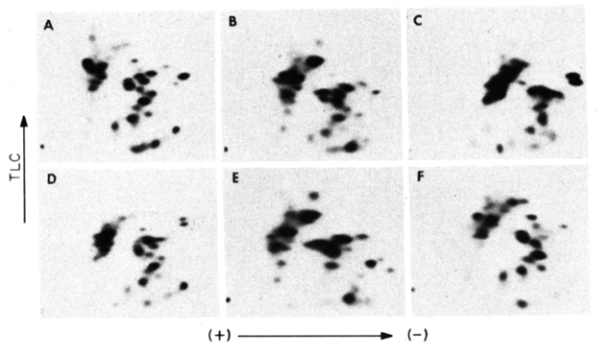

FIGURE 3: ^{125}I-Labeled peptide mapping of cleavage products resulting from digestion of type V collagen by thrombin. Bands indicated A–F in Figure 2 were cut from the gel, radioiodinated, and digested with proteinase K. The cleavage products were resolved by electrophoresis at pH 1.7, followed by thin-layer chromatography (TLC) on cellulose plates. Spots were visualized by fluorescent autoradiography. Origin was in lower left corner. Directions of electrophoresis, (+) → (–), and ascending thin-layer chromatography have been indicated.

molecular weight. This estimate was based in part on the position of migration of TC^B fragments (M_r 25 000), produced by the action of vertebrate collagenase on type I collagen; these fragments migrate with thrombin on a 7.5%/12.5% gel. There appeared to be an abrupt and reproducible transition in the structure of type V collagen at 34 °C which rendered it sensitive to limited proteolysis by thrombin. The fragments were stable for as long as 6 h at temperatures as high as 38 °C. All the other enzymes tested, including mast cell protease (Sage & Bornstein, 1979), trypsin, chymotrypsin, and elastase, failed to cleave type V collagen at temperatures below 37 °C. This collagen was also found not to be a substrate for vertebrate collagenase (Sage & Bornstein, 1979).

When placental type V collagen, composed of α1, α2, and α3 chains, was incubated with thrombin, the major cleavage products appeared as a triplet which comigrated with type I collagen chains and with the doublet produced by thrombin cleavage of type V collagen from amnion and chorion (Figure 2). The low molecular weight fragments present in Figure 1 (M_r ~10 000) are not seen in Figure 2. It has been observed that these fragments are occasionally lost from the gel during the staining process.

The fragments generated by thrombin (labeled D, E, and F in Figure 2) were subjected to ^{125}I-labeled peptide mapping and compared to maps of the starting material (labeled A, B, and C in Figure 2). This technique, which includes cleavage of the radioiodinated chains by use of proteinase K, produces a unique fingerprint for each collagen chain type (Sage et al., 1981a). The results of this experiment are shown in Figure 3. The largest fragment (panel D), which comigrated with α1(I), appeared to be a mixture of both α1(V) (panel A) and α2(V) (panel C), although the majority of the material in panel D appeared to have arisen from the α1(V) chain. The fragment represented in panel E was most similar to the α3(V) chain (panel B), and the cleavage product which comigrated on $NaDodSO_4$–polyacrylamide gel electrophoresis with α2(I) (panel F) appeared to be derived solely from the α1(V) chain (panel A). Similar mapping experiments performed on thrombin-cleaved type V collagen containing only the α1 and α2 chains produced the same results, in that the larger fragment appeared to be a mixture of both chains while the smaller fragment arose from the α1(V) chain alone.

Digestion of Type V Collagen with Chymotrypsin, Trypsin, and Elastase. Type V collagen was not digested by chymotrypsin, trypsin, or elastase to any appreciable extent at temperatures below 37 °C. However, limited proteolysis of type

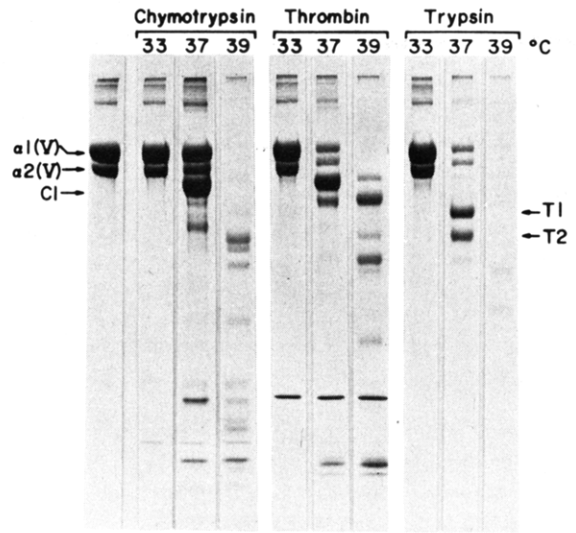

FIGURE 4: Cleavage of type V collagen by neutral proteases. Solutions of native type V collagen were incubated at 33, 37, and 39 °C for 30 min with chymotrypsin, thrombin, or trypsin at a 1:100 enzyme to substrate weight ratio. Control incubation was performed at 37 °C in the absence of enzyme. Each reaction mixture was diluted with an equal volume of sample buffer containing 50 mM DTT, and equal aliquots containing 25 μg of collagen were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on composite 4%/7.5%/12.5% slab gels. Protein was detected by Coomassie blue staining. Lane on far left represents control incubation; type V collagen chains are identified. T1 and T2 refer to trypsin cleavage products, and C1 refers to a chymotrypsin cleavage product(s).

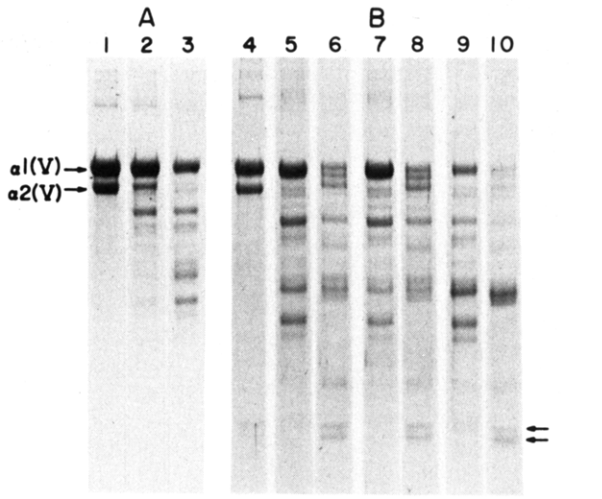

FIGURE 5: Cleavage of type V collagen by elastase followed by pepsin. Equal quantities of native type V collagen (50 μg) were incubated for various time intervals at 37, 38, or 39 °C with elastase (1:100 enzyme to substrate weight ratio). The samples were cooled to 4 or 20 °C and diluted with an equal volume of 1 M acetic acid. A 25-μg sample of each digest was subsequently incubated with pepsin (1:10 enzyme to substrate weight ratio) at 4 or 20 °C for 21 h. The samples were lyophilized and resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on a 4%/7% slab gel under reducing conditions. Protein-containing bands were visualized by staining with Coomassie blue. (A) Incubation of type V collagen with elastase. (1) Incubation control at 37 °C; α1 and α2 chains of type V collagen are identified. (2) Elastase at 37 °C for 30 min. (3) As in lane 2 except at 39 °C. (B) Incubation of type V collagen with elastase and pepsin. (4) Control, 38 °C for 30 min followed by incubation with pepsin at 4 °C; (5) elastase at 38 °C for 15 min; (6) sample in (5) was cooled to 4 °C and digested with pepsin at 4 °C; (7) elastase at 38 °C for 30 min; (8) sample in (7) was cooled to 20 °C and digested with pepsin at 20 °C; (9) elastase at 38 °C for 30 min; (10) sample in (9) was cooled to 4 °C and digested with pepsin at 4 °C. Double arrows refer to position of migration of pepsin.

V collagen was observed at 37 °C (Figures 4 and 5A) under conditions where type I remained intact. Incubation of type V collagen with chymotrypsin at 37 °C produced a major fragment, C1 (Figure 4), which migrated very similarly to α1(I) on $NaDodSO_4$–polyacrylamide gel electrophoresis. When C1 was subjected to ^{125}I-labeled peptide mapping, it was found to be a mixture of α1(V) and α2(V) chains (not shown). C1, as it appears in Figure 4, is therefore composed of more than one band.

Incubation of type V collagen with trypsin at 37 °C produced a doublet which migrated more rapidly on $NaDodSO_4$–polyacrylamide gel electrophoresis than that generated by thrombin cleavage (Figure 4). When these fragments (T1 and T2) were mapped, it was found that T1 most closely resembled α1(V), while T2 appeared to be derived from α2(V) (not shown). At 39 °C, both the chymotrypsin- and trypsin-derived fragments were further degraded (Figure 4), producing patterns similar to those observed when denatured type V collagen was used as a substrate. In contrast, the larger fragments produced by thrombin digestion were partially retained at 39 °C, even after 4 h (Figure 4).

When type V collagen was incubated with elastase at 37 °C, a selective digestion of the α2(V) chains occurred, while the α1(V) chains remained largely intact (Figure 5A, lane 2). Longer incubations at 37 and 38 °C resulted in essentially total digestion of α2(V) with far less proteolysis of α1(V) (not shown). However, raising the reaction temperature to 39 °C caused partial digestion of α1(V) (Figure 5A, lane 3). If the type V collagen sample was first denatured briefly at 40 °C for 10 min, exposure to elastase resulted in complete proteolysis of both chains to peptides of very low molecular weight.

Evidence for the Presence of α1(V) and α2(V) Chains in the Same Molecule. Since elastase digestion under controlled conditions resulted in apparently selective removal of α2(V), we used this reaction to determine whether type V collagen composed of only α1 chains existed as a native molecule in this preparation. Type V collagen from fetal membranes, containing α1 and α2 chains in a nearly 2:1 ratio (Bentz et al., 1978), was first incubated at 38 °C for 30 min without elastase and was subsequently digested with pepsin at 4 °C. Little or no degradation was observed (Figure 5, lane 4). Incubations with elastase were then performed for different periods of time and at different temperatures. These incubations resulted in nearly complete removal of α2(V) and little or no degradation of α1(V) (Figure 5, lanes 5, 7, and 9). The digests were then incubated with pepsin either at 4 °C (lanes 6 and 10) or at 20 °C (lane 8). Intact triple-helical collagen molecules would be expected to be resistant to the action of pepsin, while those in which chain cleavages had occurred might not be capable of resisting the action of pepsin. It can be seen in Figure 5B (lanes 6, 8, and 10) that the α1(V) chains which remained after elastase digestion were subsequently degraded by pepsin. This degradation occurred at both 4 and 20 °C. It should be noted that type V collagen was not completely degraded by pepsin and that some of the α1(V) chains were cleaved into large fragments as evidenced by a band migrating between α1(V) and α2(V). The resistance of a small fraction of type V collagen to pepsin may be due either to incomplete removal of α2(V) by elastase prior to pepsin digestion or to the ability of some molecules with nicked α2(V) chains to resist the action of pepsin at 4 or 20 °C. The extent of cleavage was quantitated by scanning densitometry. In lanes 5 and 6 (Figure 5), elastase cleavage accounted for a 6% loss of α1(V) and an 87% loss of α2(V), while pepsin cleavage accounted for an 80% loss of α1(V) and a greater than 95% loss of α2(V). These results therefore provide ev-

idence for the existence of $\alpha 1$(V) and $\alpha 2$(V) chains in the same triple-helical molecule.

Type V collagen from placenta is composed of $\alpha 1$, $\alpha 2$, and $\alpha 3$ chains in ratios of 1:0.9:0.4 (Sage & Bornstein, 1979). When this collagen was incubated with elastase, the $\alpha 2$(V) chain was selectively degraded, and subsequent pepsin digestion resulted in proteolysis of a major portion (>70%) of both $\alpha 1$(V) and $\alpha 3$(V) chains (not shown). These results suggest that at least a fraction of the starting material contained molecules in which $\alpha 2$(V) chains participated in the formation of helices with $\alpha 1$(V) and/or $\alpha 3$(V) chains.

Discussion

Several laboratories have provided evidence for the existence of two homopolymeric forms of type V collagen, $[\alpha 1(V)]_3$ and $[\alpha 2(V)]_3$ (Rhodes & Miller, 1978; Deyl et al., 1979; Haralson et al., 1980), while others have proposed that both heteropolymers and molecules with the composition $[\alpha 1(V)]_3$ are present (Bentz et al., 1978; Bornstein & Sage, 1980). The selective digestion of the $\alpha 2$(V) chain by elastase provided an opportunity to examine the molecular organization of type V collagen in fetal membranes and placenta. Since the $\alpha 1$(V) chain which remained after incubation with elastase was labile to pepsin, we surmise that limited proteolysis with elastase selectively nicks $\alpha 2$(V) chains in molecules which retain a helical structure at neutral pH. However, at acidic pH, these molecules unfold, exposing $\alpha 1$(V) chains to digestion by pepsin. We therefore conclude that a major fraction of type V collagen in fetal membranes contains $\alpha 1$(V) and $\alpha 2$(V) chains in the same molecule, presumably with a chain composition $[\alpha 1(V)]_2\alpha 2(V)$. An analogous observation was made for placental type V collagen which contains $\alpha 2$(V) chains in association with $\alpha 1$(V) and/or $\alpha 3$(V) chains.

The enzyme used in this study, porcine elastase 1, is a neutral serine endopeptidase and hydrolyzes peptide bonds at amino acids having short aliphatic side chains, preferentially with an alanine N terminal to the cleaved bond. Granulocyte elastase is capable of solubilizing native types I and II collagen by cleaving in the regions of telopeptides, thus removing sequences containing cross-links at the end of molecules (Starkey et al., 1977). Its action on native type III collagen is similar to that of vertebrate collagenase (Gadek et al., 1980), while native type IV collagen was cleaved into several fragments (Uitto et al., 1980). Recent studies on the degradation of the adhesive cell-surface protein fibronectin by granulocyte elastase have suggested that alterations in tissue morphology at sites of inflammation may be due in part to the disruption of cell–cell and cell–connective tissue matrix interactions which involve both fibronectin and collagen (McDonald et al., 1979; McDonald & Kelley, 1980). Because elastases exhibiting elastolytic and collagenolytic activity have been described in polymorphonuclear leukocyte granules and in aortic smooth muscle cells, this family of enzymes has been postulated to play an integral role in the degenerative changes accompanying acute phase inflammation and arteriosclerosis (Kobayashi & Nagai, 1978; Bourdillon et al., 1980).

In contrast to the effects of elastase, we find that type V collagen undergoes a different type of limited cleavage by trypsin and chymotrypsin at 37 °C (Figure 4). Both the $\alpha 1$(V) and $\alpha 2$(V) chains were digested by chymotrypsin to fragments of similar size which comigrated with $\alpha 1$(I) on NaDodSO$_4$–polyacrylamide gel electrophoresis. In contrast, trypsin cleavage produced a doublet which migrated on NaDodSO$_4$–polyacrylamide gel electrophoresis with the TC^A fragments of type I collagen produced by the action of human skin collagenase ($M_r \sim 70000$). Miller et al. (1976) have reported that trypsin cleaved native type III collagen at 15 °C near the collagenase-sensitive site. In our experiments, type I collagen was not digested by elastase, chymotrypsin, or trypsin at 37 °C, although type IV collagen did exhibit limited cleavage under the same conditions.

The significance of the susceptibility of soluble type V collagen to these neutral serine proteases is unclear. The stability of pepsin-extracted collagen in solution is likely to differ from that of a collagen that has been processed in vivo and exists as part of an extracellular matrix. It is probable that the cleavages produced by neutral proteases such as trypsin, chymotrypsin, and elastase in pepsin-solubilized type V collagen at 37 °C resulted from a localized unfolding of the triple helix and subsequent exposure of labile sequences, a process which might not readily occur in vivo.

Type V collagen, however, also exhibited an unusual sensitivity to thrombin at 34 °C (Figure 1), although no cleavage was observed at 22 °C during a 42-h incubation with an enzyme to substrate ratio of 1:40 (data not shown). For thrombin to be considered as a "type V collagenase", the reaction should satisfy several criteria: (1) limited and specific fragments should be produced, (2) the collagen substrate should be native, and (3) very low (physiologic) enzyme to substrate ratios should be effective. The production of fragments as shown in Figure 1 which are stable at temperatures as high as 38 °C for 6 h fulfills the first criterion.

The question of native conformation in this system is difficult to assess, but the data of Bentz et al. (1978), showing a T_m (midpoint melting temperature) of 37 °C for fetal membrane type V collagen and of 35 °C for renatured $\alpha 1$(V) chains [very similar to type I collagen and $\alpha 1$(I) chains], suggest that the type V collagen incubated with thrombin at 34 °C was not completely unfolded. In addition, heat-gelation experiments at 37 °C have been reported not to denature the type V triple helix (Trelstad & Carvalho, 1979). However, Rhodes & Miller (1978) have shown a biphasic melting curve for placental type V collagen (containing the $\alpha 3$ chain) with an inflection at 33–35 °C, suggesting that this collagen could be partially unfolded in this temperature range. Presumably, the susceptibility of type V collagenase to thrombin at 34 °C reflects a subtle localized unfolding of the protein.

The α-thrombin used in this study exhibited potent coagulant and esterase activity and has a specificity for peptide bonds between Arg and Gly residues. At physiologic concentrations of approximately 1 unit/mL, it has been reported to clot fibrinogen, cleave prothrombin and $\alpha 1$-antithrombin, and activate factor XIII [plasma transglutaminase; Mosher et al. (1979)]; however, it cleaves few other proteins at this concentration, including plasma proteins and fibronectin (D. Mosher, unpublished experiments; Teng & Chen, 1976). The concentrations which produced degradation of type V collagen were as low as 1 μg/mL (2.0 units/mL), although most incubations were performed with 2.4 μg/mL (4.8 units/mL) enzyme, representing a 1:400 enzyme to substrate ratio by weight. Approximately 90% of the type V collagen was cleaved in 5–10 min, or (2.5–5) $\times 10^2$ μmol of collagen (μmol of thrombin)$^{-1}$ h^{-1}, as compared to the rate reported for degradation of fibrillar type I collagen by human skin fibroblast collagenase, approximately 25 μmol of collagen (μmol of collagenase)$^{-1}$ h^{-1} (Welgus et al., 1980).

In addition to its codistribution with type IV collagen in certain basement membranes (Roll et al., 1980), type V collagen has been found to be preferentially associated with several kinds of cell layers (Pöschl & von der Mark, 1980). Vascular-derived type V collagen was localized pericellularly

to smooth muscle cells and pericytes (Gay et al., 1980). In addition, types V and III have been identified as the principal collagens associated with bovine aortic endothelial cell layers (Sage et al., 1981b). Endothelial cells are highly polarized both in vitro and in vivo and present a nonthrombogenic surface to the bloodstream. At the site of endothelial injury, the formation of thrombin occurs as an integral step during hemostasis and thrombosis. Platelet adherence, followed by the release reaction and aggregation, occurs when the endothelial cell surface is perturbed, exposing pericellular matrix and subendothelium which contain types III and V collagen. Fibrillar collagen, including type V, is a very potent thrombogenic substance and may bind multivalently to receptor sites at the platelet cell surface (Chiang et al., 1980; Barnes et al., 1980; Santoro & Cunningham, 1980).

In addition to its role as a zymogen activator in the coagulation cascade, thrombin also binds to specific receptors on both platelets and endothelial cells (Awbrey et al., 1979). The enzyme causes platelet aggregation and serotonin release, but on endothelial cells it stimulates secretion of prostacyclin, a potent anticoagulant which may serve to protect the endothelial cell layer from occlusion by platelets (Hoak et al., 1980). The present report, which describes another function for thrombin as a collagenolytic enzyme specific for type V, suggests a possible role for this enzyme during injury to endothelial and other tissues. Thrombin has been reported to release cell-surface and pericellular fibronectin from cultured fibroblasts (Mosher & Vaheri, 1978), and further experiments have described the recovery of both intact fibronectin and fragments of procollagen from fibroblast extracellular matrices after exposure to low concentrations of enzyme (Keski-Oja & Vaheri, 1980). Thrombin-stimulated release of fibronectin (or other proteins) from cell surfaces and pericellular matrices could be accomplished by cleavage of type V collagen which would disrupt protein–protein interactions. Release of type V collagen from the subendothelium may be necessary for endothelial cell migration during wound repair or for smooth muscle cell proliferation at the site of a vessel lesion.

Acknowledgments

We thank Dr. Deane Mosher for making his initial observations on thrombin cleavage known to us. Appreciation is due Drs. Walt Kisiel and William Canfield for providing the highly purified thrombin used in these studies and Dr. Dietrich Hörlein for the fetal membrane type V collagen. We also thank Donna Stewart for typing the manuscript.

References

Awbrey, B. J., Hoak, J. C., & Owen, W. G. (1979) *J. Biol. Chem. 254*, 4092–4095.

Barnes, M. J., Bailey, A. J., Gordon, J. L., & MacIntyre, D. E. (1980) *Thromb. Res. 18*, 375–388.

Bentz, H., Bächinger, H. P., Glanville, R., & Kühn, K. (1978) *Eur. J. Biochem. 92*, 563–567.

Bornstein, P., & Sage, H. (1980) *Annu. Rev. Biochem. 49*, 957–1003.

Bourdillon, M. C., Brechemier, D., Blaes, N., Derquette, J. C., Hornebeck, W., & Robert, L. (1980) *Cell Biol. Int. Rep. 4*, 313–320.

Brown, R. A., Shuttleworth, C. A., & Weiss, J. B. (1978) *Biochem. Biophys. Res. Commun. 80*, 866–872.

Burgeson, R. E., El Adli, F. A., Kaitila, I. I., & Hollister, D. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2579–2583.

Chiang, T. M., Mainardi, C. L., Seyer, J. M., & Kang, A. H. (1980) *J. Lab. Clin. Med. 95*, 99–107.

Chung, E., Rhodes, R. K., & Miller, E. J. (1976) *Biochem. Biophys. Res. Commun. 71*, 1167–1174.

Crouch, E. C., & Bornstein, P. (1979) *J. Biol. Chem. 254*, 4197–4204.

Crouch, E. C., Sage, H., & Bornstein, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 745–749.

Deyl, Z., Macek, K., & Adam, M. (1979) *Biochem. Biophys. Res. Commun. 89*, 627–634.

Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M., & Finlayson, J. S. (1977) *J. Biol. Chem. 252*, 3587–3598.

Gadek, J. E., Fells, G. A., Wright, D. G., & Crystal, R. G. (1980) *Biochem. Biophys. Res. Commun. 95*, 1815–1822.

Gay, S., Gay, R. G., & Miller, E. J. (1980) *Arthritis Rheum. 23*, 937–941.

Haralson, M. A., Mitchell, W. M., Rhodes, R. K., Kresina, T. F., Gay, R., & Miller, E. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5206–5210.

Hoak, J. C., Czervionke, R. L., Fry, G. L., & Smith, J. B. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 2606–2609.

Horwitz, A. L., Hance, A. J., & Crystal, R. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 897–901.

Keski-Oja, J., & Vaheri, A. (1980) *Int. Congr. Cell Biol., 2nd*, 419; Abstr. No. D1247.

Kobayashi, S., & Nagai, Y. (1978) *J. Biochem. (Tokyo) 84*, 559–567.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Liotta, L. A., Abe, S., Gehron-Robey, P., & Martin, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2268–2272.

Mainardi, C. L., Dixit, S. N., & Kang, A. H. (1980) *J. Biol. Chem. 255*, 5435–5441.

McDonald, J. A., & Kelley, D. G. (1980) *J. Biol. Chem. 255*, 8848–8858.

McDonald, J. A., Baum, B. J., Rosenberg, D. M., Kelman, J. A., Brin, S. C., & Crystal, R. G. (1979) *Lab. Invest. 40*, 350–357.

Miller, E. J., Finch, J. E., Chung, E., Butler, W. T., & Robertson, P. B. (1976) *Arch. Biochem. Biophys. 173*, 631–637.

Mosher, D. F., & Vaheri, A. (1978) *Exp. Cell Res. 112*, 323–334.

Mosher, D. F., Schad, P. E., & Kleinman, H. K. (1979) *J. Clin. Invest. 64*, 781–787.

Pöschl, A., & von der Mark, K. (1980) *FEBS Lett. 115*, 100–104.

Rhodes, R. K., & Miller, E. J. (1978) *Biochemistry 17*, 3442–3448.

Roll, F. J., Madri, J. A., Albert, J., & Furthmayr, H. (1980) *J. Cell Biol. 85*, 597–616.

Sage, H., & Bornstein, P. (1979) *Biochemistry 18*, 3815–3822.

Sage, H., Woodbury, R. G., & Bornstein, P. (1979) *J. Biol. Chem. 254*, 9893–9900.

Sage, H., Pritzl, P., & Bornstein, P. (1981a) *Collagen Rel. Res. 1*, 3–15.

Sage, H., Pritzl, P., & Bornstein, P. (1981b) *Biochemistry 20*, 436–442.

Santoro, S. A., & Cunningham, L. W. (1980) *Thromb. Haemostasis 43*, 158–162.

Shuppan, D., Timpl, R., & Glanville, R. W. (1980) *FEBS Lett. 115*, 297–300.

Starkey, P. M., Barrett, A. J., & Burleigh, M. C. (1977) *Biochim. Biophys. Acta 483*, 386–397.

Teng, N. N. H., & Chen, L. B. (1976) *Nature (London) 259*, 578–580.
Trelstad, R. L., & Carvalho, A. C. A. (1979) *J. Lab. Clin. Med. 93*, 499–505.
Uitto, V.-J., Schwartz, D., & Veis, A. (1980) *Eur. J. Biochem. 105*, 409–417.
Welgus, H. G., Jeffrey, J. J., Stricklin, G. P., Roswit, W. T., & Eisen, A. Z. (1980) *J. Biol. Chem. 255*, 6806–6813.
Woodbury, R. G., & Neurath, H. (1980) *FEBS Lett. 114*, 189–196.
Woolley, D. E., Glanville, R. W., Roberts, D. R., & Evanson, J. M. (1978) *Biochem. J. 169*, 265–276.

Amino Acid Sequence of p15 from Avian Myeloblastosis Virus Complex†

Robert T. Sauer,* David W. Allen, and Hugh D. Niall

ABSTRACT: The complete amino acid sequence of the p15 *gag* protein from avian myeloblastosis virus (AMV) complex has been determined by sequential Edman degradation of the intact molecule and of peptide fragments generated by limited tryptic cleavage, cleavage with staphylococcal protease, and cyanogen bromide cleavage. AMV p15 is a single-chain protein containing 124 amino acids. The charged amino acids tend to be clustered in the primary structure. p15 contains a single cysteine at position 113 which may be essential for the p15 associated proteolytic activity. However, p15 shows no appreciable sequence homology with papain or other classical thiol proteases.

Retroviruses of the avian leukosis-sarcoma group are enveloped ribonucleic acid (RNA)[1] tumor viruses displaying the characteristic morphology of C-type particles (Tooze, 1973). The internal structural proteins of these viruses are encoded by the viral *gag* gene and are designated p27, p19, p15, and p12 (August et al., 1974) since their monomer molecular weights are approximately 27 000, 19 000, 15 000, and 12 000, respectively (Fleissner, 1971). *gag* proteins from avian myeloblastosis virus (AMV) complex, the Prague strain of Rous sarcoma virus, avian myelocytamostosis virus (MC-29), and avian sarcoma virus B77 have been isolated and partially characterized (Niall et al., 1970; Fleissner, 1971; Herman et al., 1975; Fletcher et al., 1975; Hunter et al., 1978; Reynolds et al., 1978; Palmiter et al., 1978; Wiesemann et al., 1978). The homologous *gag* proteins from these different species of avian retroviruses are very similar in amino acid composition, molecular weight, and, where studied, amino acid sequence. The *gag* proteins are synthesized as a precursor polyprotein (Pr76) of 76 000 molecular weight which is subsequently cleaved to generate the four known *gag* proteins (Vogt & Eisenman, 1973; Vogt et al., 1975).

The structural locations of the *gag* proteins within the virion and the general morphology of the retroviruses have been recently reviewed [see Montelaro & Bolognesi (1978) and references cited therein]. p12 is a phosphoprotein which interacts with the viral RNA to form the centrally located, electron-dense ribonucleoprotein complex of the virus. p27 molecules form a core shell surrounding the ribonucleoprotein, and p19 molecules form an ill-defined structure between the core shell and the viral membrane. A small number of p19 molecules are also found associated with the viral genome (Sen & Todaro, 1977) and may be involved in control of intracellular processing of the RNA (Leis et al., 1978). The structural role of the p15 molecule in the avian retroviruses is poorly understood. p15 is found with p27 in core structures, but its recovery is low and variable when compared with that of p27 (Bolognesi et al., 1973) and it has been termed "core associated".

In contrast to its uncertain structural role, recent evidence suggests an important regulatory role for p15 in the viral life cycle. Highly purified preparations of p15 contain an associated protease activity which cleaves the Pr76 polyprotein precursor (von der Helm, 1977; Dittmar & Moelling, 1978; Vogt et al., 1979). At least two of the mature *gag* proteins can be generated by p15-mediated cleavage of Pr76, and the proteolytic activity appears to be moderately specific for Pr76 although certain other proteins also act as substrates when denatured (Vogt et al., 1979; Dittmar & Moelling, 1978). The idea that p15 may be an essential protease is supported by the analysis of a temperature-sensitive mutant (LA 3342) of avian sarcoma virus (Hunter et al., 1976). This conditional mutant apparently codes for an altered p15 protein (Rohrschneider et al., 1976), and at the nonpermissive temperature (41 °C) produces aberrant, noninfectious virions containing reduced amounts of certain *gag* proteins and new proteins that appear to be intermediates in the cleavage of Pr76. Cleavage of Pr76 in LA 3342 infected cells is slower at 41 °C than at the permissive temperature (Hunter et al., 1976). It is unlikely that the LA 3342 defect defines a cis-acting site where proteolysis occurs since LA 3342 can be complemented at the nonpermissive temperature by a nondefective avian leukosis virus (Hunter et al., 1976). Moreover, certain defective retroviruses such as MC-29 and avian erythroblastosis virus (AEV) produce *gag*-related fusion proteins lacking p15 (Bister et al., 1977; Hayman et al., 1979). In nonproducer cell lines, these fusion proteins are not cleaved as would be expected if functional p15 were necessary for such processing. The *gag*-related fusion proteins from AEV and MC-29 can be

† From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (R.T.S.), the Hematology Section, Veterans Administration Medical Center, Minneapolis, Minnesota 55417 (D.W.A.), and the Howard Florey Institute, University of Melbourne, Parkville, Australia 3052 (H.D.N.). *Received October 17, 1979; revised manuscript received October 3, 1980.* This work was supported in part by grants form the American Cancer Society (NP-27J), the National Institutes of Health (AI-15706), and the Athwin Foundation and by a contract from the National Cancer Institute (NIH-NCI-E-73-3265).

[1] Abbreviations used: RNA, ribonucleic acid; AMV, avian myeloblastosis virus; $NaDodSO_4$, sodium dodecyl sulfate; LC, liquid chromatography; TPCK, tosylphenylalanine chloromethyl ketone; CM, carboxymethyl.

0006-2960/81/0420-3784$01.25/0