The Recognition Sites of the Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ within Collagen IV Are Protected against Gelatinase A Attack in the Native Protein*

(Received for publication, May 21, 1996, and in revised form, August 19, 1996)

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The susceptibility of three different solubilized forms of type IV collagen to gelatinase A cleavage and the concomitant effects on cell and integrin binding have been assessed. Dithiothreitol-solubilized Engelbreth-Holm Swarm (EHS) type IV collagen with disrupted intramolecular disulfide bonds in the CB3[IV] region was cleaved N-terminally to the CB3[IV] region into the two characteristic 100–300-nm fragments at 30 °C and was totally degraded at 37 °C. This was reflected in the partial or total loss of the $\alpha_2\beta_1$ and $\alpha_2\beta_2$ integrin binding sites within this region. The ability of gelatinase A to cleave EHS type IV collagen preparations with intact interchain disulfide bonds in CB3[IV] only occurred at higher temperatures. Furthermore, no effect on binding of cells or isolated integrins to the gelatinase-treated collagen could be detected after treatment at 37 °C. Dimero- and trimeric collagen IV of human placenta with intact disulfide bonds in the CB3[IV] region was not degraded at all by gelatinase A at 37 °C.

Gelatinase A treatment of reduced and denatured CB3[IV] showed many susceptible peptide bonds. Disulfide-stabilized triple-helical CB3[IV] could only be cleaved at the N and C termini, where partial unraveling of the helix had occurred. We conclude that gelatinase A is unlikely to significantly cleave the intact, stabilized triple-helical region of CB3[IV] in vivo, which protects the major recognition sites for the integrins $\alpha_2\beta_1$ and $\alpha_2\beta_2$ of type IV collagen.

The macromolecular organization as well as the biomechanical stability of basement membranes is mainly determined by the network of type IV collagen (1). The molecules of the major isoform of collagen IV, consisting of two $\alpha_1$(IV) chains and one $\alpha_2$(IV) chain, are 400 nm long and bear a globular domain at the C terminus (2, 3). They are present in all basement membranes in the form of a network, in which the molecules aggregate with their like ends (4) and interact laterally with the triple-helical domains (5). At the N terminus, the triple-helical end regions of four molecules overlap by 25 nm and this arrangement is covalently connected by intermolecular disulfide bonds and lysine derived aldimine cross-links (6). At the C terminus, two molecules aggregate to form a hexameric complex, which is stabilized by intermolecular disulfide bonds (7).

The degradation of the stable type IV collagen network of basement membranes is an integral feature of remodeling and repair processes, and tumor cell spreading (8). Fessler et al. (9) showed that a mouse tumor matrix metalloproteinase (MMP) is able to cleave intact triple-helical collagen IV derived from cell culture into two fragments 100 and 300 nm in length. Subsequently, purified members of the MMP family, notably gelatinase A (MMP2) and gelatinase B (MMP9) have been shown to degrade type IV collagen preparations in vitro (10–16). This supported the concept that the gelatinases may be thought of as type IV collagenases (17).

The interactions of cells with extracellular matrix components are largely mediated by the integrin family of receptors, which are transmembrane heterodimeric glycoproteins composed of non-covalently associated $\alpha$ and $\beta$ subunits (18). Cellular adhesion to type IV collagen occurs mainly via the $\alpha_2\beta_1$ and $\alpha_2\beta_2$ integrins (2, 19). The recognition sites for $\alpha_2\beta_1$ and $\alpha_2\beta_2$ within collagen IV have been located in the immediate vicinity of the gelatinase A cleavage site, about 100 nm from the N terminus of the molecules. This region is stabilized by intramolecular disulfide bonds between the three $\alpha$ chains and can be isolated as a triple-helical cyanogen bromide fragment, CB3[IV], allowing detailed studies of the structure of the conformation dependent recognition sites (2, 20, 21).

Because of the close vicinity of the gelatinase A cleavage site to the recognition sites for $\alpha_2\beta_1$ and $\alpha_2\beta_2$ binding, the question arose as to whether gelatinase A treatment impairs the ability of collagen IV to interact with $\alpha_2\beta_1$ and $\alpha_2\beta_2$. We therefore incubated different collagen IV preparations, isolated from the murine Engelbreth-Holm Swarm (EHS) tumor and from human placenta, with gelatinase A and subsequently investigated the degradation products and their ability to bind cell-associated and purified $\alpha_2\beta_1$ and $\alpha_2\beta_2$. Collagen IV molecules, in which the stabilizing intramolecular disulfide bonds within the CB3[IV] region had been reduced, were cleaved at 25 °C to 30 °C by gelatinase A into the two characteristic fragments 100 and 300 nm long (9). The ability to bind $\alpha_2\beta_1$ and $\alpha_2\beta_2$ was, however, hardly impaired by this enzyme treatment. Incubation at 37 °C destroyed the molecules completely. Collagen IV molecules with intact disulfide bonds in the CB3[IV] region

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¶ The abbreviations used are: MMP, matrix metalloproteinase; EHS, Engelbreth-Holm-Swarm; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; BSA, bovine serum albumin.
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more were stable to cleavage with gelatinase A. At 30°C no cleavage could be observed, but at 37°C the molecules were not only split at the gelatinase A-specific cleavage site, but also slowly degraded from the C-terminal end of the triple-helical domain. However, the ability to interact with the two integrins α3β1 and α5β1 remained unchanged. Their recognition sites seemed to be especially stabilized by the intramolecular disulfide bonds of the CB3[IV] region.

MATERIALS AND METHODS

Isolation of Collagen IV Preparations and the Integrins α3β1 and α5β1—Intact collagen IV molecules were isolated from the murine EHS tumor by a procedure published previously (22). Under the reducing conditions used for extraction, intermolecular disulfide bonds at the N and C termini of the collagen IV molecules were broken such that mainly individual molecules were solubilized. Native collagen IV molecules also contain intramolecular disulfide bonds within the CB3[IV] region of the triple-helical domain, and possibly also within the N-terminal telopeptides. With variation from batch to batch, these bonds were either completely reduced (preparation A) or remained intact (preparation B). In some cases, mixtures of both were observed. It is unclear whether the intramolecular disulfide bonds remained intact during extraction in presence of DTT in preparation B, or whether these bonds were reformed under the oxidative conditions of further purification.

Preparation B can be converted to preparation A by incubation with 2 mM DTT. Preparation A can be oxidized to preparation B using a mixture of reduced to oxidized glutathione of 1:5. Preparation A can be distinguished from preparation B by SDS-PAGE carried out under non-reducing and reducing conditions.

Dimeric collagen IV was extracted from human placenta after mild treatment with bacterial collagenase according to Timp et al. (4). The purified collagen IV dimers still contained traces of collagenase, which partly degraded the dimers during incubation at 37°C for 24 h. The bacterial enzyme was removed by molecular sieve chromatography on an agarose 5.0m (Bio-Rad) column (110 × 2.5 cm) in 2 mM urea, 50 mM Tris/HCl, pH 7.6, and 0.2 M NaCl.

The trimeric fragment CB3[IV] was isolated after CNBr treatment of pepsin-digested tetrameric collagen IV from human placenta. Fragment F1, which comprises almost the entire triple-helical segment of CB3[IV], was isolated after limited tryptic digestion of CB3[IV]. Both procedures have been described previously (2).

The isolation of the integrins α3β1 and α5β1 was performed as described elsewhere (2, 20). α3β1 was extracted from human placenta and purified by affinity chromatography on a column of CB3[IV]-linked Sepharose. α5β1 was extracted from human platelets and purified using a collagen I Sepharose column.

Treatment of the Collagen IV Preparation with Gelatinase A—Recombinant human progelatinase was expressed from myeloma cells and purified as described previously (23). Type IV collagen substrate was dissolved in a buffer containing 25 mM Tris/HCl, pH 7.5, 100 mM NaCl, and 10 mM CaCl2. The following steps were performed in siliconized reaction tubes in order to minimize loss of proteins due to nonspecific protein absorption. To activate the progelatinase A, 10 mM 4-aminophenylmercuric acetate (Sigma) was added to the progelatinase A solution to a final concentration of 2.5 mM. The mixture was incubated at 25°C for 1 h. After the activation of gelatinase A, the solution of collagen was added at an enzyme-substrate ratio of 1:8. After incubation for 17 h at 30°C and 37°C, respectively, the digestion was stopped either by adding 500 mM EDTA solution to a final concentration of 40 mM, or, for the cell attachment assays, with a 3 mM excess of recombinant human TIMP-1, a specific inhibitor of MMPs. The degradation of type IV collagen with gelatinase A was assessed by SDS-PAGE in a 5–12% polyacrylamide gradient gel according to Laemmli (24). The gels were stained either with Coomassie Blue or with silver according to standard protocols.

To investigate the specificity of gelatinase A for denatured α1(IV) chains, CB3[IV] was reduced and carboxymethylated under reducing conditions (2). The incubation with activated gelatinase A (enzyme to substrate ratio 1:30) at 25°C and 30°C for 12 h was performed as described above. After stopping the digestion with EDTA, the peptide mixture was separated on a C18 reverse phase column (0.46 × 25 cm; Vydac, Hesperia, CA), using a gradient of 0–42% acetonitrile in 0.1% trifluoroacetic acid in 160 min at a flow rate of 0.25 ml/min, and the individual fractions obtained were analyzed by Edman degradation.

To test the ability of gelatinase A to cleave peptide bonds in the disulfide-stabilized triple-helical region, the intact fragment F1 was treated with gelatinase as described above. After incubation, the disulfide bonds were reduced and carboxymethylated, the peptide mixture separated on a C18 reverse phase column, and the individual fractions analyzed by Edman degradation.

Treatment of Collagen Preparation A with Purified Bacterial Collagenase and Determination of the Cleavage Site—Collagenase from Clostridium histolyticum, Type IA (Sigma) was separated into α1, α2, γ, δ, ε, and ζ collagenases according to Bond and Van Wart (25). 0.2 mg/ml preparations of A in 50 mM Tris/HCl, pH 7.6, 5 mM CaCl2 was added to collagen, at a substrate to enzyme ratio of 40.1, at 4°C for 16 h. Digestion was stopped with 0.5 M EDTA. For sequencing of the α1(IV) and α3(IV) chains of the C-terminal three-quarter long molecular fragment, the digest was separated using SDS-PAGE under reducing conditions. The single protein bands reflecting α1(IV) and α3(IV) were transferred to immobilized membrane (28) and subsequently sequenced by Edman degradation on a gas phase sequencer (model 470A; Applied Biosystems Inc., Foster City, CA).

Binding of α3β1 and α5β1 Integrins to Immobilized Type IV Collagen—The different preparations of type IV collagen before and after gelatinase A treatment were coated onto 96-well tissue culture plates at seven different coating concentrations in 0.1 M acetic acid at 4°C overnight. Like all seven coating concentrations used in the binding studies (20 µg/ml through 0.31 µM), similar results were obtained. Representative data measured at a coating concentration of 10 µg/ml are shown in Fig. 4. After coating with collagen, acetic acid was removed by careful washing with TBS (50 mM Tris/HCl, pH 7.4, and 150 mM NaCl). Nonspecific protein binding sites on the plastic surface were then blocked with 1% w/v heat-denatured bovine serum albumin (BSA) for 2 h at room temperature. The α3β1 and α5β1 integrins dissolved at a concentration of 3.6 µg/ml in buffer A (TBS, 1 mM MgCl2) containing 25 mM octylglucoside were allowed to bind to the immobilized collagen for 1.5 h at room temperature. Nonspecific binding of the integrins to the collagen was measured in the presence of 10 µg/ml EDTA.

After washing three times with buffer A containing octylglucoside, the amount of bound integrins was determined in an enzymelinked immunosorbent assay using an anti-β1-integrin antiserum, as described previously (20). The specific binding of each integrin was determined as the difference of the total and nonspecific binding measured in the absence and presence of EDTA, respectively. On average, nonspecific binding was less than 20% of total binding. The binding of integrin to the gelatinase A-digested collagen substrate was compared with that of the non-digested substrate, which had been incubated without gelatinase A. Neither α3β1 nor α5β1 integrin bound to gelatinase A itself, which was checked with immobilized gelatinase A.

Binding of the Fragment F1 Before and After Treatment with Gelatinase A to the Immobilized Integrins—0.6 µg α3β1 and α5β1 integrin, respectively, dissolved in buffer A were immobilized onto a microwell plate at 4°C overnight. After washing twice with buffer A, the microwells were coated with buffer B (1% w/v solution of BSA in buffer A) for 2 h at room temperature to block nonspecific protein binding sites on the plastic surface. Various concentrations of F1 fragment without and with prior degradation by gelatinase A at 25°C and 30°C were dissolved in buffer B and allowed to bind to the immobilized integrins for 1.5 h at room temperature. The collagen fragment was diluted to concentrations that were far below those of the immobilized integrin and the detecting antibodies, in order to be the limiting factor in this assay. After removing unbound collagen fragments by washing three times with buffer B, the bound collagen fragments were detected in an enzyme-linked immunosorbent assay using the monoclonal antibody CIV22 directed against CB3[IV] as described (20). This antibody does not interfere with the binding of the two integrins α3β1 or α5β1 to CB3[IV] or F1 (21). Nonspecific binding was measured in the presence of 10 µg/ml EDTA. The binding of collagen fragments to BSA-coated microwells was taken as the blank. The determinations were performed in duplicate.

Cell Culture and Attachment Assays—HT1080 (human fibrosarcoma) and RuGlI (rat glioblastoma) were routinely cultured in Dulbecco's modified Eagle's medium, supplemented with penicillin (50 units/ml), streptomycin (50 µg/ml), and 10% fetal calf serum (Globepharm, Surrey, UK). Cells were subcultured 2 days before attachment assays, performed essentially as described previously (2). The different preparations of type IV collagen before and after gelatinase A treatment were coated onto 96-well tissue culture plates as a 1 µg/ml solution in phosphate-buffered saline, pH 7.2, at 4°C overnight. Free binding sites were blocked with 1% heat-denatured BSA in phosphate-buffered saline for 2 h at 4°C. Cells were seeded onto coated wells at 6 × 104 cells/well in serum-free medium and allowed to attach for 35 min at 37°C. Attached cells were washed twice with serum-free medium and
then fixed with 3.7% formaldehyde in 0.15 M NaCl. Cells were stained with 1% methylene blue in 0.01 M borate buffer, pH 8.5. After washing extensively with distilled water, the dye adsorbed to the cells was solubilized with a solution of 0.05 M HCl, 50% ethanol and the optical density was read at 630 nm. The attachment of cells to the gelatinase A-digested collagen substrate was compared with the non-digested substrate, which had been incubated without gelatinase A. Cells did not attach to gelatinase A itself, or to the TIMP-1 used to stop the cleavage reaction. This was determined using immobilized gelatinase A and TIMP-1. Rotary shadowing and electron microscopy of preparation A before and after gelatinase treatment were done as described (27).

RESULTS

Collagen IV Preparations—Monomeric collagen IV molecules were isolated from the murine EHS tumor (22). Two different preparations were obtained (Fig. 1) as judged by comparative SDS-PAGE (Fig. 4): preparation A in which the intramolecular disulfide bonds of the CB3[IV] region and of the N-terminal telopeptides are split by reduction. The intrachain disulfide bonds within the NC1 domains remained intact under the conditions used. In preparation B the three a(IV) chains are connected by disulfide bonds in the CB3[IV] region and possibly also in the area of the telopeptides. Dimeric collagen IV was isolated from human placenta after treatment with bacterial collagenase, whereby the 100-nm-long N-terminal region of the triple-helical domain has been removed. The intramolecular disulfide bonds of the CB3[IV] region are intact and the C-terminal end of the triple-helical domain is sealed by the hexameric NC1 complex. N-terminal telopeptides and the CB3[IV] region with intact intramolecular disulfide bonds are shaded.

![Fig. 1. Schematic representation of the three collagen IV preparations used. Preparations A and B are collagen IV molecules isolated under reducing conditions from the murine EHS tumor. In preparation A the intramolecular disulfide bonds of the CB3[IV] region and of the N-terminal telopeptides are split by reduction. The intrachain disulfide bonds within the NC1 domains remained intact under the conditions used. In preparation B the three a(IV) chains are connected by disulfide bonds in the CB3[IV] region and possibly also in the area of the telopeptides. Dimeric collagen IV was isolated from human placenta after treatment with bacterial collagenase, whereby the 100-nm-long N-terminal region of the triple-helical domain has been removed. The intramolecular disulfide bonds of the CB3[IV] region are intact and the C-terminal end of the triple-helical domain is sealed by the hexameric NC1 complex. N-terminal telopeptides and the CB3[IV] region with intact intramolecular disulfide bonds are shaded.](https://example.com/fig1.png)

![human COL(IV)-dimer:](https://example.com/humancoliv.png)

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of decreasing length. Within the individual fragments, the three α chains were often of different length, thus leading to an unseparable mixture. Upon SDS-PAGE analysis, a smear of trimeric fractions was observed, which separated only after reduction of the intramolecular disulfide bonds into several individual bands (Fig. 3d). The dimeric collagen IV preparation did not show any alterations after gelatinase A treatment, either at 30 or at 37 °C (Fig. 3e).

Interaction with the Integrins α1β1 and α2β1—Since the cleavage sites of gelatinase A on type IV collagen were located in the vicinity of the N terminus of CB3[IV], which contains the recognition sites for α1β1 and α2β1, it was necessary to investigate to what extent gelatinase treatment impairs the capacity of collagen IV to bind these integrins. Before gelatinase A treatment, all three collagen IV preparations were capable of interacting with α1β1 and α2β1. After digestion of preparation A with gelatinase A at 30 °C, the integrin binding varied from unchanged to slightly reduced, with the α2β1 recognition site apparently more sensitive to cleavage (Fig. 4). Treatment at 37 °C destroyed the binding sites to a much higher extent. Preparation B with intact intramolecular disulfide bonds was found to be resistant to gelatinase A. Neither α1β1 nor α2β1 binding was reduced after incubation at 30 °C (Fig. 4). After digestion at 37 °C the molecules were partly degraded (Fig. 3d), but the binding of both integrins was barely impaired (Fig. 4).

The human dimeric collagen IV preparation still contained traces of bacterial collagenase, which during incubation overnight at 37 °C reduced the binding capacity of α2β1 integrin and, to a lesser extent, of α1β1 (not shown). After removing the enzyme by molecular sieve chromatography, the purified dimers were subjected to gelatinase A digestion. As with preparation B, no effect was observed on the binding of α1β1 and α2β1 after incubation at 30 °C or at 37 °C (Fig. 4).

Attachment of the Human Fibrosarcoma Cell Line HT1080 and the Rat Glioma Cell Line RuGLi—The attachment of cells to gelatinase-cleaved type IV collagen was monitored to complement the information obtained from the isolated integrin experiments. HT1080 cells bind to type IV collagen through α2β1 integrin and do not express α1β1 integrin. RuGLi cells express α1β1 but not α2β1 integrin (2, 20). The differential expression of integrins by these cell lines facilitated the study of integrin binding sites on the cleaved collagen. HT1080 and RuGLi adhesion to preparation A and preparation B was unaffected by gelatinase treatment at 30 °C (Fig. 5). Treatment of preparation A at 37 °C, however, greatly reduced both HT1080 and RuGLi cell adhesion. Adhesion to preparation B remained unaffected by gelatinase treatment at 37 °C, reflecting the greater resistance of preparation B to digestion due to intact intramolecular disulfide bonds. Treatment of the dimers with gelatinase A at 37 °C barely altered HT1080 or RuGLi adhesion. These results correlated with the data obtained with the isolated α2β1 and α1β1 integrins.

The Cleavage Sites of Gelatinase within Denatured CB3[IV]
and the Triple-helical Fragment F1—Attempts to determine the gelatinase cleavage site within preparation A did not generate identifiable sequences. The cleaved \( \alpha_1(IV) \) and \( \alpha_2(IV) \) chains of the 300-nm-long C-terminal fragment did not show a unique N-terminal sequence. In order to gain information regarding the specificity of gelatinase A for denatured and native collagen IV, two experiments were carried out. First, CB3[IV] was digested with gelatinase A in the denatured state, after reduction of the stabilizing disulfide bonds and carboxymethylation of the free sulphydryl groups. The peptide mixture resulting from the incubation with gelatinase A was separated by reversed phase chromatography on a C18 column and the individual peaks were subjected to Edman degradation. Triple sequences \((G-X-Y)_{n}\) were cleaved between \(G\) and \(X\), if position \(X\) was occupied by leucine or isoleucine. Hydroxyproline in position \(Y\) prevented cleavage. Cleavage of peptide bonds N-terminal to Leu or Ile was also found in non-triple-helical areas where peptide bonds Ala-Leu or Asp-Ile were split. Also, to some extent, peptide bonds between Gly and polar charged amino acid residues, such as Asp, Glu, Arg, and Lys, were split. In two cases, cleavage between \(X\) and \(Y\) positions was also observed (Fig. 6).

To test the ability of gelatinase A to cleave triple-helical type IV collagen, fragment F1, which contains the triple-helical part of CB3[IV], was incubated with gelatinase A at 30°C. After reduction and carboxymethylation, the peptide mixture was separated and analyzed as described above. No cleavage occurred in the region between the two disulfide knots of CB3 (Fig. 6). Cleavage occurred only in the N- and C-terminal region of F1 between Gly and Arg (\(\alpha_1\) 428–429), Gly and Lys (\(\alpha_2\) 431–432), Gly and Leu (\(\alpha_2\) 440–441), and Gly and Leu (\(\alpha_1\) 539–490) where the triple-helical structure seemed to be somewhat relaxed. It is striking that the Gly-Leu (\(\alpha_2\) 440–441) bond was cleaved in spite of the stabilizing hydroxyproline residue in the neighboring \(Y\) position, an effect that has not been observed after treatment of the denatured (IV) chains. The remaining triple-helical fragment still contained the recognition site of \(\alpha_1\beta_1\) between the disulfide bond and the C-terminal recognition site of \(\alpha_2\beta_1\). The N-terminal recognition site of \(\alpha_2\beta_1\) was cleaved off (Fig. 6). This is in agreement with the capacity of F1 to bind the \(\alpha_1\beta_1\) and \(\alpha_2\beta_1\) before and after treatment with gelatinase A (Fig. 7). The binding of \(\alpha_1\beta_1\) to F1 remained unaltered, whereas the binding of \(\alpha_2\beta_1\) to F1 was reduced to some extent.

**DISCUSSION**

The CB3[IV] region of collagen IV is of special interest. It is the only segment of the triple-helical domain that is stabilized by intramolecular disulfide bonds, and it contains the recognition sites for the integrins \(\alpha_2\beta_1\) and \(\alpha_2\beta_1\) (2). Since it has been observed that the triple-helical domain of collagen IV can be cleaved by bacterial collagenase (4) and by a collagen IV-specific tumor collagenase in the immediate vicinity of the N-terminal region of CB3[IV] (9), we have investigated to what extent the capacity of collagen IV to bind \(\alpha_2\beta_1\) and \(\alpha_2\beta_1\) is modified by treatment with gelatinase A.

The ability of a 68-kDa metalloproteinase to cleave collagen IV into two fragments was first shown by Sato et al. (11) and Fessler et al. (9), who treated cell-derived native collagen IV molecules at 35°C with a partially purified enzyme from metastatic mouse PMT sarcoma. Both gelatinase A (MMP2) and gelatinase B (MMP9) have been reported to cleave EHS-derived type IV preparations into two fragments, comprising one and three quarters of the intact molecule under specific conditions (12, 13, 15, 28). Activity always appeared to be temperature-sensitive, although to different degrees, ranging from neglible activity at 30°C to rapid and complete degradation at 37°C (16, 29, 30). The study of Mackay et al. (31) showed a rapid transition from no detectable cleavage of EHS-type IV collagen at 30°C to complete breakdown at 37°C, with little detection of distinct intermediates.

To initiate our study, we reassessed the action of gelatinase A against type IV collagen in our laboratories. Although most type IV collagen is prepared from the EHS tumor in ostensibly the same way, in the presence of reducing agents (22) we have found that the extent of reformation of the disulfide bonds may vary from that seen in preparation A (unstable) to that in preparation B (stable). In our experience, commercial preparations of EHS-derived type IV collagen are similar to ours and can vary significantly in the extent of disulfide bonding. In collagen IV preparations, which are more like the native protein with intramolecular disulfide bridges within CB3[IV], the
temperature of the specific gelatinase cleavage would be shifted to higher temperatures.

During preparation of collagen IV from human placenta and other tissues, bacterial collagenase preferentially cleaves the collagen IV network at an N-terminal site, close to the CB3[IV] region. Following this treatment, dimeric molecules can be extracted from the tissue. Dimers in solution are susceptible to further attack by bacterial collagenase. From these results one may assume that the C-terminal three quarters of type IV collagen are particularly protected by the supermolecular structure of collagen IV and that the cleavage site N-terminal to CB3[IV] is more exposed to proteolytic attack and thus may also be accessible to cells. Similarly in the case of soluble collagen IV, this particular region will be preferentially cleaved under mild conditions by bacterial collagenase and gelatinase A. Our data show that the status of the disulfide bonds within the CB3[IV] region and the NC1 domain may be critical in the conformational stability of soluble type IV collagen and hence in gelatinase A susceptibility. Thus, at 30 °C gelatinase A cleaved the monomeric molecules of preparation A, N-terminal to CB3[IV] into the typical one and three quarter fragments. At 37 °C the molecules were completely degraded. In molecules of preparation B with the disulfide-stabilized CB3[IV] region, specific cleavage of this area was shifted to higher temperatures, and in addition, at 37 °C, further digestion from the N terminus prevented. Under these conditions, however, a slow digestion of the triple-helical molecule from the C terminus was observed. In contrast, dimeric collagen molecules were found to be completely stable against gelatinase at 37 °C. Here both ends of the triple helix are sealed: the N terminus by CB3[IV], and the C terminus by the hexameric NC1 complex formed by end-to-end aggregation of two molecules (4, 6).

It is striking that gelatinase A, in contrast to bacterial collagenase, is unable to attack the triple-helical domain of the dimeric collagen at 37 °C, although the triple helix, in particular in the neighborhood of non-triple-helical interruptions, should be relaxed at this temperature. This may in part be caused by the different specificity of the two enzymes. In the relaxed regions, bacterial collagenase attacks all Pro-Gly and Hyp-Gly bonds, which occur frequently along the α chains. Gelatinase A cleaves mainly between Gly and X, if position X is occupied by hydrophobic or polar charged residues. However, not all of these bonds are sensitive; in addition, hydroxyproline in position Y prevents gelatinase A cleavage of the Gly-X bond. Thus, bacterial collagenase will find many more cleavable peptide bonds in a given relaxed region than gelatinase A and is therefore able to attack collagen IV at lower temperatures. Neither gelatinase nor bacterial collagenase are able to cleave peptide bonds protected in an intact triple helix. Between 10 and 15 °C, collagen I molecules in solution are not attacked within the triple-helical domain. Digestion occurs slowly via the relaxed ends and stops in polar regions with low numbers of Pro-Gly bonds (34).

It is an open question whether the recognition sites of the CB3[IV] region, which have been characterized using collagen molecules in solution, are accessible when incorporated into the macromolecular network. The data observed on the resistance or sensitivity of the different collagen IV preparations to gelatinase A relative to bacterial collagenase allow some conclusions to be drawn on the proteolytic sensitivity of type IV collagen in vivo. Collagen IV molecules in the native network seemed to be well protected against proteolysis. Only the 60-nm-long stretch between the N-terminal region of the molecules, involved in overlapping intermolecular aggregation, and the CB3[IV] region appears to be sensitive to proteolytic attack (43). It is interesting that this stretch is rich in non-triple-helical interruptions (33). Whether gelatinase A alone can cleave at this site when collagen is incorporated into a network is not clear and has to be proven. One has to take into account that in vivo gelatinase A will be accompanied by other MMPs, which

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2 A. Ries and K. Kühn, unpublished data.
somewhat relaxed triple-helical end regions of F1 were partly
remained stable against gelatinase A. In contrast, the
isolated integrin reflected the situation occurring in cell attach-
ment to collagen IV. We addressed this point using the human
fibrosarcoma cell line HT1080 and a rat glioblastoma cell line
RuGLi, since these lines have been shown to interact with
collagen IV via the integrins αβ1 and αβ2, respectively (2, 20).
Other integrins expressed by these cells did not seem to be
involved. The results of the cell attachment assays supported
the data from the integrin binding experiments.

This resistance of the integrin recognition sites against pro-
teolytic attack may have some implications for in vivo situa-
tions, e.g. for the penetration of tumor cells through basement
membranes. The receptors αβ1 and αβ2 at the surface of cells
may remain occupied by the resistant recognition sites even
when the collagen IV network is largely degraded. They may
thus prevent further interaction of cells with intact molecules
of the surrounding macromolecular network and may modify
the ability of the cells to move through basement membranes.

On the other hand, one could speculate that excessive amounts
of protease-resistant recognition sites may inhibit invasion by
blocking too many of the integrin sites needed for adhesion and
migration.

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