Characterization of a Type IV Collagen Major Cell Binding Site with Affinity to the $\alpha1\beta1$ and the $\alpha2\beta1$ Integrins

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Abstract. The aim of this investigation was to identify the domains of type IV collagen participating in cell binding and the cell surface receptor involved. A major cell binding site was found in the trimeric cyanogen bromide-derived fragment CB3, located 100 nm away from the NH_2 terminus of the molecule, in which the triple-helical conformation is stabilized by interchain disulfide bridges. Cell attachment assays with type IV collagen and CB3 revealed comparable cell binding activities. Antibodies against CB3 inhibited attachment on fragment CB3 completely and on type IV collagen to 80%. The ability to bind cells was strictly conformation dependent.

Four trypsin derived fragments of CB3 allowed a closer investigation of the binding site. The smallest, fully active triple-helical fragment was $(150)_3$-amino acid residues long. It contained segments of 27 and 37 residues, respectively, at the NH_2 and COOH terminus, which proved to be essential for cell binding.

By affinity chromatography on Sepharose-immobilized CB3, two receptor molecules of the integrin family, $\alpha1\beta1$ and $\alpha2\beta1$, were isolated. Their subunits were identified by sequencing the NH_2 termini or by immunoblotting. The availability of fragment CB3 will allow for a more in-depth study of the molecular interaction of a short, well defined triple-helical ligand with collagen receptors $\alpha1\beta1$ and $\alpha2\beta1$.

TYPE IV collagen is the major collagenous constituent of basement membranes. It consists of two $\alpha1$(IV) chains and one $\alpha2$(IV) chain, each ~1,700 amino acid residues long (8). The molecules are 390 nm long with the globular NC1 domain found at the COOH terminus. In the extracellular matrix, type IV collagen forms a complex nonfibrillar network. In a manner not completely understood the molecules are covalently cross-linked via their NH_2 termini and COOH termini, respectively (45), and also arranged laterally to form segments of superhelices in which two or three triple helices are involved (51).

This network, in which other constituents of basement membranes, such as laminin or heparan sulfate proteoglycan are specifically incorporated, acts as mechanically stable support for cells of the basement membrane zone (44). In vitro experiments have shown that type IV collagen is a highly specific substrate that interacts with endothelial (35), epidermal (32), and mesenchymal cells (3, 7), including transformed and metastatic tumor cells (3, 12, 15, 34, 47). The cell surface receptors responsible for the interaction with type IV collagen appear to belong mainly to the $\beta1$ or VLA subgroup of the integrin receptor family that is specifically involved in interactions with constituents of the extracellular matrix (21, 27, 30). The cell binding sites of type IV collagen are conformation dependent and have been ascribed to the triple-helical domain of the molecule (3), although some reports describe cell binding to denatured molecules as well (39).

Here we present the isolation of a triple-helical fragment, approximately $(150)_3$ amino acid residues long and located 100 nm away from the NH_2 terminus of the molecule, which contains a major cell binding site of type IV collagen. Its triple-helical conformation is stabilized by intramolecular disulfide bonds (8, 13). Polyclonal antibodies prepared against the fragment inhibited 70–80% of cell attachment capacity to type IV collagen. Integrins of the $\alpha1\beta1$ and $\alpha2\beta1$ type from human tissues and cell lines bind specifically to this triple-helical fragment.

Materials and Methods

Isolation of Type IV Collagen

Tetrameric type IV collagen was prepared according to reference 45. Minced human placental material (obtained from Behringwerke, Marburg, Germany) from fresh human placenta, washed blood free with 0.4 M sodium chloride, was suspended in 0.5 M acetic acid and treated with porcine pepsin (1:2000 [wt/wt]; Boehringer, Mannheim, Germany) at 4°C for 16 to 20 h. The protein was precipitated from the supernatant with 0.7 M NaCl, again dissolved in 0.03 M Tris/HCl, pH 7.6, containing 0.2 M NaCl, and the

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tetrameric type IV collagen separated by repeated precipitation with 1.7 M NaCl. For further purification, type IV collagen was dissolved in 0.03 M Tris/HCl, pH 8.6, containing 2 M urea and 0.2 M NaCl and applied to DEAE-cellulose (DE52; Whatman Inc., Clifton, NJ) in a batch procedure. The nonbinding fraction was dialyzed against 0.1 M acetic acid and lyophilized. All procedures were carried out at 4°C. In type IV collagen samples prepared in this way a part of the methionine residues was oxidized and not available for CNBr cleavage. To minimize oxidation of the methionine residues, fresh placenta was used as starting material for the preparation of type IV collagen, and the procedures described above were carried out under nitrogen to exclude oxygen as far as possible.

Intact type IV collagen from mouse was isolated from the Engelbreth-Holm-Swarm tumor by the procedure of Kleinman et al. (26). The NH_2-terminal 7S domain of human type IV collagen was prepared as described earlier (28). The NC1 domain was isolated from human placenta using a two step collagenase treatment described previously (33).

Preparation of the CNBr Peptide CB3

Pepsin-derived human type IV collagen was dissolved in 70% formic acid (10 mg/ml), flushed with nitrogen and incubated with CNBr (protein/CNBr 1:1 [wt/wt]) for 4 h at 37°C (13). Subsequently, CNBr was removed under vacuum overnight; the remaining solution was diluted with 5 volumes of water and lyophilized.

The CNBr-derived peptide mixture was separated on an agarose 1.5 m column (Bio-Rad Laboratories, Richmond, CA) (5 × 110 cm) equilibrated with 0.05 M Tris/HCl, pH 7.5, containing 1 M $CaCl_2$ and 0.02 M sodium azide at a flow rate of 100 ml/h. The triple-helical, trimeric CNBr peptide CB3 eluting at a molecular mass range of 80–100 kD was collected and rechromatographed under the same conditions.

Reduction and Carboxymethylation

A solution (5–10 mg/ml) of CB3 in 6 M guanidine.HCl, 0.05 M Tris/HCl, pH 8.1, and 0.002 M EDTA, was flushed with nitrogen and placed in a 60°C water bath for 30 min to denature the triple helix. DTT (50 mol per mole disulfide) was added, the tube flushed briefly with nitrogen and maintained at 50°C for 4 h. After cooling the solution to room temperature, an aqueous iodoacetic acid solution (twofold molar excess over DTT) was added. After alkylation for 20 min in the dark, the reagents were removed by dialysis against 0.1 M acetic acid.

Preparation of Tryptic Fragments of CB3

CB3 was suspended in 0.02 M Tris/HCl, pH 8, at a concentration of 2 mg/ml and heated to 70°C for 30 min to dissolve the protein. To reform the triple-helical structure of the fragment, the solution was kept at 20°C overnight. Trypsin digestion (trypsin-TPCK; Worthington Biochemical Corp., Freehold, NJ) was performed at 20°C for 2 h and terminated by adding acetic acid and subsequent lyophilization.

The tryptic digest was first separated on a TSK 3000 SW ultropac column (7.5 × 600 mm; Pharmacia-LKB, Uppsala, Sweden), which was equilibrated at a flow rate of 0.15 ml/min with 0.2 M ammonium acetate containing 0.1% trifluoroacetic acid. The fragments pooled were further chromatographed on a Mono Q HR 5/5 (Pharmacia) anion exchanger, using 0.02 M Tris/HCl, pH 8 (solvent A) and 0.02 M Tris/HCl, pH8, 2 M NaCl (solvent B), both containing 2 M urea, at a flow rate of 0.5 ml/min with a gradient of 2.5–16% B in 50 min. Fragment 2 was further purified on a Mono S HR 5/5 (Pharmacia) cation exchange column equilibrated with 0.1% trifluoroacetic acid containing 2 M urea, adjusted to pH 2.5 with NaOH (solvent A). Elution was accomplished with 0.1% trifluoroacetic acid, 2 M NaCl, and 2 M urea, pH 2.5 (solvent B), using a linear gradient of 4–25% B in 30 min at a flow rate of 0.5 ml/min. The pooled fragments were dialyzed against 0.5 M acetic acid.

Analytical Methods

SDS-PAGE followed established protocols (29). For molecular mass estimates, runs were calibrated with globular proteins (Bio-Rad Laboratories) and CNBr digests of type I collagen, under reducing and nonreducing conditions. For fluorography of gels containing radiolabeled proteins we used Kodak XAR-5 film.

Amino acid compositions and protein concentrations were determined after hydrolysis with 6 M HCl (24 h, 110°C) on an analyzer (LC5001; Biotronic Wissenschaft Liche Geräte GmbH, Puchheim, Germany).

Amino acid sequences were determined by Edman degradation on a gas phase sequencer (model 470A; Applied Biosystems Inc., Foster City, CA) either from purified material or from single protein bands transferred to polyvinylidene difluoride membranes (Immobilon; Millipore Continental Water Systems, Bedford, MA) (31) by semi-dry electroblotting with a Fast-Blot apparatus (Biometra, Göttingen, Germany) after SDS-PAGE under reducing conditions. The blotting buffer was 50 mM sodium borate at the anode and 50 mM sodium borate with 0.1% SDS at the cathode. Both buffers contained 10% methanol. Blotting was done at 0.8 mA/cm^2 for 1 h.

Immunochemical Methods

Rabbit antisera against CB3 were raised according to standard protocols (43) using 500 μg antigen per injection. The IgG fraction of the serum was isolated by ammonium sulfate precipitation and subsequent chromatography on protein A-Sepharose or DEAE-cellulose (DE 52; Whatman Inc., Clifton, NJ). The immunoreactivity of the purified IgG fraction was verified by ELISA.

For the identification of the β_1 integrin subunit, the proteins were blotted onto nitrocellulose sheets (Bio-Rad Laboratories) and then immunostained as in reference 46. The antiserum against human β_1 isolated from blood platelets was a gift of Dr. K. von der Mark, Erlangen, Germany.

Cells and Cell Culture

The following cell lines were used: HT1080 (human fibrosarcoma), A 375 (human melanoma), A 431 (human epidermoid), HBL-100 (human mammary epithelia), SCL I (human skin carcinoma), SCL II (human skin carcinoma), Saos-2 (human osteosarcoma), RuGLi (rat glioblastoma), RN22 (rat schwannoma) and primary mouse myoblasts (3, 5). Cells were grown to confluency in DMEM, supplemented with glutamine (300 μg/ml), penicillin (400 U/ml), streptomycin (50 μg/ml), and 10% FCS (Gibco Laboratories, Grand Island, NY). For propagation and cell attachment assays, cells were harvested with 0.05% trypsin, 0.02% EDTA in PBS, pH 7.2. For roller bottle cultures (3027; Falcon Labware, Oxnard, CA), 1 mM pyruvate and 15 mM Hepes were used as additional supplements.

Surface Labeling of Cells

10^8 cells grown in roller bottles were pelleted and their surfaces labeled with Na ^{125}I (37 Megabequerel sp act 481 MBq/μg, Amersham Buchler, GmbH, Braunschweig, Germany) using Iodo-beads iodination reagent (Pierce Chemical Company, Rockford, IL). After labeling, cells were washed three times in TBS, pH 7.2 containing 1 mM each $CaCl_2$, $MgCl_2$, and $MnCl_2$ (wash buffer).

Attachment and Spreading Assays

Attachment assays followed the method described by Aumailley et al. (4). Tissue culture plates (96 multiwell plates; Costar, Cambridge, MA) were coated by adsorption of the proteins overnight at 4°C. Free binding sites on the plastic were blocked with 1% BSA. The amount of protein adsorbed to the wells was determined in separate experiments by using ^{125}I-labeled protein as tracers and counting the radioactivity after solubilization with 2 M NaOH. Labeling was performed with iodo-beads (Pierce Chemical Co.) following the manufacturer's instructions. The coating efficiency of CB3 and fragments 1–4 was demonstrated to be comparable to that of intact collagen type IV from human or mouse (3–5%).

Freshly suspended cells (see above) in serum-free medium (4 × 10^5 cells/ml) were plated onto coated wells (0.1 ml/well) and allowed to attach for 30 min at 37°C. For assays carried out in the presence of cycloheximide, this was added to culture dishes 2–4 h before testing, and into the attachment medium at a concentration of 25 μg/ml.

At the end of the attachment period, the medium was removed, the attached cells were washed with medium, fixed in 70% ethanol and stained (41) with 0.1% crystal violet in water (20). After washing extensively with distilled water, the dye adsorbed to the cells was solubilized with 0.2% Triton X-100 (0.05 ml/well) and optical density was read with an ELISA reader (MR 600; Dynatech; Denkendorf, Germany) at 570 nm. A linear correlation of cell numbers and optical density of released dye has been shown previously (4).

To assess inhibition of cell attachment by antibodies against CB3, protein coated dishes were incubated with different dilutions of the antibody in PBS for 1 h before adding the cell suspension. Dilutions of the IgG fraction of rabbit nonimmune serum were used as controls. To assess inhibition of cell attachment by synthetic peptides, equal volumes of cell suspensions were mixed with different dilutions of peptides and added immediately to the

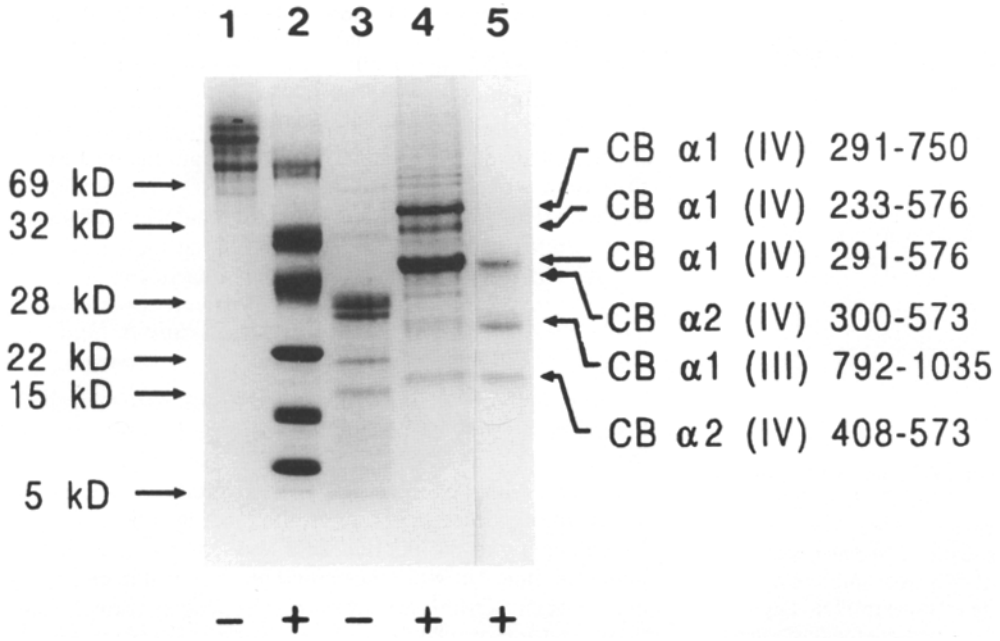


Figure 1. SDS-PAGE of the triple-helical CNBr peptide CB3 isolated from pepsin-derived human type IV collagen. Lanes *1* and *4*, CB3 from type IV collagen with partially oxidized methionine residues; lane *5*, CB3 from type IV collagen prepared under nitrogen; lane *2*, low molecular mass protein standard; lane *3*, CNBr peptides from type I collagen: (−) without and (+) with DTT. Arrows indicate relative molecular mass values of the CNBr peptides from type I collagen. Proteins were stained with Coomassie brilliant blue. The bands in lanes *4* and *5* were blotted and characterized by Edman degradation. Numbers in brackets denote NH_2- and COOH-terminal residues of the individual peptides. Whereas the NH_2 termini were directly detected by sequencing, the COOH termini were inferred from the relative molecular mass of the polypeptides and the positions of the methionine residues.

wells (4). Control experiments contained equal amounts of solvent. The synthetic peptides used were: GRGDS (Protogen AG, Läufelfingen, Switzerland); ARGDPGF, ARGDP*GF (P* = hydroxyproline) and SRGDTG (all peptides were synthesized by Dr. Jäger, Martinsried, Germany).

Receptor Extraction from Placenta, Platelets, and Cells

Placenta and platelets were extracted according to (19) with slight modification. Fresh human placenta was freed from chorion and amnion membranes, cut into small pieces and washed in TBS containing 1 mM $MnCl_2$, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml antipain by stirring and filtration through a nylon net. The tissue was homogenized in this buffer with an Ultraturax and freed from blood and serum proteins by repeated centrifugation (five times, 1,000 *g*, 10 min) and resuspension. The insoluble placental material was extracted by adding an equal volume of extraction buffer (washing buffer containing 50 mM octylglucoside) and stirring for 4–6 h at 4°C. After centrifugation at 27,000 *g* for 30 min, the supernatant was stored at −80°C.

Outdated human platelets were washed once with 20 mM Tris/HCl, pH 8, 1 mM EDTA, 0.9% glucose, and centrifugation at 1,000 *g* for 10 min. The pellet was washed twice with TBS containing 2 mM $MgCl_2$ and protease inhibitors as above by stirring for 1 h at 4°C. Finally, the pelleted platelets were extracted with TBS containing 100 mM octylglucoside, 2 mM $MgCl_2$, 1 mM $MnCl_2$, and protease inhibitors. The suspension was centrifuged at 32,000 *g* for 30 min. The supernatant was stored at −80°C for affinity chromatography.

Trypsinized cells were collected by centrifugation (200 *g*, 10 min) and washed three times with TBS containing 2 mM $MgCl_2$, 1 mM $MnCl_2$, and protease inhibitors as above. After pelleting, cells were extracted with wash buffer containing 100 mM octylglucoside (1 ml/ml packed cell volume) for 20 min on ice with frequent shaking. Extraction was terminated by centrifugation (16,000 *g*, 10 min) (19).

Receptor Isolation by Affinity Chromatography

This is described in reference 19. Peptide CB3 was coupled to CNBr-activated Sepharose (Pharmacia LKB, Freiburg, Germany) according to the manufacturer's instructions. The concentration of bound CB3 was 3.5 mg/ml Sepharose. Placental extracts were passed over a CB3-Sepharose column (10 ml bed volume) with a flow rate of 5 ml/h. The column was washed with TBS containing 25 mM octylglucoside, 1 mM $MnCl_2$, and protease inhibitors as above (80 ml) and then with 20 ml of washing buffer containing 300 mM NaCl. Bound protein was eluted with TBS containing 25 mM octylglucoside, 10 mM EDTA and protease inhibitors. Finally, the column was washed with elution buffer containing 1.85 M NaCl.

Extracts of cells or platelets were incubated with equal volumes of CB3-Sepharose (equilibrated in TBS, 25 mM octylglucoside, 2 mM $MgCl_2$, 1 mM $MnCl_2$, and protease inhibitors as above) overnight by rotating at 4°C. The Sepharose was then packed into a column (2 × 5 cm) and washed as above. Bound protein was eluted with TBS containing 25 mM octylglucoside and 15 mM EDTA. Eluted samples were made 20 mM in $MnCl_2$ to inactivate EDTA.

Column runs were monitored at 280 nm. Aliquots of the collected samples were precipitated by adding 5 vol of acetone (−20°C). After 10 min precipitates were collected by centrifugation (16,000 *g*, 10 min), dried under nitrogen, and dissolved in electrophoresis buffer with or without 8 mM DTT or 200 mM in mercaptoethanol.

Results

Isolation of the Triple-helical Cyanogen Bromide Fragment CB3

As starting material for the isolation of the cell binding site we used pepsin-derived type IV collagen from human placenta. This preparation consists of tetramers in which the molecules are covalently cross-linked via their NH_2 terminal 7S domains. They contain intact triple-helical domains. Only the COOH terminal NC1 domain which was degraded by pepsin is missing (45). CNBr cleavage of the tetrameric type IV collagen led to a complex peptide mixture which was separated on an Agarose 1.5 m column (not shown). Upon testing individual peptide fractions in cell attachment assays, only one fraction in the range of M_r = 80–100 kD was active. SDS-PAGE of this fraction after rechromatography revealed triple-helical CNBr peptides in which the individual α-chain fragments were held together by disulfide bridges, since several well separated bands appeared after reduction (Fig. 1). These reduced bands were characterized by blotting and sequencing of the NH_2 termini. The trimeric CNBr peptide isolated from tetrameric type IV collagen prepared

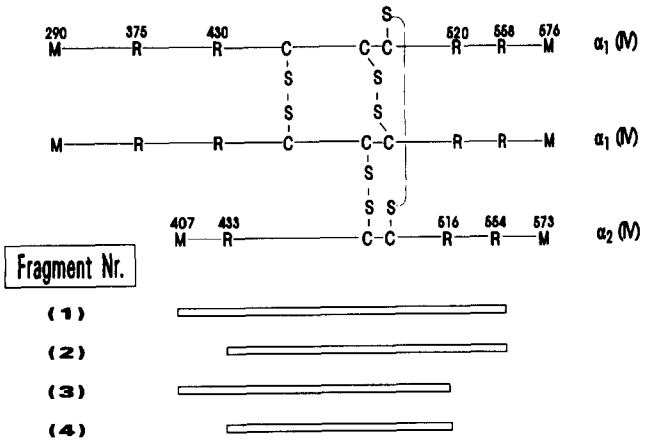


Figure 2. Scheme of the CNBr peptide CB3 and its tryptic fragments. The triple-helical conformation is stabilized by intramolecular disulfide bridges. The heterogeneity of CB3 shown in Fig. 1 (lanes *1* and *4*) is caused by partial oxidation of the methionine residues 290 and 576 (α1) and 407 and 573 (α2), preventing CNBr cleavage. The positions of some of the arginine residues as putative trypsin cleavage sites are indicated. Numbers are the position numbers of the aligned α1(IV) and α2(IV) chains (8). They do not coincide with residue numbers of the single α-chains. *M*, methionine; *C*, cysteine; *R*, arginine; *G*, glycine; *D*, aspartic acid; *T*, threonine; *P*, proline. Disulfide bridges are tentative. The bars below represent the triple helical parts of the four tryptic fragments: fragment *1*, $[\alpha1(IV)376\text{-}558]_2\alpha2(IV)408\text{-}554$; fragment *2*, $[\alpha1(IV)431\text{-}558]_2\alpha2(IV)434\text{-}554$; fragment *3*, $[\alpha1(IV)376\text{-}520]_2\alpha2(IV)408\text{-}516$; fragment *4*, $[\alpha1(IV)431\text{-}520]_2\alpha2(IV)434\text{-}516$. Numbers give the position of the NH_2- and COOH-terminal residue of the individual peptides. NH_2 termini were directly determined by sequencing, COOH termini were inferred from the relative molecular mass of the peptides and the potential-trypsin cleavage sites. Sequence of the NH_2- and COOH-terminal triple-helical segments of fragment 1 important for cell attachment is the following. Position 408–433: *α1(IV)*, PGERGEKGDRGFPGTSLPGPSGRDGLP; *α2(IV)*, MGPKGFIGDPGIPALYG-GPPGPDGKR; and position 517–553: *α1(IV)*, KGDRGLPGRDGVAGVPGPQGTPGLIGQPGAKGEPGEF; *α2(IV)*, KGDKGDPGQHGLPGFPGLKGVPGNIGAPGPKGAKGDS.

from fresh placenta under conditions where oxidation of methionine residues was largely prevented (see Materials and Methods) revealed after reduction the expected monomeric peptides CBα1(IV) 291–576 and CBα2(IV) 408-573 (Fig. 2, Fig. 1, lane *5*). An additional band between CBα1(IV) and CBα2(IV) was identified as a COOH-terminal CNBr peptide of the α1(III) chain which started at position 789 and had a 220-amino acid residue long triple-helical sequence (2). It arose from a contamination with type III collagen and could be removed by chromatography on a Mono Q column. This peptide had, in contrast to the entire type III collagen molecule, no cell binding capacity (not shown). The CNBr peptide fraction obtained from tetrameric type IV collagen with partly oxidized methionine residues revealed a peptide mixture (Fig. 1, lanes *1* and *4*). Beside the expected monomeric peptides, the double peptides CBα1(IV)291–750, CBα1(IV) 233–576, and CBα2(IV)300–573 appeared because of incomplete cleavage due to a fraction of oxidized methionine residues at position 290 and 576 in the α1(IV) and 407 in the α2(IV) chain. For cell attachment experiments only CB3 preparations with a content of <10% of the double peptides were used.

Trypsin treatment of CB3 preparations and subsequent chromatography on a TSK 3000 column resulted in four major peaks containing large fragments (Fig. 3 *a*). They were further purified by ion exchange chromatography on a Mono Q column (not shown), and then run on SDS-PAGE without and with reduction (Fig. 3 *b*). The individual bands observed were blotted and their exact location along the α1(IV) and α2(IV) chain determined by sequence analysis (Fig. 2).

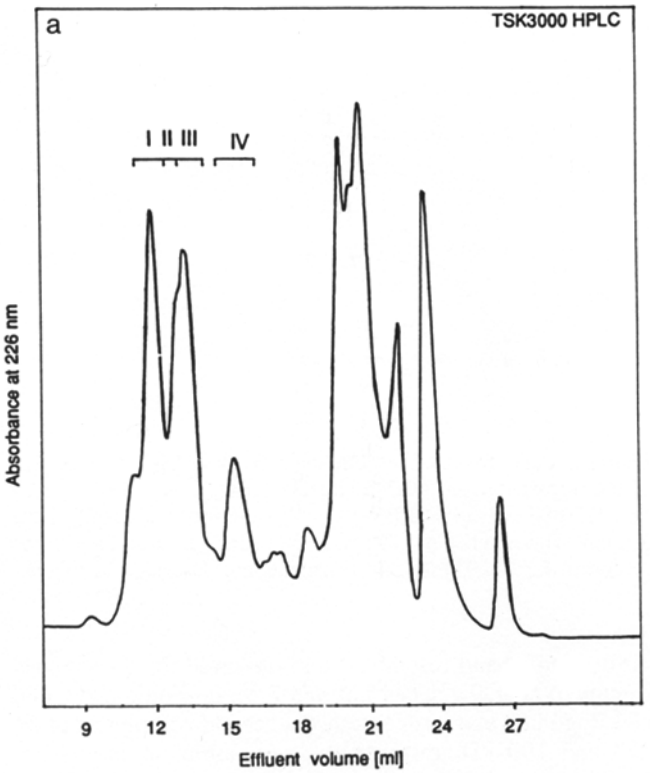
The Cell Attachment Capacity of the Triple-helical Fragment CB3

Several different cell lines were tested to compare the cell attachment capacity of pepsin-derived type IV collagen and the triple-helical CNBr peptide CB3 (see Materials and Methods). Although the ability to attach to type IV collagen varied among different cell lines, the binding activity to type IV collagen and the peptide CB3 was comparable for any given cell line. No difference could be observed in cell binding between pepsin-derived type IV collagen from human tissue and intact type IV collagen isolated from the Engelbreth-Holm-Swarm mouse tumor (not shown). The experiments carried out with the human fibrosarcoma cells HT1080 are described in detail as an example of the cell lines assayed.

HT1080 cells show almost identical dose-response profiles in their attachment to type IV collagen and peptide CB3 (Fig. 4). Complete destruction of the triple-helical conformation of CB3 by reduction of disulfide bridges under denaturing conditions and subsequent carboxymethylation abolished cell attachment. Since unfolded collagenous polypeptides crosslinked by covalent bonds easily reform the triple helix, reductive cleavage of the disulfide bridges was essential for complete and irreversible denaturation of CB3 (6, 14). The two terminal regions of the type IV collagen molecule, the 7S and the NC1 domain did not show cell binding (not shown).

The sequence Arg-Gly-Asp (RGD) is essential for the interaction of extracellular components such as fibronectin and vitronectin with cell receptors of the β1 integrin subfamily (23, 36). Since α1(IV) and α2(IV) from human type IV collagen contain three and seven RGD sequences, respectively (8, 22), we used RGD containing synthetic peptides in inhibition assays of HT1080 cell attachment to type IV collagen and CB3. None of the peptides ARGDPGF, ARGDP*GF, SRGDTG, and RGDV, all sequences present in type IV collagen, inhibited cell attachment. Only the peptide GRGDS, a sequence not present in type IV collagen, showed weak inhibitory activity (Fig. 5). However, at a concentration of 500 μg/ml that inhibited the attachment of HT1080 cells to fibronectin almost completely, still >80% cell attachment to type IV collagen and CB3 was observed.

Polyclonal antibodies raised against CB3 were used to inhibit cell binding to CB3 and type IV collagen. Under conditions where the attachment of HT1080 cells to CB3 was completely inhibited, type IV collagen still showed a cell attachment corresponding to 20–30% of the control (Fig. 6). Since the whole antiserum or IgG purified by DEAE chromatography contained components that appeared to mediate additional cell attachment, only the IgG fraction purified by affinity chromatography on a protein A column could be used for these experiments.



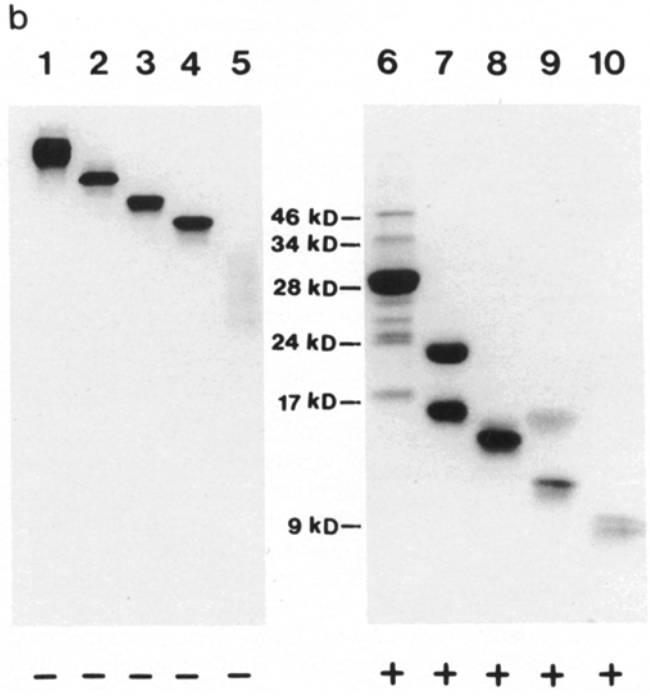


Figure 3. Separation and PAGE analysis of tryptic fragments. (*a*) Separation of a tryptic digest of the triple-helical CNBr peptide CB3 by molecular sieve HPLC on a TSK 3000 column (7.5 × 600 mm) equilibrated with 0.2 M ammonium acetate containing 0.1% trifluoroacetic acid. (*b*) SDS-PAGE of fragments *1–4* after rechromatography on a Mono Q HR 5/5 column. CB3 (lanes *1* and *6*), fragment 1 (lanes *2* and *7*), fragment 2 (lanes *3* and *8*), fragment 3 (lanes *4* and *9*), fragment 4 (lanes *5* and *10*). (−) Without and (+) with DTT. Relative molecular mass of the main CNBr-derived peptides are indicated. The individual bands of the reduced preparations (lanes *6–10*) were blotted and sequenced. The results are listed in Fig. 2.

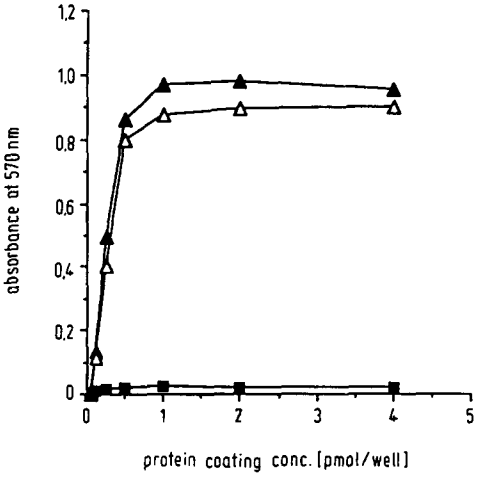


Figure 4. Cell binding activity of the triple-helical CNBr peptide CB3 (△), pepsin-derived tetrameric type IV collagen (▲), and CB3 reduced under denaturing conditions and carboxymethylated (■). Tissue culture multi-well plates were coated with indicated amounts of substrate. The remaining protein binding sites on the plastic were blocked with BSA and the wells were incubated with HT1080 cells (4×10^4 cells/well) at 37°C for 30 min. After washing, the number of cells attached was determined after staining with crystal violet as described in Materials and Methods. The values shown are the average of triplicates with blank values substracted.

Of the four trypsin-derived fragments of CB3, only fragment 1, which comprises almost the entire triple-helical part of CB3 (see Fig. 2), showed an attachment capacity similar to CB3 (Fig. 7). The smallest fragment, fragment 4, with a triple-helical segment from positions 433 to 516 was, however, inactive (Fig. 7). Extension of the NH_2 terminus of this inactive segment to position 408, as in fragment 3, restores the cell binding activity to 80% of the value found for

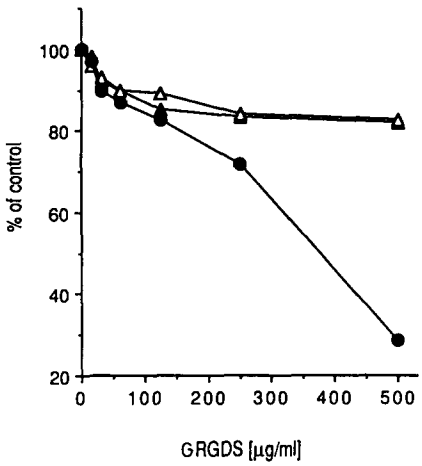


Figure 5. Inhibition of cell attachment to type IV collagen (▲), CB3 (△), and fibronectin (●) by the synthetic peptide GRGDS. HT1080 (4×10^5 cells/ml) were mixed with the peptide and 0.1 ml immediately added to the culture wells coated with 1 pmol protein per well. After incubation at 37°C for 30 min the number of cells was determined as described in the legend to Fig. 4. The number of cells attached on each substrate in the absence of GRGDS was set to 100%.

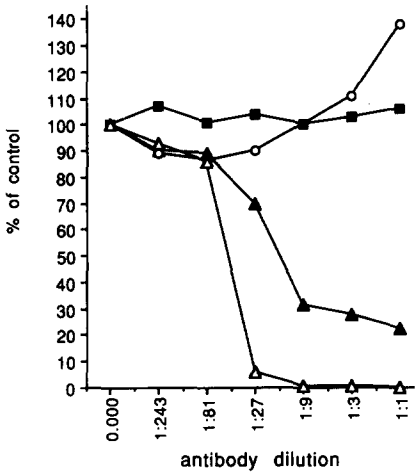


Figure 6. Inhibition of cell attachment to type IV collagen and CB3 by antibodies against CB3. Antibodies were added in indicated dilutions to the wells coated with 1 pmol of protein and incubated at 22°C for 1 h before the addition of 4 × 10^4 HT1080 cells to each well. After 30 min at 37°C the number of cells attached on each substrate was determined as described in the legend to Fig. 4. The number of cells attached on each substrate in the absence of anti-CB3 IgG was set to 100%. Only IgG purified by DEAE chromatography and affinity chromatography on protein A inhibited cell binding to type IV collagen (▲) and to CB3 (△). Purification of IgG on a DEAE-cellulose column was not sufficient to remove all adventitious serum components mediating cell attachment (○). A purified IgG fraction from a rabbit preimmune serum was used as control (■).

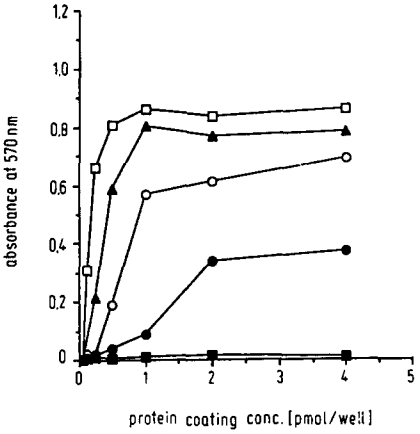


Figure 7. Comparison of cell binding activity of CB3 (□) with that of the trypsin-derived CB3 fragments 1 (▲), 2 (●), 3 (○), and 4 (■). HT1080 cells (4 × 10^4 cells/well) were incubated at 37°C for 30 min. The number of cells was determined as described in the legend to Fig. 4. The tryptic fragments are described in Fig. 2.

fragment 1. Fragment 2, in which the inactive segment is extended towards the COOH terminus to position 558 has 40% of the cell binding activity of fragment 1. We also measured the time dependence of cell attachment at a given protein concentration (1 pmol) of CB3 and the trypsin-derived fragments and confirmed the differences in cell binding activity of the trypsin-derived fragments of CB3. The tryptic fragments 1, 2, and 3 did not only promote attachment but also spreading of the HT1080 cells (Fig. 8). Synthetic peptides representing the sequence of the NH_2-terminal triple-helical part of fragment 1 (α1(IV) and α2(IV) positions 408–433 [Fig. 2]) were used for cell attachment inhibition experiments. Neither the individual peptides nor the 2:1 mixture of α1(IV) and α2(IV) peptides showed an inhibition effect with up to 20-fold molar excess over the attachment substrates.

Identification of Cell Receptors of the Integrin Family Responsible for the Binding of Cells to the Fragment CB3

HT1080 and Rugli cells, which attached to and spread to a comparable extent on type IV collagen and CB3, were extracted with an octylglucoside and Mn^{2+} containing buffer (19). The extracts were passed through an affinity column of fragment CB3 immobilized on Sepharose. After washing, the column was eluted with EDTA buffer and the eluates subjected to SDS-PAGE. Both preparations revealed a similar polypeptide with an apparent M_r of 116 kD. After reduction, the molecular mass and decreased electrophoretic mobility of this band resembled that observed for the integrin subunit β1 (Fig. 9 *a*, lanes *5*, *6*, and *7*, *8*). In addition, HT1080 and Rugli cell extracts contained a second component of M_r 150 and 190 kD, respectively, resembling in their electrophoresis mobility the integrin subunits α2 and α1 (Fig. 9 *a*, lanes *5*, *6* and *7*, *8*) (27, 36). In a separate experiment the extract of human HBL-100 cells, surface-labeled with ^{125}I was applied to a CB3-Sepharose affinity column. The EDTA eluate was submitted to SDS-PAGE. Three bands with relative molecular mass identical to the α1, α2, and β1 subunits were observed, suggesting the presence of the α1β1 and α2β1 integrins (Fig. 9 *b*). The two receptors were also isolated from placenta and blood platelets, and subjected to SDS-PAGE (Fig. 9 *a*, lanes *1*, *2* and *3*, *4*). The separated bands were blotted and identified by Edman degradation. The amino-terminal sequences determined for the 190- (FNVDVKNSMTF) and the 150-kD (YNVGLPEAKIFSGP) band were identical to those of the α1 and α2 integrin subunits, respectively (24, 40, 41). Probably due to a blocked NH_2 terminus, the 120-kD β1 band did not yield a sequence. The identity of the β1 subunit was therefore proven by Western blot analysis with an antiserum against human β1 (not shown). The β1 subunits isolated from placenta and platelets showed slightly different electrophoretic mobilities (Fig. 9 *a*, lanes *1*, *2* and *3*, *4*). This seemed to be due to a different carbohydrate moiety. After deglycosylation they exhibited identical mobility (not shown). Using immobilized, intact murine type IV collagen for affinity chromatography of placenta and platelet extracts, essentially the same results were obtained as with a CB3 column. Except for the integrins α1β1 and α2β1, no additional type IV collagen-binding integrins could be observed under the conditions used (not shown).

Discussion

Type IV collagen is responsible not only for the macromolecular organization and the biomechanical stability of basement membranes; along with laminin, nidogen, and heparansulfate proteoglycan it is also involved in the interac-

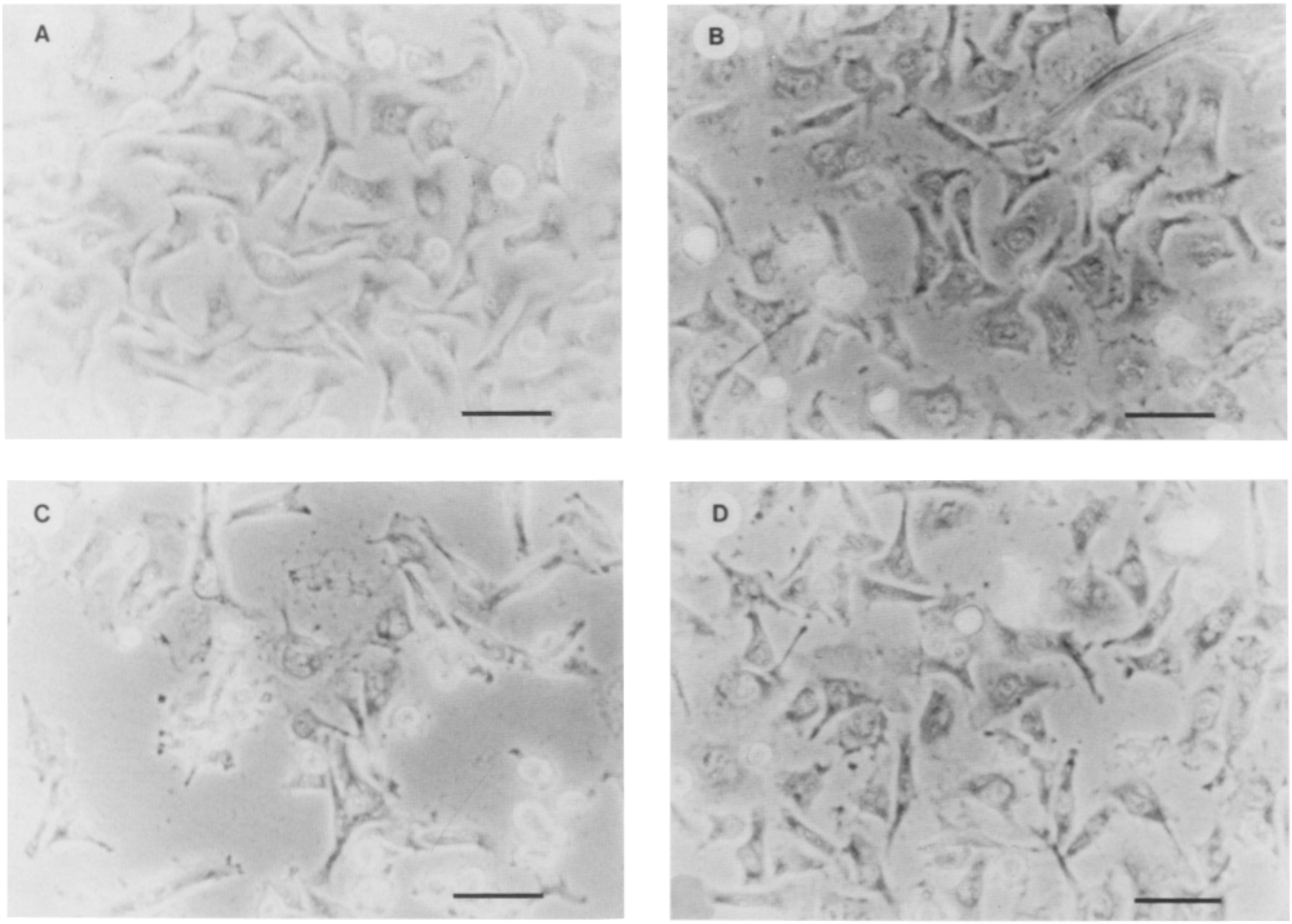


Figure 8. Spreading of HT1080 cells on substrates of CB3 (*A*) and the tryptic fragments 1 (*B*), 2 (*C*), and 3 (*D*). All wells were coated with 4 pmol/well. Pictures were taken after 60 min of exposure to the coats. Bars, 100 μm.

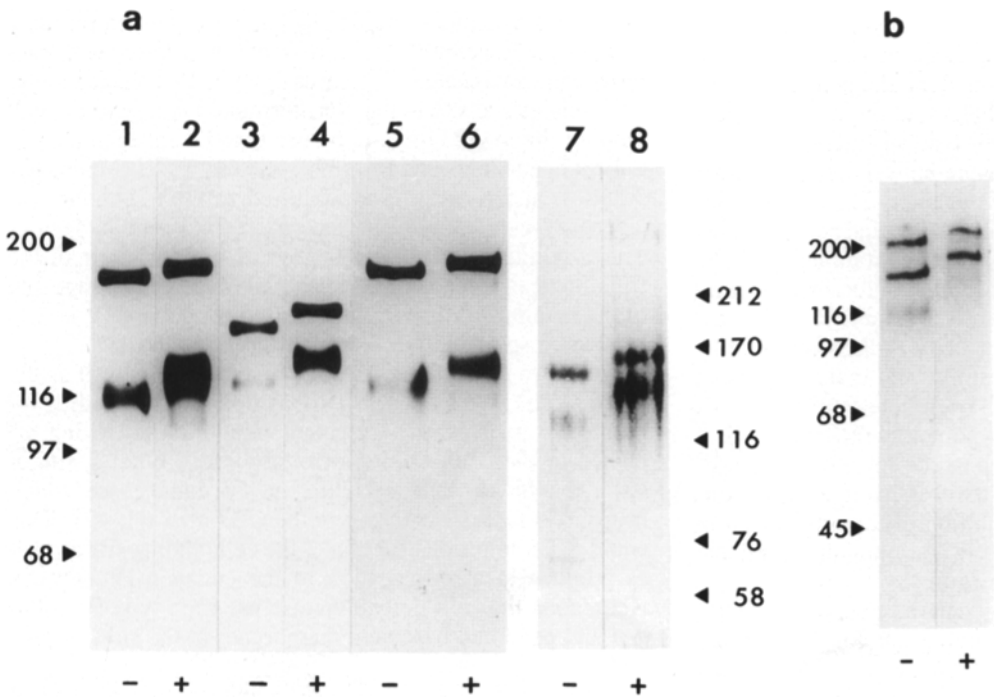


Figure 9. SDS-PAGE of type IV collagen binding proteins purified by affinity chromatography on a CB3-Sepharose column. (*a*) Octylglucoside-containing cell extracts were applied to a CB3 column. After washing, the columns were eluted with 10 mM EDTA, aliquots of the eluates were precipitated and redissolved in the same buffer without (−) and with (+) DTT. The proteins were separated in a 5–12 (lanes *1–6*) or a 7.5% (lanes *7* and *8*) polyacrylamide gel and stained with silver. Lanes *1* and *2*, human placenta; lanes *3* and *4*, human platelets; lanes *5* and *6*, rat glioblastoma (Ru-Gli); lanes *7* and *8*, human fibrosarcoma (HT1080). Arrows indicate relative molecular mass marker proteins. (*b*) Human mammary epithelial cells were surface labeled with Na ^{125}I, extracted with octylglucoside, and affinity chromatographed on a CB3-Sepharose column. The 10 mM EDTA eluate was separated on a 7.5% polyacrylamide gel with (+) and without (−) DTT and subjected to fluorography. Arrows indicate relative molecular mass of marker proteins.

tion of basement membranes with cells (44). The aim of our investigations was to identify domains of type IV collagen participating in cell binding and the cell surface receptors involved. We found a $(150)_3$ amino acid residue long triple-helical segment, ~100 nm away from the NH_2-terminal end of the molecule, which appears to possess the major cell-binding sites of type IV collagen. Antibodies against CB3, which blocked cell binding of the fragment completely, inhibited cell attachment to type IV collagen up to 80%. The terminal 7S and NC1 domains did not show cell attachment under the experimental conditions used. To what extent other regions of the triple-helical domain of the type IV molecule interact with cells is not clear, since we failed to prepare larger triple-helical segments that did not contain the CB3 region.

Affinity chromatography of cell extracts with the immobilized CB3 fragment was used to identify the cell receptors involved in type IV collagen binding. Two different integrin types, $\alpha 1\beta 1$ and $\alpha 2\beta 1$, were found to interact specifically with CB3. These two integrins are the typical collagen receptors (27, 37, 48). They mediate cell binding to type I as well as to type IV collagen, and there are reports that at least $\alpha 2\beta 1$ interact also with types II, III and VI collagen (38, 48). In addition, it has been found that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ have affinity for laminin (10, 18, 25). Thus ligand binding of these two integrins does not appear to be very specific. Whether this is due to a binding domain with an extremely broad specificity or to the presence of several distinct, more specific domains, is not known.

In our experiments, the binding of CB3 to both integrins was strictly dependent on the triple-helical conformation. There are, however, reports that integrins also interact with denatured collagen. This may be due to the fact that the sequence RGD occurs relatively frequently in the triple-helical areas of collagen. After unfolding of the triple helix, RGD sequences become exposed and may then be able to interact with different integrins such as $\alpha 5\beta 1$, $\alpha_v\beta 1$, or $\alpha_{IIb}\beta 3$ $\alpha_v\beta 3$, known as RGD-dependent fibronectin or vitronectin receptors (1). However, in intact triple helices the glycine residues, which occupy every third position along the peptide chains, are hidden in the center of the helix and are not accessible for receptors. A typical example is type VI collagen (3). The native molecules with an intact triple-helical domain interact with cells in a RGD-independent manner. The unfolded individual α chains of type VI collagen also bind cells, but now cell binding can be inhibited by RGD-containing synthetic peptides. The question is whether interaction of cells with unfolded collagen is physiologically relevant. It can be assumed that in vivo, denatured collagen will be removed by proteolytic enzymes relatively rapidly and that it is present in the extracellular matrix only in negligible amounts. In this respect it is striking that cell attachment to the triple-helical fragment CB3 can be inhibited up to 20% by 500 μg/ml synthetic peptide RGDS, in spite of the fact that it does not contain a RGD sequence. Thus inhibition of cell attachment in the presence of high amounts of RGD containing synthetic peptides may lead to unspecific results.

Cell binding to the four trypsin derived fragments of CB3 revealed two sequence regions important for binding the $\alpha 2\beta 1$ containing HT1080 cells ~50 nm away from each other, whereby the NH_2-terminal site appeared to be the more important one. Experiments with isolated integrins will be necessary to decide whether $\alpha 2\beta 1$ interacts with two neighboring triple-helical segments or whether additional collagen binding proteins at the surface of the HT1080 cells are involved. Synthetic peptides with the $\alpha 1$(IV) and $\alpha 2$(IV) sequences of the NH_2-terminal cell binding site of CB3 did not show any binding or inhibitory effect, neither in large molar excess, nor in mixtures. This corroborates our finding that the activity to interact with cells is strongly dependent on the triple-helical structure and that both polypeptide chains are important constituents of the binding site.

Recently it has been reported (9) that a synthetic peptide of 15 amino acid residues, representing an $\alpha 1$(IV) sequence located near the COOH-terminal end of the triple-helical domain of the type IV collagen molecule, promoted adhesion and spreading of a murine melanoma cell. Another synthetic peptide with a sequence of $\alpha 1$(IV) in the vicinity of the CB3 fragment seemed to promote attachment of human keratinocytes (50). In both cases the cell receptors are unknown. Staatz et al. (39) have observed that the $\alpha 2\beta 1$ integrin, which is responsible for the Mg^{+2}-dependent adhesion of platelets to type I collagen, interacts not only with the triple-helical type I collagen molecule, but also with the unfolded $\alpha 1$(I) and $\alpha 2$(I) chains and the 147-residues long CNBr peptide $\alpha 1$(I)CB3. In earlier experiments (16) it was shown that adhesion of platelets to type I and III collagen depends on the triple-helical conformation. Only one CNBr peptide of type III collagen, $\alpha 1$(III)CB4, in location and sequence homologous to $\alpha 1$(I)CB3, also revealed platelet adhesion activity, but in comparison to the activity of the triple-helical molecule only in a 500–1,000-fold molar excess. Comparison of the sequences of $\alpha 1$(I)CB3 and $\alpha 1$(III)CB4 with the amino acid sequence of the $\alpha 1$(IV) and $\alpha 2$(IV) chains of fragment CB3 did not reveal an obvious homology.

The question arises whether the cell binding site of the CB3 region, discovered in soluble collagen molecules is also accessible to cells in type IV collagen when it is incorporated in the extracellular matrix. There is evidence that the CB3 region is accessible in tissue. Treatment of human placenta and murine Engelbreth-Holm-Swarm tumor tissue with bacterial collagenase at 20°C cleaves the type IV collagen molecule only at one site in the NH_2-terminal vicinity of the cell binding site in CB3 (49). In mouse type IV collagen the initial cut occurs between Pro (390) and Gly (391) (Mann, K., A. Ries, and K. Kühn, unpublished results). Similar collagenase treatment of dissolved type IV collagen molecules causes additional cleavages at several other regions of the triple-helical domain. It is interesting that the cleavage site of the mammalian metalloproteinase collagenase IV is also located close to the NH_2-terminal area of CB3 (17).

The close proximity of the cell binding site as well as the cleavage site of collagenase IV may be used by invading tumor cells to penetrate basement membranes. Having attached to the collagen network via the cell binding site of CB3, the tumor cells secrete type IV collagenase which hydrolyzes type IV collagen in the CB3 area (11, 42). This could result in the destruction of the cell binding site as well as produce local degradation of the collagen IV network whereupon the cells detach themselves from type IV collagen and penetrate basement membranes at the sites of proteolysis.

The isolation of a relatively short and stable triple-helical segment, which bears the binding site for the typical collagen

receptors $\alpha1\beta1$ and $\alpha2\beta1$, provides for the first time the opportunity to investigate in more detail those regions of the $\alpha1$, $\alpha2$, and $\beta1$ subunits of integrins which are responsible for the interaction with a stiff, rod-like collagen ligand.

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