The Cell Adhesion Domain of Type XVII Collagen Promotes Integrin-mediated Cell Spreading by a Novel Mechanism*

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Type XVII collagen (BP180) is a keratinocyte transmembrane protein that exists as the full-length protein in hemidesmosomes and as a 120-kDa shed ectodomain in the extracellular matrix. The largest collagenous domain of type XVII collagen, COL15, has been described previously as a cell adhesion domain (Tasanen, K., Eble, J. A., Aumaillé, M., Schumann, H., Baetge, J., Tu, H., Bruckner, P., and Bruckner-Tuderman, L. (2000) J. Biol. Chem. 275, 3093–3098). In the present work, the integrin binding of triple helical, human recombiant COL15 was tested. Solid phase binding assays using recombinant integrin α1I, α2I, and α10I domains and cell spreading assays with αβ1 and αβ1-expressing Chinese hamster ovary cells showed that, unlike other collagens, COL15 was not recognized by the collagen receptors. Denaturation of the COL15 domain increased the spreading of human HaCaT keratinocytes, which could migrate on the denatured COL15 domain as effectively as on fibronectin. Spreading of HaCaT cells on the COL15 domain was mediated by αβ₁ and αβ₁ integrins, and it could be blocked by RGD peptides. The collagen α-chains in the COL15 domain do not contain RGD motifs but, instead, contain 12 closely related KGD motifs, four in each of the three α-chains. Twenty-two overlapping, synthetic peptides corresponding to the entire COL15 domain were tested; three peptides, all containing the KGD motif, inhibited the spreading of HaCaT cells on denatured COL15 domain. Furthermore, this effect was lost by mutation from D to E (KGE instead of KGD). We suggest that the COL15 domain of type XVII collagen represents a specific collagenous structure, unable to interact with the cellular receptors for other collagens. After being shed from the cell surface, it may support keratinocyte spreading and migration.

The collagens are a family of extracellular matrix proteins (1). Among the 19 different collagen subtypes that have been identified, types XIII and XVII are the only transmembrane proteins (2, 3). Type XVII collagen (BP180) is hemidesmosomal transmembrane protein that is mutated in junctional epidermolysis bullosa and is targeted by autoantibodies in blistering skin diseases (4–6). It occurs in two forms: as a full-length transmembrane protein and as a distinct, soluble ectodomain that is shed from the cell surface by a furin-mediated, proteolytic process (7, 8). This collagen possesses intracellular and transmembrane domains that are of 560 and 23 amino acids in length, respectively. In addition, it has an extracellular domain of 914 amino acids that contains multiple noncollagenous interruptions, dividing it into 15 collagenous subdomains (3). The longest of these subdomains, COL15, consists of 242 amino acids (residues 567–808 of collagen XVII). Thus, it is much larger than any of the other collagenous domains, which vary from 14 to 45 residues in length (3).

Most collagen subtypes can be recognized by a group of integrin-type cell adhesion receptors. Although all collagen-receptor integrins share the common α subunits resulting in four heterodimers: αβ₁, αβ₁, αβ₁, and αβ₁ integrins, the collagen receptors do not recognize the putative binding motif, arginine-glycine-aspartic acid (RGD). In contrast, they require the triple helical structure of native collagen. Different from other matrix receptor integrins, the collagen receptors have an independently folding protein structure called the I domain (inserted domain) (9–12). This 200-amino acid structure is located between the second and the third NH₂-terminal repeated sequences in the ectodomain of an α subunit. The I domain adopts a structure called the “Rossmann fold” configuration in which several β sheets are surrounded by α helices and support a divalent metal-binding site, referred to as the metal ion-dependent adhesion site (13). Both the α and αI domains are known to be essential for the primary recognition of the collagens (14, 15).

The published experiments have revealed interesting differences in the recognition of different collagens by αβ₁ and αβ₁ integrins (16, 17). All collagens tested so far have been recognized by at least one of these two receptors (16–19). Still, the cellular receptors for many collagen subtypes remain unidentified. Here, we show that the largest collagenous domain, COL15, in type XVII collagen (20) is an exception to the established trend. However, human HaCaT keratinocytes can use their RGD-dependent integrins to spread on COL15 and COL15 as a migration substrate. Although COL15 does not contain an RGD motif, it has numerous, highly related KGD sequences, and, as previous studies have shown, snake venom poisons can bind integrins through this sequence (21, 22).
Integrins in Cell Spreading on Type XVII Collagen

Materials and Methods
Production and Purification of Recombinant COL15 Domain of Collagen XVII—Human kidney 293-EBNA cells (Invitrogen, Groningen, the Netherlands) constitutively expressing EBNA-1 protein from Epstein-Barr virus were grown in Dulbecco’s modified Eagle’s/Nutrient mix F-12 medium (Life Technologies, Inc.) containing 10% fetal calf serum (Life Technologies, Inc.) and 0.35 g/ml puromycin (Sigma). One million cells/10-cm culture dish were transfected with 25 μg of an expression vector, pCPE-Col15, coding for the amino acids 567–907 of human collagen XVII (for details see Ref. 20) using the calcium phosphate method. Following a selection with 0.5 μg/ml puromycin (Sigma), the transfected cells were grown to confluency, washed twice with phosphate-buffered saline, and switched to the same serum-free medium consisting of 50 μg/ml ascorbic acid (Fluka, Deisenhofen, Germany). To maintain sufficient levels of ascorbic acid in the medium, 10 μl of freshly made ascorbic acid stock solution (5 mg/ml) per ml of medium was added every 24 h. The media were collected every 48 h, centrifuged, and switched to the same serum-free medium containing 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin-G, and 100 μg/ml streptomycin. 20 μg of expression plasmid consisting of Col15 cDNA in pAW (23) was used to transfect cells by electroporation (0.3 kV, 960 microfarad, 0.4-cm cuvette in RPMI plus 1 mM sodium pyruvate, 2 mM l-glutamine, without serum). Integrin α1 cDNA in pLEN (20 μg; Ref. 9) was cotransfected with 1 μg of pAWneo2. Similarly, 20 μg α2β2/pAW was used. Transfected cells were plated and allowed to recover for 1 day in culture medium. G418 (Life Technologies, Inc.) was added to the medium at a concentration of 1 mg/ml. G418-resistant clones were selected for 1–2 weeks, isolated, and analyzed for their expression of α1 or α2 integrin. The cell surface expression levels of the integrins were checked using anti-integrin antibodies (12F1 for α2 integrin, a gift from Dr. V. Woods, UCSD; SR-84 for α1 integrin, a gift from Dr. W. Rettig, Boehringer Ingelheim) and flow cytometry (17).

Transfection of Chinese Hamster Ovary Cells to Express α1β2 or α1β6 Integrins—CHO1 cells obtained from the American Type Culture Collection (Manassas, VA) were used as hosts for transfection and expression of integrin α1 or α2 subunits (17). Integrin α1 cDNA (9) was a gift from Dr. E. Marcantonio (Columbia University, New York, NY), and α2 cDNA (10) was a gift from Dr. M. Hemler (Dana-Farber Cancer Research Center, Boston, MA). CHO cells were grown in α-minimum essential medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin-G, and 100 μg/ml streptomycin. 20 μg of expression plasmid consisting of α1 cDNA in pAW (23) was used to transfect cells by electroporation (0.3 kV, 960 microfarad, 0.4-cm cuvette in RPMI plus 1 mM sodium pyruvate, 2 mM l-glutamine, without serum). Integrin α1 cDNA in pLEN (20 μg; Ref. 9) was cotransfected with 1 μg of pAWneo2. Similarly, 20 μg α2β2/pAW was used. Transfected cells were plated and allowed to recover for 1 day in culture medium. G418 (Life Technologies, Inc.) was added to the medium at a concentration of 1 mg/ml. G418-resistant clones were selected for 1–2 weeks, isolated, and analyzed for their expression of α1 or α2 integrin. The cell surface expression levels of the integrins were checked using anti-integrin antibodies (12F1 for α2 integrin, a gift from Dr. V. Woods, UCSD; SR-84 for α1 integrin, a gift from Dr. W. Rettig, Boehringer Ingelheim) and flow cytometry (17).

1 The abbreviations used are: CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; GST, glutathione S-transferase; MBP, maltose-binding protein.

Figure 1. Collagen receptor integrins, α1β2 and α2β6, or the collagen binding α1I, α2I, and α2I domains cannot recognize the COL15 domain of type XVII collagen. Microtiter plate wells were precoated (5 μg/cm²) with rat type I (I) collagen, mouse type IV (IV) collagen, or either native (COL15) or denatured (dCOL15) human recombinant COL15 domain of collagen type XVII overnight. In spreading studies CHO cells (10,000 cells/well) expressing either α1β2 (A) or α2β6 (B) integrins were allowed to attach and spread on collagens for 120 min and fixed after incubation. The total number of attached and spread cells were counted. In binding studies α1I (C), α2I (D), and α2I (E) domains (10 μg/ml), produced and purified as fusion proteins, were allowed to attach on collagens for 1 h. Anti-GST or anti-MBP (for α1I) antibody was added and incubated for 1 h. Europium-labeled protein-G was added and incubated for 1 h. The incubations mentioned above were done in the presence of 2 mM MgCl₂. The wells were washed three times, and the europium signal was measured. The data shown are the means ± S.E. of three parallel measurements. BSA, bovine serum albumin.
EPITHELIAL KERATINOCTYES SPREAD EFFECTIVELY ON COL15. Micortiter plate wells were precoated (5 or 20 μg/cm²) with either native (COL15) or heat-denatured (dCOL15) human recombinant COL15 domain of collagen type XVII overnight. HaCaT cells (10,000 cells/well) were allowed to attach and spread for 120 min and fixed after incubation. The total number of attached and spread cells were counted. These data are the means ± S.E. of three to twelve parallel measurements.

Cell Spreading Assays—96-well microtiter plates were precoated by exposure (12 h, 4 °C) to 0.1 ml of PBS containing purified collagens or either native or heat-denatured COL15. COL15 was denatured for 20 min at 56 °C. Residual protein sites on all wells were blocked for 1 h at 37 °C with 0.1% heat-inactivated bovine serum albumin in PBS. Three cell lines were used: a mixture of CHO clones expressing the αβ₂ integrin (CHO-α₂β₂), monoclonal CHO cells expressing the αβ₅ integrin (CHO-αβ₅), and an immortalized human keratinoctye cell line (HaCaT) that was obtained from Dr. N. E. Fusenic (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Semiconfluent culture cells were treated with 0.01% trypsin and 0.02% EDTA. Trypsin activity was inhibited by washing the cells with 0.2% soybean trypsin inhibitor. The cells were suspended in serum-free Dulbecco’s modified Eagle’s medium or serum-free α-minimum essential medium containing 0.1 mg/ml cycloheximide to avoid endogenous matrix synthesis. The cells were added to microtiter plates (10⁴ cells/well) and allowed to attach and spread for 120 min. The role of specific integrin subunits in cell adhesion to COL15 was tested by adding to cell suspensions, function-blocking antibodies (2 μg/ml) against either the β₁, collagen receptor, or α₅ (American Type Culture Collection), L230 α₁ (Serotec), or α₂ (Chemicon) subunits. In inhibition studies, either linear or cyclic synthetic peptides were added to the cell suspensions. Linear peptides were used at concentrations of 0.01, 0.1, or 0.7 mM. The cyclic RGD peptide used to inhibit integrin function was GACRGDCLGA, which contained a covalent bond between the cysteins. A similar RGE peptide (GACRGECGLGA) was used as a nonfunctional control. These peptides were produced as described and were used at 500 μM. After incubation, the medium containing nonadhered cells was poured out; no additional washes were performed. The cells were fixed for 30 min with a solution of 30% formaldehyde and 5% sucrose. The cells in at least three parallel fields in each of three wells were analyzed using phase contrast microscopy. The total number of cells attached and the percentage of spread cells were counted. A spread cell was characterized as one having a clearly visible ring of cytoplasm around the nucleus.

LATERAL CELL MIGRATION ASSAYS—A 24-well cell culture cluster (Costar) was precoated for 2 h at 37 °C with 0.5 ml of PBS, pH 7.4, containing human fibronectin (5 μg/cm²; Roche Molecular Biochemicals) or either native or heat denatured COL15 (5 μg/cm²). Semiconfluent HaCaT cell cultures were detached with 0.01% trypsin and 0.02% EDTA. Trypsin activity was inhibited by washing the cells with 0.2% soybean trypsin inhibitor (Sigma). The cells were suspended in serum-free Opti-Prep 1 medium (Life Technologies, Inc.), and 2 × 10⁴ cells/well were transferred into a custom-made stainless steel cylinder with an opening of 2.8 mm in diameter in the center. The cells were allowed to attach to the substrate for 2 h at 37 °C. The cylinders were removed, nonadhered cells were washed away, and the adherent cells were allowed to migrate for 2 or 4 days (d) at 37 °C. The cells were fixed with 2% paraformaldehyde, stained with 5% crystal violet, and washed with distilled water. The rate of migration was estimated by measuring the surface area (mm²) covered by the cells. The data shown are the means ± S.E. of three to six parallel measurements.

Production of Human Recombinant Integrin α₁I and α₁I Domains as Fusion Proteins—cDNAs encoding for α₁, α₁, and α₂ domains were generated by PCR as described earlier (17) using human integral α₁, and α₂ cDNAs as templates. Vectors pGEX-4T-3 and pGEX-2T (both Amersham Pharmacia Biotech) were used to generate recombinant glutathione S-transferase (GST) fusion proteins of human α₁ and α₂ domains, respectively. Competent Escherichia coli BL21 cells were transformed with the plasmids for protein production. LB medium (500 ml; Biokar) containing 100 g/ml ampicillin was inoculated with 50 ml of overnight culture of either BL21/pα₁ or BL21/pα₂, and the cultures were grown at 37 °C until the A₆₀₀ of the suspension reached 1.0–2.0. An inducer, isopropyl-β-D-thiogalactopyranoside, was added, and the cells were allowed to grow for an additional 4–6 h before harvesting by centrifugation. Pelleted cells were resuspended in PBS, pH 7.4, and then lysed by sonication followed by addition of Triton X-100 to a final concentration of 10%. After incubation for 30 min, the suspensions were centrifuged, and the supernatants were pooled. Glutathione-Sepharose (Amersham Pharmacia Biotech) was added to the lysate, which was incubated at room temperature for 30 min with gentle agitation. The lysate was then centrifuged, the supernatant was removed, and glutathione-Sepharose with bound fusion protein was transferred into disposable chromatography columns (Bio-Rad). The columns were washed with PBS, and the fusion proteins were eluted using 30 mM glutathione.

**Fig. 2.** Epithelial keratinoctyes spread effectively on COL15. Microtiter plate wells were precoated (5 or 20 μg/cm²) with either native (COL15) or heat-denatured (dCOL15) human recombinant COL15 domain of collagen type XVII overnight. HaCaT cells (10,000 cells/well) were allowed to attach and spread for 120 min and fixed after incubation. The total number of attached and spread cells were counted. These data are the means ± S.E. of three to twelve parallel measurements.

**Fig. 3.** Denatured COL15 domain promotes the migration of human keratinoctyes. A 24-well cell culture cluster was precoated (5 μg/cm²) with 0.5 ml PBS, pH 7.4, containing human fibronectin (FN) or either native or heat-denatured (dCOL15) human recombinant COL15 domain of collagen type XVII for 2 h at 37 °C. HaCaT cells were suspended in serum-free medium, and 20,000 cells/well were transferred into a metal cylinder having a diameter of 2.8 mm. The cells were allowed to attach to the substrata for 2 h at 37 °C. The cylinders were removed, nonadherent cells were washed away, and the adherent cells were allowed to migrate for 2 or 4 days (d) at 37 °C. The cells were fixed with 2% paraformaldehyde, stained with 5% crystal violet, and washed with distilled water. The rate of migration was estimated by measuring the surface area (mm²) covered by the cells. The data shown are the means ± S.E. of three to six parallel measurements.
Native polyacrylamide gel electrophoresis was done for both recombinant proteins. The recombinant α1I domain produced was 227 amino acids in length, corresponding to sequence 123–338, whereas the α2I domain was 223 amino acids long, which corresponded to sequence 124–339. The carboxyl termini of the α1I and α2I domains contained ten and six nonintegrin amino acids, respectively. Recombinant I domains were used as GST fusion proteins for binding experiments.

Cloning and Production of the α1I Domain—The α1I domain cDNA was generated by reverse transcriptase-PCR from RNA isolated from KHOS-240 cells (human Caucasian osteosarcoma). Total cellular RNA was isolated by using a RNeasy mini kit (Qiagen). Reverse transcriptase-PCR was done using the Gene Amp PCR kit (PerkinElmer Life Sciences). The amplified construct was checked with DNA sequencing. The construct was checked with DNA sequencing. The α1I domain was produced as a fusion with maltose-binding protein (MBP) and tested to show specific binding to several fibril-forming collagens as well as to basement membrane type IV collagen in a Mg2+-dependent manner.6

Binding Assay for α1I, α1I, and α2I Domains—The coating of a 96-well high binding microtiter plate (Nunc) was done by exposure to 0.1 ml of PBS containing 5 μg/cm² (15 μg/ml) collagens or either native or heat-denatured COL15 for 12 h at +4 °C. Blank wells were coated with 0.1 ml of Delfia® Diluent II (Wallac). Residual protein absorption sites on all wells were blocked with 0.1 ml of Delfia® Diluent II (Wallac). Recombinant proteins, α1I-GST, α1I-GST, or α2I-MBP were added to the coated wells at a concentration of 10 or 15 μg/ml in Delfia® assay buffer and incubated for 1 h at +37 °C. Anti-GST antibody (Amersham Pharmacia Biotech) at a concentration of 63 ng/ml or anti-MBP antibody (New England BioLabs) diluted in 1:1000 was added to wells and incubated for 1 h at +37 °C. Europium-labeled protein-G (Wallac) was then added, and the mixtures were incubated for 1 h at +37 °C. All of the incubations mentioned above were in the presence of 2 mM MgCl2. Delfia® enhancement solution (Wallac) was added to each well, and the Europium signal was measured by time-resolved fluorometry (Victor2 multilabel counter, Wallac). At least three parallel wells were analyzed.

RESULTS

Collagen Receptor Integrins α1β1, α2β1, and α2β1 Cannot Recognize the COL15 Domain of Type XVII Collagen—Wild-type CHO cells show endogenous expression of the β1 integrin subunit, but they do not express any collagen-binding α subunits. Here, cDNAs coding for either the integrin α1 or α2 subunit were transfected into CHO cells. The expression of these exogenous integrin subunits in transfected cells was verified by flow cytometry. In our previous studies the cell spreading assay has turned out to be a more sensitive and reproducible method to study integrin function than the cell attachment assay. CHO-α1β1 and CHO-α2β1 cells were allowed to spread for 120 min on microtiter wells that had been precoated with type I or type IV collagen or the largest collagenous domain of type XVII collagen, COL15. The total number of attached and spread cells were counted. Spread cells were described as those with cytoplasm around the nucleus and with either a flattened, circular shape with a string-of-pearls-like plasma membrane structure or a fibroblast-like morphology. Nonspread cells were characterized by either a round or a splinter-like shape and a yellow shimmer when viewed under a phase contrast microscope. After the 120-min incubation, more of the CHO-α1β1 cells on type IV collagen had spread (62 ± 3%) than those on type I (18 ± 3%; Fig. 1A). In contrast, CHO-α2β1 cells spread faster on type I collagen; 88 ± 3% had spread versus 75 ± 6% on type IV collagen. These values are consistent with our previous observations.6 Both α1β1- and α2β1-expressing cells attached and spread on COL15, although the spreading was much slower than on either types I or IV collagen. After incubation only 14 ± 6% of CHO-α1β1 cells and 17 ± 6% of CHO-α2β1 cells had spread on COL15. The spreading of vector-transfected CHO cells was at the same level (15 ± 4%), suggesting that COL15 was not recognized by α1β1 or α2β1, but probably by some other cell adhesion receptor expressed on CHO cells.

Recombinant human integrin α1I, α2I, and α2I domains were produced as fusion proteins in E. coli and purified. The fusion proteins were characterized using both SDS- and native polyacrylamide gel electrophoresis (not shown). A solid phase assay was used to investigate the binding of αI domains to type I collagen and native and denatured COL15. Because the collagen-binding activity of integrin I domains has been shown to be Mg2+-dependent, the assays were carried out in the presence of 2 mM MgCl2. Binding levels of each αI domain to bovine serum albumin were used as background controls. The fusion proteins of each αI domain were applied to immobilized COL15, and europium-labeled protein G was linked to the bound αI domain via an anti-GST or anti-MBP antibody. The binding

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Footnote:

6 M. Tulla, O. T. Pentikäinen, T. Viitasalo, J. Kapylä, U. Impola, P. Nykvist, L. Niissinen, M. S. Johnson, and J. Heino, submitted.
Integrins in Cell Spreading on Type XVII Collagen

The spreading of HaCaT cells is inhibited by synthetic peptides derived from the COL15 domain

Microtiter plate wells were precoated (5 μg/cm²) with heat-denatured human recombinant collagen fragment 15 of collagen type XVII overnight. HaCaT cells (10,000 cells/well) were allowed to attach and spread for 120 min in the presence of synthetic peptides (0.7 mM). Cells were fixed after incubation, and total number of attached and spread cells were counted. The peptides inhibiting more than 90% are indicated (+).

| Peptide Sequence | Inhibition > 90% |
|------------------|-----------------|
| P1               | GPDKDGSFDPG     |
| P2               | GPDKDRPFPGTPG   |
| P3               | GPGPGHPGPGPCP   |
| P4               | GPKGLKSGVDPGM   |
| P5               | GMSGPQGRGREGP   |
| P6               | GPMGRPEGPGPDS   |
| P7               | GSGERGGERAAGEP  |
| P8               | EPFPKPGPGPVGPS  |
| P9               | SVFPKGSSGSPGPQ  |
| P10              | PDPFPGPLQLQG    |
| P11              | LRGVEGLPGVKGD   |
| P12              | GVKGDRGFVPGPFGDQ|
| P13              | GPKGDQGKPGPGRL  |
| P14              | GLTPGMPNMGLP    |
| P15              | LPFGVPEPGKAG    |
| P16              | KGMAGPAGDGHL    |
| P17              | GHQPGREPQGLT    |
| P18              | LCGMPGIRQPGP    |
| P19              | PGSGDPGSGGGL    |
| P20              | GLTPQGPGQGPL    |
| P21              | LPFGPGRPGXK     |
| P22              | KEGEPAGPKI      |

FIG. 5. Antibodies against αv and α5 integrins can block HaCaT cell spreading on COL15. Microtiter plate wells were precoated (5–20 μg/cm²) with either native (COL15) (C) or heat-denatured (dCOL15) (A and B) human recombinant collagen fragment 15 of collagen type XVII overnight. HaCaT cells (10,000 cells/well) were allowed to attach and spread for 120 min in the presence of either function blocking antibodies (1–5 μg/ml) against β1 (B), αv (B and C), or αv (A–C) integrin subunits. The cells were fixed after incubation, and the total number of attached and spread cells were counted. The data shown are the means ± S.E. of three parallel measurements.

was measured using time-resolved fluorescence as the number of counts/s. Integrin α1I (Fig. 1C), αvI (Fig. 1D), and α10I (Fig. 1E) domains bound to the fibril-forming collagen used as a positive control (type I collagen for α1I and α10I domains and type II collagen for α10I domain). The binding of each domain to either native or denatured COL15 was insignificant. Importantly, this suggests that COL15 is one of the very few collagens that cannot be recognized by the collagen receptors or their corresponding domains.

Denatured Unlike Native COL15 Domain Promotes the Migration of Human HaCaT Keratinocytes—Human HaCaT keratinocytes were tested in cell spreading assays on both denatured and native COL15. Spreading on the native COL15 fragment at concentrations of 5 and 20 μg/cm² was 27 ± 6 and 36 ± 5%, respectively (Fig. 2). Although HaCaT cell spreading was slow on native COL15, it became significantly faster after denaturation. The spreading on denatured COL15 at concentrations 5 and 20 μg/cm² was 31 ± 6 and 53 ± 2%. Because COL15 has a relatively low melting temperature (26 °C; Ref. 20), it is probable that at room temperature a portion of native collagen is partially denatured and that only the denatured COL15 mediates cell adhesion.

The capability of keratinocytes to migrate on COL15 was tested using coated cell culture wells. Migration was measured in serum-free conditions after 2 or 4 days. Most cells plated on native COL15 detached during the first 2 days, and no migration could be measured. Meanwhile, HaCaT cells stayed attached on denatured COL15, and cell migration during the first 4 days was comparable with that on fibronectin (Fig. 3). In 2 days, HaCaT cells migrated 9.3 ± 1.7 mm² on denatured COL15 domain. Within 4 days, the extents of migration on denatured COL15 and fibronectin were 10.8 ± 1.8 and 10.3 ± 1.5 mm², respectively. Thus, denatured COL15 forms an excellent matrix for keratinocyte migration.

The Spreading of HaCaT Keratinocytes on COL15 Domain Is Mediated by αvβ1 and αv Integrins and Is Inhibited by RGD Peptides—Similarly to the nonactivated basal keratinocytes in skin, the HaCaT cells express the following integrins: αvβ1 collagen receptor and laminin receptors αvβ3 and αvβ5. However, they also have integrins that can usually be seen only in activated skin keratinocytes: fibronectin receptors α5β1, α5β2, and αvβ2, and vitronectin receptor αvβ5. (24, 25). Here, antibodies against β1 integrin (5 μg/ml) could inhibit 75% of HaCaT cell spreading on denatured COL15 (Fig. 4A). This is in accordance with the previous report showing that anti-β1 antibody can block cell adhesion to both native and denatured COL15 (20). However, in our experiments the inhibition was not complete. A cyclic RGD peptide (26), unlike a control peptide with an RGE sequence, could effectively inhibit cell spreading (Fig. 4B). In this experiment, when no synthetic peptide was present, 30 ± 9% of HaCaT cells spread on denatured COL15. In the presence of the RGE peptide, 41 ± 17% of cells spread.
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Fig. 6. The spreading of HaCaT cells is inhibited by the KGD motif containing peptides. Microtiter plate wells were precoated (5 μg/cm²) with heat-denatured (dCOL15) human recombinant COL15 domain of collagen type XVII overnight. HaCaT cells (10,000 cells/well) were allowed to attach and spread for 120 min in the presence of synthetic COL15-derived peptides in the concentration of 0.01 or 0.1 mM (A). In B, the most effective peptide (P11) was mutated (+mutP11) to contain KGE sequence instead of KGD and tested at a concentration of 0.1 mM. The cells were fixed after incubation, and the total number of attached and spread cells were counted. The data shown are the means ± S.E. of three parallel measurements. BSA, bovine serum albumin.

When the RGD peptide was present, spreading decreased to only 12 ± 4% of attached cells, which was equivalent to bovine serum albumin background (Fig. 4B). Thus, the data indicate that the main cellular receptors for COL15 are the RGD-dependent β₁ integrins. In HaCaT cells this suggests the involvement of α₁β₁ and α₁β₁. This was further supported by the fact that anti-α₁ integrin antibody could partially (about 40%) inhibit HaCaT cell adhesion to COL15 (Fig. 5A) and that specific antibodies against integrin α₂ and α₃ subunits had no effect (data not shown). The involvement of α₂β₁ integrin was confirmed using anti-α₁ antibodies (Fig. 5B). In this experiment the inhibition by anti-α₁ was about 50%, compared with 70% inhibition by anti-β₁ (antibody concentration, 2 μg/ml). When anti-α₂ and anti-α₃ antibodies were used together to inhibit cell spreading, they were slightly more effective than anti-α₁ alone (Fig. 5B), but they were not more effective than anti-β₁. This result was duplicated in a separate experiment, suggesting that the β₁ heterodimers of these α subunits (α₁β₁ and α₁β₁) each contribute to COL15 recognition. On native COL15 the anti-α₂ and anti-α₁ combination inhibited cell spreading by about 74%, suggesting that HaCaT cells, despite the slower spreading rate, use the same integrins to spread on native COL15 as on denatured COL15 (Fig. 5C). To conclude, α₁β₁ and α₁β₁ seem to be the most important receptors of COL15. The potent effect of RGD peptides makes it unlikely that nonintegrin receptors are involved despite the fact that none of the anti-integrin antibodies or their combinations could block cell spreading completely.

The data strongly suggest that, despite the fact that COL15 does not contain any RGD motifs, the recognition mechanism is very similar. COL15 does not contain any of the integrin recognition motifs described previously in other matrix proteins. Instead, each one of the three α-chains in COL15 contains four highly RGD-related KGD motifs. KGD is found in snake venom proteins (21, 22) in which it can interact with RGD-dependent integrins, suggesting that COL15 uses the same mechanism. Here, we tested the entire sequence of COL15 domain as 22 overlapping, short (12–14 amino acids) peptides in cell adhesion assays (Table I). At first, the ability of the peptides to inhibit cell spreading on the COL15 was tested with a relatively high concentration (0.7 mM; Table I). The five peptides blocking cell spreading were tested in lower concentrations (0.1 and 0.01 mM). Of these, the three best inhibitors all contained the KGD motif (Fig. 6A). The peptide showing the strongest inhibition was mutated to have a glutamate residue instead of the aspartate (KGE). This mutation abolished the activity of the peptide, confirming the importance of the KGD motif (Fig. 6B).

DISCUSSION

The I domain-containing integrins mediate cell adhesion to native fibrillar collagens. After denaturation, the RGD motif in collagen α-chains is accessible by a different subset of integrins, namely the fibronectin and vitronectin receptors (27). Native collagen seems to have multiple binding sites for both α₁β₁, α₁β₁, and α₁β₁ integrins. However, α₁β₁ seems to be a better receptor for them than for the fibrillar collagens (16, 30). The receptors for other collagen subtypes, such as the fibril-associated collagens with interrupted triple-helices or transmembrane collagens, are less well known.

Both transmembrane collagens XIII and XVII are proteolytically released from cell surface to the extracellular matrix (7, 8, 31), which allows them to function as matrix proteins, contributing to cell adhesion and migration. We have recently shown that the α₁β₁ integrin, rather than α₁β₁, is a cellular receptor for type XIII collagen (17). No receptor for type XVII collagen has been identified. Structurally, type XIII and type XVII are different; type XIII collagen has three relatively large collagenous domains, whereas type XVII has only one large and 14 short collagenous domains. The largest collagenous domain of collagen XVII, COL15, has been shown to support keratinocyte adhesion (20). Here, we have shown that collagen receptors α₁β₁, α₁β₁, and α₁β₁ do not mediate this interaction. So far, all other collagen subtypes have been able to bind to at least one of these receptors, although the collagenous triple helix alone is not sufficient to support cell adhesion (19). Interestingly, COL15 does not contain a GPOGER sequence. It has a similar GKGGER sequence, indicating that the GER motif is not sufficient for collagen binding alone.

Type XVII collagen is shed from the cell surface (7, 8), which
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...may lead to the denaturation of COL15. In vitro recombinant COL15, when it is not a part of entire type XVII, will denature at body temperature (20). Denaturation also makes it a cell adhesion ligand. This interaction seems to be mediated by a fibronectin receptor, α5β1 integrin, and by fibronectin/vitronectin receptors containing the αv subunit, especially αvβ3 Integrin α5β1 binds specifically to the RGD motif in fibronectin. The αv integrins can recognize, in addition to the RGD-containing ligands, several other proteins. These include matrix metalloproteinase-2 (33), a disintegrin ADAM 23 (24), the fibrinogen γ-chain (35), and the COOH-terminal domain of tenasin C (35). Synthetic peptides derived from these proteins can block the corresponding interactions suggesting the putative binding sites. None of these sequences contain any similarity to COL15. Snake venom proteins, such as barbourin (21) and ussuristatin 2 (22), have an RGD-like motif, namely KGD. KGD, however, still be sufficient for cell adhesion (35). Actually, the lower avidity of type XVII collagen may mediate the migration of wound keratinocytes, especially if it is denatured. An interesting possibility is that after tissue injury, the platelets may also bind to the denatured type XVII collagen with a high avidity. This hypothesis was not studied before.

The KGD motif has been shown to be a part of the mechanism used by certain snake venom toxins to inhibit platelet function. Here, the same motif is proposed to mediate integrin binding to a matrix protein and to represent a novel mechanism of collagen recognition.

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