Collagen XVI Harbors an Integrin α1β1 Recognition Site in Its C-terminal Domains*

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Collagen XVI is integrated tissue-dependently into distinct fibrillar aggregates, such as D-banded cartilage fibrils and fibrillin-1-containing microfibrils. In skin, the distribution of collagen XVI overlaps that of the collagen-binding integrins α1β1 and α2β1. Basal layer keratinocytes express integrin α2β1, whereas integrin α1β1 occurs in smooth muscle cells surrounding blood vessels, in hair follicles, and on adipocytes. Cells bearing the integrins α1β1 and α2β1 attach and spread on recombinant collagen XVI. Furthermore, collagen XVI induces the recruitment of these integrins into focal adhesion plaques, a principal step in integrin signaling. Of potential physiological relevance, these integrin-collagen XVI interactions may connect cells with specialized fibrils, thus contributing to the organization of fibrillar and cellular components within connective tissues. In cell-free binding assays, collagen XVI is more avidly bound by α1β1 integrin than by α2β1 integrin. Both integrins interact with collagen XVI via the A domain of their α subunits. A tryptic collagen XVI fragment comprising the collagenuous domains 1–3 is recognized by α1β1 integrin. Electron microscopy of complexes of α1β1 integrin with this tryptic collagen XVI fragment or with full-length collagen XVI revealed a unique α1β1 integrin-binding site within collagen XVI located close to its C-terminal end.

Collagen XVI belongs to the fibril-associated collagens with interrupted triple-helices (FACIT) family of collagens (1, 2) and is composed of 10 collagenuous domains, designated as COL1–10, which are flanked by 11 noncollagenous (NC) regions (3). It constitutes a minor component of the extracellular matrix (ECM) of skin and cartilage. Despite its integration into small heterotypic D-banded fibrils of cartilage, collagen XVI is not generally a component of D-banded fibrils, which contain several types of collagens and further constituents, i.e. small leucine-rich proteoglycans and noncollagenous glycoproteins, in an alloy-like mixture (4, 5). In a tissue-specific manner, collagen XVI is incorporated into structurally and functionally discrete matrix aggregates, such as in specialized fibrillin-1-rich microfibrils in skin. Collagen XVI preferentially associates with that part of the microfibrillar apparatus lacking an amorphous elastin core and is localized in the dermal epidermal junction (DEJ) zone of the papillary dermis (4). Here the microfibrils insert perpendicularly into the basement membrane. This location suggests that collagen XVI plays an active role in anchoring microfibrils to basement membranes. Despite its occurrence in the dermis, it is not only produced by fibroblasts but also by keratinocytes, similarly to type VII collagen (6, 7). However, the mechanism by which these collagens are transported across the basement membrane and incorporated into the mesenchymal stroma is yet unknown. Although many matrix proteins self-assemble and thus contribute to the organization of supramolecular networks, cells also affect the architecture of the ECM networks by grasping and moving ECM proteins. Most of these cell-matrix interactions are mediated via integrins, a huge family of cell adhesion molecules (reviewed in Ref. 8). Integrins consist of two subunits, α and β. Both subunits associate with their N-terminal domains to form the integrin head, which harbors the divalent cation-dependent binding site for its extracellular matrix ligand. Both subunits span the plasmalemma once, and with their short C-terminal cytoplasmic domains they interact with the actin cytoskeleton. By connecting the ECM with the cytoskeleton, integrins are not only responsible for mechanical anchorage of the cell but also transfer signals from the ECM into the cell and vice versa (8–10). Thus, via integrins, the components of the ECM not only induce cellular reactions, such as cell adhesion, spreading, migration, and gene activation, in an outside-in fashion, but can also be remodeled by the cells in an inside-out manner (11).

Four collagen-binding integrins have been described so far (12, 13). Although the integrins α10β1 and α11β1 are mainly found on differentiated chondrocytes (14) and mesenchymal progenitor cells of developing skeletal muscle tissue (15), respectively, the two integrins, α1β1 and α2β1, show a broader tissue distribution. Integrin α1β1 is expressed in various tissues, e.g. in smooth muscle cells surrounding small blood vessels, in neuronal tissue, epithelial cells, and (pre)adipocytes (12, 16, 17). Integrin α2β1 is not only the collagen-binding integrin
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on platelets and involved in collagen-induced platelet activation and blood clotting, but it is also localized in mesenchymal cells and in epithelial cells abutting the basement membrane (12, 18). Its high expression level on basal layer keratinocytes is drastically reduced during their terminal differentiation and concomitant loss of basement membrane contact (19).

Until now, no direct interaction with cells has been reported for collagen XVI, nor has any cell surface adhesion molecule yet been identified that may mediate such a cellular interaction. Similar tissue distribution of the integrins, \( \alpha_1 \beta_1 \) and \( \alpha_2 \beta_1 \), and of collagen XVI in skin prompted us to investigate the potential interaction of this FACIT molecule with these collagen-binding integrins. Here we demonstrate that cells can interact with collagen XVI via the integrins \( \alpha_1 \beta_1 \) and \( \alpha_2 \beta_1 \). Furthermore, we narrowed down the putative \( \alpha_1 \beta_1 \) integrin-binding site within a C-terminal tryptic fragment of collagen XVI, which includes the domains COL1–3.

**EXPERIMENTAL PROCEDURES**

**Materials**—RuGli cells were provided by Dr. F. Ruggiero (Institute de Biologie et Chimie des Protéines, CNRS UMR 5086, Lyon, France). Both RuGli and HT1080 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) under 5% CO\(_2\). For the assays, cells were harvested with 0.5 mM EDTA in PBS and washed with DMEM.

The monoclonal antibody (mAb) Ha31/8 against rat integrin \( \alpha_1 \) subunit was purchased from Pharmingen. The mAb AGF-1 directed against the human integrin \( \alpha_1 \) subunit was a generous gift from Dr. H. Gardner (Biogen, Cambridge, MA). Monoclonal antibodies against the integrins were from different sources: P1E6 from Chemicon (Hampshire, UK); JA202, JA208, JA215, JA218, and JA221 with well characterized epitopes (20, 21) were kindly provided by Dr. D. Tuckwell (University of Manchester, School of Biological Sciences, Manchester, UK). Hybridoma cells producing mAb 9EG7 against the human integrin \( \beta_1 \) subunit were provided by Dr. D. Vestweber (University of Münster, Münster, Germany). Generation of antibodies directed against recombinant collagen XVI is described elsewhere (22). Antibodies were purified by affinity chromatography on protein A and G columns according to standard procedures. The anti-vinculin mAb hVinc, purified from ascites (Sigma), was covalently labeled with digoxigenin using digoxigenin-N-4-(hydroxysuccinimidyl) ester (Roche Diagnostics) in a 400-fold molar surplus. The antibody JA221 was biotinylated with a 500-fold molar excess of biotin-hexanoic-N-4-(hydroxysuccinimidyl) ester (Pierce). Recombinant expression and purification of human collagen XVI and of soluble \( \alpha_2 \beta_1 \) integrin have been described previously (22, 23).

**Immunofluorescence Staining of Skin Sections**—Cryosections of adult murine skin were fixed for 10 min at 4 °C with cold acetone and incubated for 1 h at 37 °C in blocking solution consisting of 5% goat serum (Dako Serotec, Düsseldorf, Germany) and 1% BSA in Complete Mini® protease inhibitor mixture (Roche Diagnostics) dissolved in PBS. When biotinylated primary antibodies were used, sections were subsequently saturated with the Biotin Blocking System X0590 (Dako) according to the manufacturer’s instructions. After washing with PBS, sections were incubated with the following primary antibodies in blocking solution without BSA for 2 h at 37 °C: biotinylated murine mAb AGF-1, the murine monoclonal antibody JA221, diluted 1:1,000 and 1:100, respectively, and a protein A-purified polyclonal antibody raised against recombinant collagen XVI in guinea pig, diluted 1:50. After washing with PBS, the appropriate secondary antibodies were applied in blocking solution without BSA for 1 h at 37 °C as follows: AlexaFluor 488-conjugated streptavidin, AlexaFluor 488-conjugated anti-(mouse IgG) antibodies, and AlexaFluor 555-conjugated anti-(guinea pig IgG) antibodies (all from Molecular Probes, Leiden, The Netherlands), each at a final concentration of 5 μg/ml. After washing with PBS, sections were counterstained with the nuclear dye DAPI (Molecular Probes) for 7 min at 300 nM in PBS and subsequently mounted with Fluoromount (Dako). Micrographs were taken with a laser scanning microscopic unit C1 (Nikon GmbH, Düsseldorf, Germany) at the indicated magnifications.

**Cell Adhesion Assay with Recombinant Collagen XVI**—Multicwell plates were coated with recombinant collagen XVI dissolved in PBS at 4 °C overnight. Nonspecific protein-binding sites were blocked with 1% BSA in PBS for 2 h at room temperature. RuGli and HT1080 cells were added to each well at a cell density of 0.5 × 10\(^6\) cells/ml in DMEM, supplemented with either 1 mM MnCl\(_2\) or 10 mM EDTA. Cells were allowed to adhere for 30–45 min at 37 °C under 5% CO\(_2\). After a single wash with PBS, adherent cells were fixed with 70% (v/v) ethanol for 7 min, stained with 0.2% (w/v) crystal violet (Sigma) for 30 min, and thoroughly rinsed with water to remove excess dye. Cell-absorbed crystal violet was extracted with 70% ethanol, and absorbance was measured at 560 nm. Background values of cells attached to BSA were subtracted.

For mAb inhibition studies, the multicwell plates were coated with 5 μg/ml recombinant collagen XVI in PBS. The cells were preincubated with the mAbs for 20 min before they were added to the wells. All other steps were carried out as in the adhesion assay described above.

**Immunofluorescence Staining of Collagen-binding Integrins in Cells**—The wells of a chamber slide (Greiner, Frickenhausen, Germany) were coated with recombinant collagen XVI at 40 μg/ml, with the collagen IV fragment CB3[IV] at 2 μg/ml, with collagen I at 10 μg/ml, or with fibronectin at 5 μg/ml (Invitrogen) at 4 °C overnight. After washing with PBS, the wells were blocked with 0.1% heat-denatured BSA in PBS for 1 h at room temperature. RuGli and HT1080 cells suspended in DMEM with or without 0.1 mM MnCl\(_2\) were added at a density of 200,000 and 50,000 cells/ml into the substrate-coated wells. For incubation after 2 h at 37 °C under 5% CO\(_2\), adherent wells were washed with PBS supplemented with 2 mM each of MgCl\(_2\) and CaCl\(_2\), with or without 0.1 mM MnCl\(_2\), and fixed with 2% formaldehyde in the same buffer for 7 min at room temperature. After triple washing with PBS, cells were permeabilized and blocked with blocking buffer (0.5% saponin, 2.5% methanol, 2% horse serum in PBS) for 1 h. RuGli cells were stained with rabbit antiserum directed against the integrin \( \alpha_1 \) subunit (24) and murine anti-vinculin mAb hVinc (Sigma), diluted in blocking buffer 1:500 and 1:250, respectively, for 1.5 h. These primary antibodies were then detected with AlexaFluor 568-
conjugated anti-(rabbit IgG) antibodies and AlexaFluor 488-conjugated anti-(mouse IgG) antibodies (both from Molecular Probes). These secondary antibodies were diluted 1:400 and 1:500 in blocking buffer and incubated for 1.5 h. HT1080 cells were incubated for 1.5 h with 5 μg/ml biotinylated JA221 and 10 μg/ml digoxigenin-labeled mAb hVinc, both in blocking buffer. Detection was performed by incubation for 1.5 h with AlexaFluor 555-conjugated streptavidin (Molecular Probes) and fluorescein-labeled anti-digoxigenin antibodies (Roche Diagnostics) at 10 μg/ml, both dissolved in blocking buffer. Incubation steps with both primary and secondary antibodies were followed by three washing steps with blocking buffer for 15 min each. Finally, all cells were shorty rinsed with water and mounted in Fluoromount G (Molecular Probes). Pictures were taken with a confocal laser scanning microscope (Nikon PCM 2000) with at least six pictures averaged to minimize the background noise.

**Generation, Production, and Purification of a Soluble α1β1 Integrin**—The cDNA encoding the full-length human integrin α1 subunit including the 28-amino acid rat signal sequence was kindly provided by Dr. E. E. Marcantonio (Columbia University, New York) as an insert in the pLEN-vector, as described previously (25). A 3119-bp sequence, which codes for the signal sequence and the amino acids 1–1008 of the mature integrin α1 ectodomain, was excised from this pLEN-integrin α1 plasmid using Sall and BamHI. The BamHI- and AgeI-flanked DNA-fragment, 340 bp in length, which codes for amino acids 1009–1113 of the ectodomain, and a short spacer stretch containing a factor X cleavage site, was generated by PCR using the pLEN-integrin α1 plasmid as template and the following primer pair: forward primer, 5' CCC CAT ATC TTT GAG GAT CCT TTC 3', and reverse primer, 5' GCC ACC GGT TCT TCC TTC GAT CCC TTC TGG CAC TCT GCC CGG TAG 3', with the restriction sites of BamHI and AgeI, respectively, underlined. The insect cell expression vector pUC-HygMT-α3Fos (26) was cleaved by Sall and AgeI. By a triple ligation, the Sall-BamHI fragment and the BamHI-AgeI-flanked PCR amplicon, encoding the N- and C-terminal parts, respectively, of the integrin α1 ectodomain were directionally introduced into the cleaved pUC-HygMT vector and thus joined to its C-terminal Fos zipper sequence via a factor X cleavage site. The PCR-derived DNA sequence was verified by sequencing. The pUC-HygMT-α1Xjun plasmid encoding the integrin β1 ectodomain with a factor X cleavage site and the dimerizing leucine zipper motif of Jun was derived from the pUC-Hyg-β1jun plasmid (26) by inserting the factor X cleavage site-coding sequence. Both constructs were cotransfected into *Drosophila* Schneider cells, which were then selected by hygromycin. After two rounds of subcloning by limited dilution, the clone 2H4 was established for α1β1 integrin production.

To produce soluble α1β1 integrin, the clone 2H4 was grown in spinner flasks to a density of 12 × 10^6/ml. After induction with 0.8 mM Cu^{2+} ions for 5 days, the cell supernatant was harvested, concentrated by ultrafiltration (Amicon Millipore, Schwabach, Germany), and loaded onto a CB314 [IV] column. Soluble α1β1 integrin was eluted by 10 mM EDTA. Its concentration and purity were determined by BCA test (Pierce) and SDS-PAGE.

**Integrin Binding to Collagen XVI**

Integrin Binding to Collagen XVI and Inhibition by mAb—Recombinant collagen XVI dissolved at 20 μg/ml in PBS was coated onto a multiwell plate at 4 °C overnight. After washing with PBS, the wells were blocked with 1% heat-inactivated BSA in TBS, pH 7.4 (50 mM Tris/ HCl, pH 7.4, 150 mM NaCl), supplemented with 2 mM MnCl₂. The recombinant soluble integrins α1β1 and α2β1 were dissolved in the same buffer, supplemented with either 1 mM MnCl₂ or 1 mM MnCl₂ together with the integrin-activating mAb 9EG7 or 10 mM EDTA alone (nonspecific background). After binding to collagen XVI, unbound integrins were washed off twice with HEPES-washing buffer (50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, and 1 mM MnCl₂). Bound integrins were fixed with 2.5% glutarddehyde in HEPES-washing buffer for 10 min and detected by ELISA using a rabbit antiserum directed against the integrin β1 subunit and subsequently an alkaline phosphatase-conjugated anti-(rabbit IgG) antibody (Sigma) as described previously (23). Affinity constants were calculated from the titration curves as described previously (24).

For antibody inhibition studies, wells coated with 20 μg/ml collagen XVI were incubated with constant concentrations of the integrins α1β1 (100 nM) or α2β1 (300 nM). The anti-integrin α1-mAb AGF-1 was used at different concentrations. Binding of soluble α2β1 integrin to collagen XVI was individually challenged with a panel of anti-α2 integrin mAbs each at 600 nM. Bound integrins were detected after fixation by the ELISA procedure described above.

**Limited Trypsin Digestion of Collagen XVI**—0.5 mg of recombinant collagen XVI, dissolved in PBS, was digested with trypsin (Sigma) at an enzymesubstrate ratio of 1:100 in the presence of 5 mM CaCl₂ for 30 min at 20 °C. After stopping the reaction with 1 μg/ml aprotinin, the digestion mixture was diluted with 20 mM sodium phosphate buffer, pH 5.6 (Mono S-buffer A), and applied to a Mono S column (Amersham Biosciences). The integrin-binding triple-helical fragment with an SDS-PAGE-determined molecular mass of each chain of about 64 kDa was eluted in a linear gradient from 0 to 1 M NaCl in Mono S buffer A at 0.5 ml/min.

**Cross-linkage of Soluble α1β1 Integrin with the Triple-helical Tryptic Collagen XVI Fragment**—Soluble α1β1 integrin was dialyzed four times against 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 10 μM MnCl₂. Then the integrin was mixed with the Mono S eluate fraction containing the tryptic collagen XVI fragment. The mixture containing 0.1 mg/ml (360 nM) integrin and about 5 μg/ml (26 nM) trypptic fragment was supplemented with MgCl₂ and MnCl₂ to final concentrations of 1 mM and 10 μM, respectively. After addition of bis-(sulfo-N-hydroxy-succinimidyl)-suberate (BS²) (KMIF, Lohmar, Germany) to 0.2 mM, the mixture was incubated for 2 h at 20 °C. The cross-linking reaction was stopped with 5 mM Tris/HCl, pH 8.0. To purify the complex of α1β1 integrin and the triple-helical collagen XVI fragment, the solution was diluted with 20 mM Tris/HCl, pH 8.9 (Mono Q buffer A) and loaded onto a Mono Q column (Amersham Biosciences). The complex was separated from noncross-linked proteins in a gradient of 0–0.5 M NaCl in Mono Q buffer A. Eluate fractions that contained the covalent complex of α1β1 integrin and tryptic collagen XVI
fragment were identified by Western blotting using antibodies specifically directed against either integrin or collagen XVI.

Negative Staining of the Covalent Complex Consisting of \( \alpha 1\beta 1 \) Integrin and the Tryptic Collagen XVI Fragment or Full-length Collagen XVI—The Mono Q-eluate fractions containing the covalent complex of \( \alpha 1\beta 1 \) integrin and tryptic collagen XVI fragment were submitted to negative staining. 5-\( \mu l \) aliquots of cross-linked protein solutions were adsorbed for 1 min on carbon-coated grids, which had been rendered hydrophilic by glow discharge at low pressure beforehand. Adsorbed samples were washed with 2 drops of water and stained with 2 drops of 0.75\% (w/v) uranyl formate. Specimens were visualized in a Jeol JEM 1230 electron microscope operated at an accelerating voltage of 60 kV. Images were recorded with a Gatan Multiscan 791 CCD camera. Contour length analysis was carried out with Digital-Micrograph software (Gatan Inc.).

Electron Microscopy of Complexes of \( \alpha 1\beta 1 \) Integrin and Full-length Collagen XVI—Soluble \( \alpha 1\beta 1 \) integrin was purified on a Mono Q column (Amersham Biosciences) in a 20 mM ammonium bicarbonate buffer containing 0.5 mM MgCl\(_2\) and 20 \( \mu M \) MnCl\(_2\) by a gradient of 0–500 mM NaCl. Collagen XVI was dialyzed against 200 mM ammonium bicarbonate buffer, pH 7.9. Both binding partners were allowed to react for 3 min in the presence of integrin-activating antibody 9EG7. Then they were mixed with an equal volume of 80\%(v/v) glycerol and squeezed between two freshly cleaved mica pieces. Specimens were then dried for 2 h in a high vacuum. Subsequently, they were decorated with 3 nm platinum/carbon at a low angle on a rotating
RESULTS

Collagen XVI and the Integrins α1β1 and α2β1 Show Overlapping Distribution Patterns within Skin Tissue—In adult skin, collagen XVI is characteristically confined to narrow zones below basement membranes at the dermal-epidermal junction (DEJ) of the upper papillary dermis, of hair follicles, and around fatty tissue (Fig. 1, red fluorescence), in accordance with previous results (6). To study potential interactions of dermal and epidermal cells with collagen XVI, we performed immunofluorescence stainings of adult mouse skin sections with monoclonal antibodies directed against the α subunits of the collagen-binding integrins α1β1 and α2β1. In particular, integrin α2β1 is prominently expressed by the basal layer keratinocytes lining the epidermal side of the DEJ basement membrane (Fig. 1, G–I, green fluorescence). The integrin α1β1 exhibits a much weaker and broader distribution within the epidermal cell layers compared with the restricted α2β1 integrin expression. Instead, it is found around dermal hair follicles (Fig. 1, A–C, green fluorescence) and within the adipose tissue in deeper skin layers (Fig. 1, D–F, green fluorescence). Double immunofluorescence staining with antibodies directed against collagen XVI and integrin α1β1 displayed a partial colocalization of both proteins within the adipose tissue in the deeper dermal layers (Fig. 1, D–F). Additionally, the inner sheet of dermal hair bulbs exhibited a strong α1β1 integrin signal, whereas cells of the outer root sheet are positive for collagen XVI (Fig. 1, A–C). Double immunofluorescence staining of collagen XVI and α2β1 integrin revealed an adjacent, very close location of collagen XVI with α2β1 integrin within the basement membrane zone of the DEJ (Fig. 1, G–I). Although collagen XVI appeared to be more abundant on the dermal face of the DEJ zone, opposite to the α2β1 integrin-rich basal layer keratinocytes, it is also found within the basement membrane itself forming the borderline between the dermal and epidermal compartment. The partially overlapping tissue distribution prompted us to test a putative interaction of collagen XVI with α1β1 or α2β1 integrin-bearing cells.

Integrins α1β1 and α2β1 Mediate Cell Attachment to Collagen XVI—To study potential cell contacts with collagen XVI in more detail, recombinant human collagen XVI was isolated from a 293 EBNA cell expression system in sufficient quantities for more detail, recombinant human collagen XVI was isolated from a 293 EBNA cell expression system in sufficient quantities

FIGURE 2. Cell attachment to collagen XVI and its inhibition by monoclonal antibodies against α1 and α2 integrin subunits. Rat glioma RuGli cells (A) and human fibrosarcoma HT1080 cells (B) bearing integrins, α1β1 and α2β1, respectively, were plated on collagen XVI. Cells were allowed to attach to the substratum in the presence of either 1 mM MnCl2 (diamonds) or 10 mM EDTA (circles), or in the absence of any additives (triangles). Afterward they were stained with crystal violet, the absorbance of which was detected at 560 nm. For mAb inhibition studies (C), RuGli cells (squares) and HT1080 cells (triangles) were preincubated with the mAbs Ha31/8 and P1E6, respectively, at the indicated concentrations. Subsequently they were added to wells, which had been coated with collagen XVI at 5 μg/ml. Adherent cells were detected as in A and B. Nonspecific cell attachment to BSA was subtracted from all values. All data are mean values ± S.D. of quadruplicate measurements. A representative of at least three experiments is shown.

to collagen XVI. Remarkably, adhesion of these cells in the presence of Mn2+ ions also showed an elevated plateau value, thus indicating increased adhesion strength (Fig. 2A). This activating effect was less pronounced for HT1080 cells interacting with collagen XVI (Fig. 2B).

To prove whether the attachment of RuGli and HT1080 cells to immobilized collagen XVI was mediated by binding of the integrins α1β1 and α2β1, respectively, we challenged these cellular interactions with mAbs directed against the respective integrin α subunits (Fig. 2C). Ha31/8 is a function-blocking antibody directed against the rat integrin α1 subunit, whereas the mAb P1E6 recognizes the human integrin α2 subunit. Both antibodies block binding to collagens. Although P1E6 inhibited HT1080 cell attachment to collagen XVI almost completely and very efficiently with an IC50 value of about 0.05 μg/ml (26 nM), the adhesion of RuGli cells to collagen XVI could not be entirely

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replicates were floated off on distilled water and picked up on 400 mesh copper grids. In parallel experiments, specimens were subjected to negative staining. Finally, complexes were visualized by transmission electron microscopy, as described above.

Integrin Binding to Collagen XVI
blocked by the mAb Ha31/8. Concordantly, the half-maximal inhibitory effect of Ha31/8 was also observed at a concentration of about 0.05–0.1 μg/ml (26–52 nM). Both monoclonal antibodies suppressed cell attachment to collagen XVI, indicating that cells might interact directly with collagen XVI via the collagen-binding integrins α1β1 and α2β1.

Collagen XVI Recruits Integrins α1β1 and α2β1 into Focal Adhesion Plaques—Integrin-ligand interactions do not only facilitate the mechanical anchorage of cells but also transmit signals from the extracellular matrix into the cell and vice versa. The first step in integrin-mediated signal transduction is the formation of focal contacts and focal adhesion plaques. To test whether cellular interactions with collagen XVI also result in the recruitment of collagen-binding integrins into these supramolecular structures, RuGli and HT1080 cells were seeded on immobilized collagen XVI. Double immunofluorescence staining for both the respective integrin α subunit and vinculin, a marker protein of focal adhesion plaques, demonstrated that RuGli cells not only spread on collagen XVI but also formed vinculin-positive focal adhesion plaques, which contained α1β1 integrin (Fig. 3A).

Immobilized CB3[IV], a cyanogen bromide fragment of collagen IV containing a high affinity binding site for α1β1 integrin, was used as positive control (24, 29). Collagen XVI provoked cellular effects similar to CB3[IV] as seen by the appearance of streak-like focal adhesion plaques. Cells spread on CB3[IV] seemed to have more dot-focal contacts all around the cell periphery than the cells attached to collagen XVI. The streak-like focal adhesion plaques of RuGli cells on the collagen XVI substrate increased in length in the presence of Mn²⁺, which is likely the reason for the increased adhesion strength and adhesion plateau observed under these attachment conditions (Fig. 2A). Again, the focal adhesion plaques contained both α1β1 integrin and vinculin. Conversely, cells attached to fibronectin lacked any staining for α1β1 integrin, yet showed vinculin-positive focal adhesion plaques.

FIGURE 3. Collagen XVI induces recruitment of integrins α1β1 and α2β1 into focal adhesion plaques. RuGli cells (A) and HT1080 cells (B) were seeded on collagen XVI in both the absence and presence of 0.1 mM MnCl₂ on collagen IV fragment CB3[IV] or collagen I, and on fibronectin. After 2 h, RuGli cells were fixed and double-stained with rabbit antiserum against the integrin α1 subunit and murine mAb hVinc directed against vinculin (A). Attached HT1080 cells were fixed and stained with a biotinylated mAb JA221 and with digoxigenin-labeled mAb hVinc, directed against the human integrin α2 subunit and vinculin, respectively (B). Under the laser scanning microscope at a ×600 magnification, red fluorescence revealed integrins α1β1 (A, middle row) or α2β1 (B, middle row), and green fluorescence indicated vinculin (A and B, bottom row). Their colocalization is shown as yellow color in the top row of both panels.
Similar experiments were performed with α2β1 integrinbearing HT1080 cells (Fig. 3B). They spread well on collagen XVI and formed peripheral focal contacts as well as streak-like focal adhesion plaques, which contained both α2β1 integrin and vinculin. No obvious differences in either cell spreading or size of focal adhesion plaques were observed when cells were plated in the presence of Mn²⁺ ions. On collagen I, used as positive control, HT1080 cells spread similarly and developed α2β1 integrin- and vinculin-positive focal adhesion contacts and plaques in the cell perimeter. In contrast, fibronectin, which was used as negative control, triggered the formation of vinculin-positive focal adhesion plaques, which lack α2β1 integrin. Altogether, collagen XVI was able not only to mediate cell attachment but also to elicit cellular reactions, such as cell spreading, which are triggered by the selective recruitment of the collagen-binding integrins α1β1 and α2β1 into focal adhesion plaques.

Soluble Integrins α1β1 and α2β1 Directly Interact with Collagen XVI in a Cell-free Protein Binding Assay—To prove a direct interaction of the collagen-binding integrins α1β1 and α2β1 with collagen XVI, we examined the binding of soluble integrin heterodimers to this collagen. The soluble integrins, α1β1 and α2β1, consist of the ectodomains of both integrin subunits (23). Fig. 4A shows that soluble α1β1 integrin binding to collagen XVI was dose-dependent and saturable. As indicated by a shift of the titration curve toward lower integrin concentrations, the general activation of integrins by Mn²⁺ ions could be further raised by the addition of the integrin-activating mAb 9EG7, which is directed against the integrin β1 subunit (30). From these titration curves, an apparent Kᵦ value of 101 (±44) nM could be determined for the interaction of α1β1 integrin with collagen XVI in the presence of 1 mM Mn²⁺ ions. Affinity was further increased, and the Kᵦ value was reduced to 15 (±7.3) nM, after additional activation with the mAb 9EG7.

The other collagen-binding integrin α2β1 also directly interacted with immobilized collagen XVI (Fig. 4B). However, even under optimal activation conditions in the presence of both 1 mM Mn²⁺ ions and activating mAb 9EG7, saturation of the α2β1 integrin binding signal to collagen XVI was not achieved even at a concentration of 800 nM. Although soluble α2β1 integrin seemed to bind to collagen XVI directly, the high concentrations of α2β1 indicated a very weak affinity to this collagen ligand. Therefore, a Kᵦ value could not be determined with this ELISA-type interaction assay. From the titration curve (Fig. 4B), it could only be estimated to be above 0.5 μM.

This cell-free integrin-ligand interaction assay was also used to test the inhibitory capabilities of mAbs directed against integrin α1 and α2 subunits. AGF-1 is a mAb raised against the human integrin α1 subunit. Its epitope is located within the A domain of the integrin α1 subunit and therefore blocks integrin α1β1 binding to its collagenous ligands. As shown in Fig. 5A, the mAb AGF-1 inhibited binding of soluble α1β1 integrin to collagen XVI efficiently and completely. Additionally, various mAbs against the A domain of the integrin α2 subunit were tested for their inhibitory effect on α2β1 integrin binding to collagen XVI. The mAbs JA215, JA202, and P1E6, which block the interaction of α2β1 integrin with collagen I, also inhibited α2β1 integrin binding to collagen XVI (Fig. 5B). Notably, the mAb JA221, which recognizes a ligand-induced binding site epitope exposed within the α2 A domain after ligand binding thus supporting α2β1 integrin binding to collagen I, also increased the binding signal of α2β1 integrin to collagen XVI. The mAb JA208 did not affect the binding signal to either collagen I or XVI. The mAb JA218, which inhibited α2β1 integrin
binding to collagen I only at high concentrations (data not shown), blocked its interaction with collagen XVI at much lower concentrations (Fig. 5B). This may be explained by the lower affinity of α2β1 integrin to collagen XVI compared with collagen I. Nevertheless, these antibody inhibition assays suggested that collagen XVI was recognized by both integrins α1β1 and α2β1 in a manner similar to other collagen types. As the inhibitory antibodies are directed against the A domain of the respective integrin α subunits, these data further suggested that the molecular interaction of collagen XVI is carried out by the A domains of both integrin α subunits.

A Tryptic C-terminal Fragment of Collagen XVI Harbors the Binding Sites for the Integrins α1β1 and α2β1—Recombinant collagen XVI was fragmented by limited proteolysis with trypsin at 20 °C. Because of the proteolytic stability of the triple-helical parts of collagen XVI, trypsin preferentially degrades the noncollagenous domains of collagen XVI. After separation of the tryptic digest by cation exchange chromatography, a heterogeneously appearing fragment designated as the tryptic 64-kDa collagen XVI fragment was isolated, which consisted of three α1[XVI] chain fragments with nonuniform apparent molecular masses of around 64 kDa, as estimated from SDS-PAGE (Fig. 6). The N-terminal end of this tryptic fragment was unraveled by Edman degradation to be AHPGY, which represents a tryptic cleavage site within the noncollagenous domain NC4 (Fig. 8A). The C terminus of this fragment could not be determined directly. However, the loss of the C-terminal strepII tag showed that trypsin also cleaved at the C-terminal end of this collagen XVI fragment, presumably within the NC1 domain (data not shown), and thus caused the heterogeneous masses of the chains. As judged from its apparent molecular mass, the tryptic fragment contains the collagenous domains COL1–3 including the intervening NC domains (Fig. 8B). The three chains are likely to form a collagenous triple helix. Their close vicinity explains their capability to be easily cross-linked,
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The tryptic collagen XVI fragment adopts triple-helical conformation with three chains coming in close vicinity to each other. Consequently, electron microscopy proved that this tryptic fragment is triple helical and hence visible as a thread-like molecule after negative staining (Fig. 7, red rods in B).

After its isolation, this tryptic collagen XVI fragment was incubated with soluble α1β1 integrin. The complex was covalently cross-linked with 200 μM BS3 and purified by anion exchange chromatography. Although complete purity of the complex was not achieved by this method, a broader protein band in one eluate fraction with an apparent molecular mass of 300–350 kDa was proven to be a covalently cross-linked complex of the tryptic collagen fragment and the soluble α1β1 integrin (Western blot data with antisera against collagen XVI and the integrin α1 subunit are not shown). Proteins of this eluate fraction were negatively stained and scrutinized by electron microscopy. The tryptic collagen XVI fragment appeared as a short rod of about 60 nm in length (Fig. 7), in accordance to previously published data on the structure of the entire recombinant collagen XVI (Fig. 8A) (22). In no other eluate fractions, but the one containing the complex of collagen XVI fragment and α1β1 integrin, were tadpole-like structures visualized with a globular head in the size of the α1β1 integrin heterodimer, from which a tail-like rod representing the tryptic collagen XVI fragment protruded (Fig. 7). Fig. 8B outlines a schematic representation of this tadpole-like complex. Thus, electron microscopic images of the negatively stained complexes proved a direct interaction of α1β1 integrin with collagen XVI on a molecular level. Furthermore, the α1β1 integrin-binding site within collagen XVI must be located close to one of the termini of the tryptic collagen XVI fragment.

To address the question of whether this α1β1 integrin-binding site is the only one within collagen XVI, complexes of α1β1 integrin and full-length collagen XVI were generated and visualized by electron microscopy after rotary shadowing (Fig. 7, C and D) and negative staining (Fig. 7D, inset). These images

as very low concentrations of the homobifunctional cross-linker BS3 led to an intramolecularly cross-linked dimer (at 10 μM BS3) and trimer (at 100 μM BS3) of single collagen chains with apparent molecular masses of 130 and 220 kDa, respectively (Fig. 6). However, a portion of single collagen chains remained uncross-linked even at a higher concentration of cross-linking reagent (Fig. 6), indicating that only a fraction of the tryptic collagen XVI fragment.
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**FIGURE 8.** Schematic drawing of entire collagen XVI molecule (A) and of its tryptic 64-kDa fragment (B), both in complex with an α1β1 integrin molecule (gray circle). A, collagen XVI comprises 10 triple-helical domains (COL1–10) represented by three parallel horizontal lines, which are interrupted by several noncollagenous domains (NC1–11, indicated by boxes). The N-terminal domain NC11 carries a thrombospondin-like domain (indicated by a gray star). As indicated by gray triangles below the molecule, NC domains are targets for trypsin. B, limited proteolysis with trypsin releases the C-terminal fragment of collagen XVI, which starts at amino acid 964 with the sequence AHPGL and which contains at least the collagenous domains COL1–3. A and B, the integrin α1β1 (indicated by a gray globe) binds to a unique site within the collagen XVI molecule, which is located at the N-terminal end of tryptic 64-kDa collagen XVI fragment. Its potential binding sites, GLOGER or GIKGER, presented within the homotrimeric collagenous triple-helix are also marked.

showed that collagen XVI harbors only one α1β1 integrin-binding site, which is located 74 ± 5 nm from the C terminus of the entire collagen XVI molecule as demonstrated by contour length analysis (Fig. 7E). This analysis confirmed that the unique α1β1 integrin-binding site is located in a region representing the N-terminal end of the tryptic fragment (Fig. 8).

**DISCUSSION**

In this study, we provide evidence that collagen XVI is recognized by the collagen-binding integrins α1β1 and α2β1. Although α1β1 integrin interacts with collagen XVI with high affinity, α2β1 integrin binds only weakly. The binding site of the α1β1 integrin was mapped to the C-terminal tryptic fragment comprising the collagenous domains COL1–3. So far, two different collagenous recognition sites for integrins have been characterized. As a recognition site, α1β1 integrin requires a threedimensional array of two aspartate and one arginine residues in all three chains of collagen IV, which come into close vicinity upon triple-helix formation (29, 31). Also presented within a collagenous triple helix, the integrin α2β1 recognizes the homotrimeric collagenous sequence GFOGER, with O being 4-hydroxyproline (32, 33). Variations of this homotrimeric sequence result in altered affinities for the integrins α1β1 and α2β1 (34). The integrin α1β1 preferentially recognizes the homotrimeric sequences GFOGER and GLOGER but does not tolerate the substitution of the arginine residue for a lysine residue (34). Within the amino acid sequence of collagen XVI (3), we found the analogous sequences GLQGER (1065–1070) and GIKGER (1101–1106) within the N-terminal half of the COL2 domain (Fig. 8B). Because it is the longest triple-helical domain of collagen XVI (422 amino acids), COL2 is visualized as a rod-like structure in the electron microscope after negative staining. Binding of integrin α1β1 to its N-terminal end explains the tadpole-like appearance of the covalently cross-linked complex (Figs. 7 and 8B). Therefore, we assume that α1β1 integrin binds to either or both of the collagenous integrin recognition sites found in the COL2 domain. Notably, α2β1 integrin also binds to this tryptic collagen XVI fragment. Because of its low affinity to collagen XVI, its localization was not mapped. The low affinity of α2β1 integrin is unlikely to be caused by the incomplete prolyl hydroxylation of recombinant collagen XVI, as previous studies have demonstrated that α2β1 integrin interacts with recombinant collagen I almost independently of its 4-hydroxyproline content (28). In contrast, α1β1 integrin binding to homotrimeric collagen I strongly depends on the 4-hydroxyproline content of the collagen ligand (28). In recombinant collagen XVI, about half of the available proline residues in the Y position of the collagenous GXY repeats were hydroxylated (22). Hence, we anticipate a significantly higher affinity for α1β1 integrin binding to authentic collagen XVI in vivo. Additionally, it is remarkable that α1β1 integrin avidly binds to collagen XVI, which is a homotrimeric molecule as opposed to the heterotrimeric collagen IV, the high affinity ligand of α1β1 integrin (29, 35). Another common integrin adhesion sequence, RGD, is located in the center of the COL2 domain. Yet this sequence is not accessible to RGD-dependent integrins within the collagenous triple-helical confirmation and is not recognized by α1β1 integrin, as no complex of an integrin globule centrally located within the tryptic collagen XVI fragment was ever seen under the electron microscope. This is in accordance with the RGD independence of the collagen-binding integrins, α1β1 and α2β1 (23, 29).

Combining full-length collagen XVI molecules with soluble α1β1 integrin, we demonstrated by electron microscopy that there is only one α1β1 integrin-binding site within the entire collagen XVI molecule, located 74 ± 5 nm away from its C terminus. Thus, other potential collagenous α1β1 integrin recognition sequences (34), such as GGKGER (337–342) and GKAGER (672–677) located in the collagenous domains COL10 and COL6, respectively, are not functional. Obviously, α1β1 integrin does not tolerate a substitution of the bulky hydrophobic side chain within the generic G(F/L)GER sequence for a small glycine or a basic lysine residue. As demonstrated by the electron micrographs, α1β1 integrin recognizes a site within the triple-helical rod of collagen XVI. However, because the triple helix of collagen XVI is interrupted by several rather short noncollagenous domains, we cannot exclude the possibility that the α1β1 integrin recognition site comprises also noncollagenous sequences.

By using highly purified recombinant integrins and recombinant collagen XVI (22, 23, 36), we proved in a cell-free binding assay that integrins α1β1 and α2β1 interact directly with collagen XVI. Furthermore, inhibitory monoclonal antibodies demonstrated that the two integrins utilize the A domains of their α
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subunits to interact with collagen XVI. We therefore suggest that on the molecular level the integrins α1β1 and α2β1 bind to collagen XVI in a similar mechanism as with other types of collagens (33, 37–39).

In vitro cell culture experiments provided additional evidence for a physiological role of integrin-mediated cell attachment to collagen XVI. Immobilized collagen XVI enabled divalent cation-dependent cell adhesion, which is typical of integrins. Furthermore, the collagen-binding integrins, α1β1 and α2β1, were recruited to focal contacts and focal adhesion plaques. As the formation of focal adhesion plaques is considered the first step in integrin signaling, this finding demonstrates that collagen XVI constitutes a physiological agonist of these two integrins. Thus, collagen XVI not only serves mechanically as cell attachment substratum, but it also triggers signals into the cell and induces integrin-mediated cell reactions, such as spreading and alterations in cell morphology. However, other integrin-mediated cellular reactions elicited by collagen XVI remain elusive, such as migration, differentiation, mechanical force transmission, and ECM remodeling (8). In this context, it is noteworthy that collagen XVI is integrated into distinct tissue-specific supramolecular structures (4). In cartilage, it is found as a component of D-banded fibrils, which also contain collagens II, XI, and sometimes IX. They are located mostly territorial or pericellular (4). However, it is unknown how collagen XVI is integrated into these fibrils and whether the integrin-binding site within the C-terminal part of collagen XVI is accessible to cells. Furthermore, because chondrocytes bear α10β1 integrin more abundantly than α1β1 integrin (14), a potential molecular interaction of α10β1 integrin with collagen XVI needs further investigation to understand the role of collagen XVI in cellular interactions in cartilage. Another FACIT molecule, collagen IX, which is abundant in cartilage, has recently been shown to avidly interact with each of the four collagen-binding integrins (40). Together with that study, our data suggest that a general task of FACIT molecules, which decorate the D-banded collagen fibrils and other fibrillar systems, may be to contribute a linkage between these fibrillar ECM structures and the integrin receptors on cells. Similar to collagen XVI, the integrin recognition site of collagen IX was identified to be located within a triple-helical collagen domain and formed by a collagenous sequence different from the GFOGER motif (40).

In contrast to cartilage, dermal collagen XVI preferentially associates with fibrillin-1 containing microfibrils, an entirely different supramolecular structure. In this study, we have focused on skin and provide evidence that cells may use integrins to interact with microfibrils via the FACIT collagen XVI. This is corroborated by our observation that cells bearing α1β1 and α2β1 integrins are found in tissue compartments that are adjacent and partially overlapping with the localization of collagen XVI. Although both α1β1 and α2β1 integrins were found in the stratum germinativum and spinosum of the epidermis, collagen XVI was predominantly localized to the dermal face of the basement membrane and to some extent within it. Yet, the mechanism underlying this particular distribution of collagen XVI in vivo is still unclear. Not only dermal fibroblasts but also basal layer keratinocytes residing in the stratum germinativum express collagen XVI (6). Assuming that the basal keratinocytes contribute to collagen XVI deposition on the dermal face of the basement membrane, the molecular pathway of its transfer through the basement membrane into the dermis needs to be unraveled. Based on our data we suggest that an integrin-mediated polarized collagen XVI deposition by keratinocytes may be involved in this process. Furthermore, integrins on basal layer keratinocytes may also play a role in the integration of collagen XVI into the basement membrane in a process similar to collagen IV aggregation (41, 42).

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