Integrin-mediated Cell Adhesion to Type I Collagen Fibrils*

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In the integrin family, the collagen receptors form a structurally and functionally distinct subgroup. Two members of this subgroup, α1β1 and α2β1, integrins, are known to bind to monomeric form of type I collagen. However, in tissues type I collagen monomers are organized into large fibrils immediately after they are released from cells. Here, we studied collagen fibril recognition by integrins. By an immunoelectron microscopy method we showed that integrin α1I domain is able to bind to classical D-banded type I collagen fibrils. However, according to the solid phase binding assay, the collagen fibril formation appeared to reduce integrin α1 and α2I domain avidity to collagen and to lower the number of putative αI domain binding sites on it. Respectively, cellular α1β1 integrin was able to mediate cell spreading significantly better on monomeric than on fibrillar type I collagen matrix, whereas α2β1 integrin appeared still to facilitate both cell spreading on fibrillar type I collagen matrix and also the contraction of fibrillar type I collagen gel. Additionally, α2β1 integrin promoted the integrin-mediated formation of long cellular projections typically induced by fibrillary collagen. Thus, these findings suggest that α2β1 integrin is a functional cellular receptor for type I collagen fibrils, whereas α1β1 integrin may only effectively bind type I collagen monomers. Furthermore, when the effect of soluble αI domains on type I collagen fibril formation was tested in vitro, the observations suggest that integrin type collagen receptors might guide or even promote pericellular collagen fibrillogenesis.

A fibril-forming type I collagen, a ubiquitous protein in all vertebrates, is known to provide mechanical stability for tissues and serve as a functional environment for cells (1). Depending on the physical properties of the tissue, type I collagen fibrils are arranged with different suprafibrillar architectures and diameters. Thus, narrow fibrils (~20 nm) in highly ordered arrangement occur in the cornea, where optical transparency is important, whereas large diameter fibrils (~500 nm) provide high tensile strength in mature tendon (2).

The mechanism of type I collagen fibril formation has been under extensive research for decades. In tissues, type I collagen is synthesized as a monomeric precursor, which is secreted by exocytosis into the extracellular space. In addition to the triple helical collagenous domain, the precursor contains noncollagenous C- and N-propeptides, which are linked to the triple helical domain by short sequences called telopeptides (3). After the enzymatic removal of propeptides, the solubility of collagen monomers decreases, and they spontaneously form fibrils, assisted by remaining nonhelical telopeptides (1, 4). Evidently, collagen molecules themselves contain all the information needed for fibril assembly. Therefore, in physiological conditions, acid-solubilized collagen monomers form tissue-type long fibrils with characteristic axial periodic structure also in vitro (5–7). However, self-assembly cannot by itself explain the diverse morphologies of collagen fibrils found in different tissues. In fact, several collagen binding extracellular matrix molecules, decorin, fibromodulin and lumican among others, have been found to influence the size of type I collagen fibrils (8–10).

Recently, also cell surface receptors, including integrins, were recognized to contribute to collagen polymerization indicating that the collagen fibril formation is under close cellular control (11, 12).

The integrins are a large family of cell adhesion receptors involved in cell-cell and cell-matrix interactions. In addition to their importance as anchoring molecules, these heterodimeric receptors transmit bidirectional signals that regulate many important aspects of cell behavior including proliferation, differentiation, and survival. The integrin family contains four collagen receptors that are structurally closely related (13). Two well known collagen receptors are named α1β1 and α2β1 integrins, whereas the α10β1 and α3β1 integrins constitute the latest additions to the integrin family (14–17).

Whereas all collagen receptor integrins share the common β1 integrin subunit, there are four unique α subunits, resulting in the four heterodimers. Unlike the other matrix receptor integrins, the collagen receptors have an independently folding structural domain, inserted domain (αI domain), which executes ligand recognition (10–13). This ~200-amino acid domain is located in the integrin α subunit, between the second and third N-terminal blades of the β-propeller domain. The αI domain itself adopts a “Rossmann fold” conformation, in which a central β-sheet is surrounded by α-helices forming a divergent metal binding site, referred to as MIDAS (18). Despite the structural similarity of highly conserved αI domains, the small differences present may provide specific ligand binding characteristics for different integrins (19–22).
Although αβ1 integrin is able to bind to monomers of fibril-forming collagen types I, II, III, and V, it seems to favor other subtypes such as basement membrane type IV collagen (21–25), beaded filament-forming type VI collagen (21), and type XIII transmembrane collagen (25). Instead, αβ2 integrin is the major receptor for type I collagen and other fibril-forming collagens (21, 22, 24–26). Currently, less is known about ligand binding preference of the novel α10β1 and α11β1 integrins. However, our previous results show that α10 domain resembles α1, rather than α2, in its ligand binding specificity (21), and that it may not be a better receptor for fibrillar collagen than α1 domain. Integrin α1β1 domain seems to bind most strongly to type I collagen, but it is not clear whether it can mediate high affinity interactions (22).

Integrin type collagen receptors do not differ only in their ability to recognize distinct collagen subtypes, but they also transmit divergent signals into the cell. This may explain why many cell types express several collagen receptors concomitantly. Structurally similar αβ1 and αβ2 integrins are connected to distinct signaling pathways, often leading to opposite cellular responses. Whereas αβ1 integrin seems to stimulate cell proliferation (27, 28) and act as a negative feedback regulator for collagen synthesis (26, 29, 30), αβ2 integrin inhibits the growth of some cell types (31) as well as increases matrix synthesis and turnover (26, 29, 30, 32, 33). The properties of the recently identified α10β1 and α11β1 integrins are just beginning to receive attention and still remain to be identified.

Specific recognition sites on the triple helical structures of different collagen subtypes can be recognized by a group of integrin type cell adhesion receptors (13, 34, 35). Although detailed information about integrin binding to the monomeric form of collagen is available, it has not been clear whether αI domains and the corresponding integrins can effectively support cell adhesion to complex collagen fibrils. The objective of this study is to provide further insight into αβ1 and αβ2 integrin interactions with the type I collagen fibril, the primary component of the extracellular matrix.

**EXPERIMENTAL PROCEDURES**

**Formation of Type I Collagen Fibrils**—Bovine skin type I collagen (Vitrogen®, Cohesion, or Cellon S.A.) was diluted on ice with PBS to final concentrations of 0.05, 0.1, or 0.5 mg/ml. To initiate collagen fibrillogenesis, samples were adjusted to physiological pH 7.4 with 1 M NaOH, and the temperature was increased from 4 to 37 °C. The progression of fibril formation was analyzed using a Beckman DU640 spectrophotometer by recording an optical density at 313 nm for 120 h. Finally, fibril quality was verified by transmission electron microscopy (JEM-1200EX, JEOL, Peabody, MA). For the electron microscopy analysis, fibrillogenesis of the samples was allowed to proceed between 60 min and 48 h at 37 °C. The temperature of monomeric samples was maintained below 4 °C. At each time point, collagen samples were spotted onto Formvar/carbon-coated copper electron microscopy grids for 1 h at 37 °C. Specimens were then fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 30 min at room temperature. Grids were embedded with 1.5% methyl cellulose and stained with 0.4% uranyl acetate for 10 min on ice.

**The Production of the Human Recombinant Integrin αI and αI Domains as GST Fusion Proteins—cDNAs encoding αI and αI domains were generated by PCR as described earlier (25) using human integrin αI and αI cDNAs as templates. Vectors pGEX-4T-3 and pGEX-2T (Amer sham Biosciences) were used to generate recombinant N-terminal GST fusion proteins of integrin αI and αI domains, respectively. For protein production, transformed Escherichia coli BL21 cells were grown at 37 °C in LB medium containing 100 μg/ml ampicillin until the A600 of the suspension reached 1–2. Next, the protein overexpression was induced with 0.4 mM isopropyl-β-D-thiogalactoside, and it was allowed to proceed for an additional 4–6 h at room temperature before harvesting the bacterial cells by centrifugation (5000 × g, 10 min, 4 °C). Pelleted cells were resuspended in PBS (pH 7.4) and then lysed by sonication. To permeabilize cell membranes, the disrupted bacterial cells were incubated with 1% Triton X-100 for 30 min on ice, and soluble proteins were isolated from cell debris by centrifugation (3500 × g, 10 min, 4 °C). Glutathione-Sepharose (Amershams Biosciences) was adsorbed to the supernatant. The mixture was incubated at room temperature for 30 min with gentle agitation. Glutathione-Sepharose beads with specifically bound GST fusion proteins were then collected by low speed centrifugation (500 × g, 5 min, 4 °C), and the slurry was transferred to disposable chromatography columns (Bio-Rad). The columns were washed with PBS, and fusion proteins were eluted with 30 mM reduced glutathiones (Sigma). GST—treated and αI were then analyzed by native polyacrylamide gel electrophoresis. The recombinant αI domain contains 227 amino acids, corresponding to amino acids 123–338 of the whole αI integrin subunit, whereas the recombinant αI domain is 223 amino acids in length, consisting of amino acids 124–339 of the full-length αI integrin subunit. The C terminus of the αI and αI domains contain 10 and 6 nonintegrin amino acids, respectively.

**Collagen Binding Assay for Recombinant αI and αI Domains**—To analyze the integrin binding to collagen, universal binding 96-well microtiter plates (Costar) were coated with either the monomeric or fibrillar form of type I collagen (0.5 mg/ml; Cellon S.A.) by 3.5-min exposure to UV light. Fibrillogenesis of type I collagen was allowed to proceed for 1 h at 37 °C before coating, whereas the monomeric state was maintained by keeping the collagen samples treated with Delfia® Diluent II containing BSA (Wallac) diluted 1:2 in PBS. Later the same solution was used for blocking the nonspecific protein absorption sites on all wells for 1 h at 37 °C. Next, GST-αI and GST—αI fusion proteins, at concentrations between 10 and 500 nM in Delfia assay buffer (Wallac), were allowed to bind to collagen for 1 h at 37 °C in the presence of 2 mM MgCl2. All of the solutions used thereafter were supplemented with 2 mM MgCl2. Next, unbound GST-αI domains were removed by repeated PBS wash steps, and europium-labeled Delfia® GST antibody (Wallac), diluted depending on the preparation at 1:800 or 1:2000 in Delfia® assay buffer (Wallac), was added to the wells. After 1 h of incubation at 37 °C, specimens were rinsed with PBS, and finally Delfia® enhancement solution (Wallac) was added to each well in order to dissociate the fluorescent europium label. Europium signal was determined by time-resolved fluorometry (Victor2 multilabel counter; Wallac). Each assay was performed at least in triplicate, and approximate dissociation constants were obtained by fitting the data to a Michaelis-Menten form equation. Statistical analysis was performed using two-way analysis of variance. The significance of the difference between the integrin domain binding to the fibrillar form of type I collagen was analyzed using two independent variables: the form of the collagen and αI domain concentration. Statistical significance was assigned at p < 0.05.

**Immunoelectron Microscopy Analysis of αI and αI Domain Binding to Collagen Fibrils**—Type I collagen fibrillogenesis (0.1 mg/ml Vitrogen®, Cohesion) was initiated as described above. Following 48 h of fibril formation, type I collagen fibrils were spotted onto Formvar/carbon-coated copper electron microscopy grids for 1 h at 37 °C. In order to prevent the nonspecific protein binding, the residual binding sites on grids were blocked with 5% milk powder in PBS for 15 min at room temperature. Then GST-αI at concentrations of 10, 30, 100, and 300 nM was allowed to bind to collagen fibrils attached to the grids for 1 h at 37 °C in the presence of 2 mM MgCl2. Before fixing the samples with 4% paraformaldehyde for 30 min at room temperature, unbound GST-αI was removed by washing the samples with PBS containing 2 mM MgCl2. Next, specimens were first rinsed with PBS, and then collagen-bound GST-αI domains were detected with the antibody against GST (Amer sham Biosciences) diluted at 1:400 in 5% milk powder in PBS. After 1 h, the samples were carefully washed with PBS, and bound primary antibody was localized with protein A-gold conjugate (10 nm; a kind gift from Dr. George Posthuma, University Medical Center Utrecht) diluted 1:65 in 5% milk powder in PBS. Following secondary antibody attachment for 30 min at room temperature, samples were washed with PBS and distilled water. Finally, grids were embedded with 1.5% methyl cellulose and stained with 0.4% uranyl acetate on ice for 10 min. Specimens were examined by transmission electron microscopy (JEM-1200EX, JEOL, Peabody, MA), and average numbers of bound GST-αI or GST control per collagen fibril D-period were counted from representative micrographs. Finally, the dissociation constant for GST-αI domain binding to fibrillar collagen was estimated by fitting the data to

1 The abbreviations used are: PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; GST, glutathione S-transferase; HGF, human gingival fibroblast(s); MIDAS, metal ion-dependent adhesion site; BSA, bovine serum albumin.
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In order to examine the effects of GST-α1 domains on type I collagen fibrillation, fibril assembly was monitored in the presence of GST-α1 or GST-α2 using a Beckman DU 640 spectrophotometer. Changes in turbidity were recorded at 315 nm at intervals of 1 min for 120 min. Fibrillogenesis of 330 μg type I collagen (0.1 mg/ml; Vitrogen) was analyzed in the presence of either 70 μg GST-α1-GST or GST-α2-GST at concentrations of 330, 165, 80, 70, 30, and 15 μM. The effect of GST-α1 was analyzed both in the presence of 2 mM MgCl₂ and in conditions where Mg²⁺ was made unavailable for protein binding using 2 mM EDTA as a chelating agent.

Isolation of Integrin α1, α2, and α1β1 Subunits—Integrin α1, α2 subunits (25) as well as a D219N/D292N double mutant α2 subunit. Transfected cells were maintained in α-minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin-G, 100 μg/ml streptomycin, and 0.5 mg/ml neomycin analogue G418. The human osteosarcoma cell line, Saos-2, was obtained from the ATCC, and it was also transfected to express integrin α2 subunit. Transfected cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen), 2 mM glutamine, 100 IU/ml penicillin G, and 100 μg/ml streptomycin.

Stable Transfections—Integrin α1, α2 cDNA (15) was a kind gift from Dr. E. Marcatonio (Columbia University, New York, NY), and integrin α2 cDNA (14) was from Dr. M. Hemler (Dana-Farber Cancer Research Center, Boston, MA). Integrin α2 cDNA was ligated into a modified pLNCX vector expression system (15), and integrin α2 cDNA was ligated into the pAWneo2 expression vector (36), which carries the spleen focus-forming virus long terminal repeat promoter and a neomycin resistance gene (a kind gift from Dr. A. Weiss, University of California, San Francisco, CA). Two-site-specific point mutations, D218N and D292N, were introduced into full-length α2 cDNA using the Stratagene QuikChange Site-Directed Mutagenesis kit, before the construct was transfected into CHO cells (37). CHO cells were transfected by electroporation (0.3 kV, 960 microfarads, 0.4-cm cuvette in RPMI plus 1 mM sodium pyruvate, 2 mM l-glutamine, without serum) with 20 μg of pAWneo2 carrying either wild type or mutated α2 cDNA or with 20 μg of pLEN vector carrying α1 cDNA. Construct containing α1 integrin was co-transfected with 1 μg of pAWneo2 null expression vector (25). Transfection of Saos-2 cells with the α2-pAWneo2 construct was performed using Lipofectin reagent (Invitrogen) essentially following the manufacturer’s recommendations. Control cells were transfected with pAWneo2 null expression vector. Transfected cells were then plated and allowed to recover for 1 day in culture medium and cells were then cultured using the Stratagene QuikChange Site-Directed Mutagenesis kit, added to the culture medium at 0.4–0.5 mg/ml, and G418-resistant clones were selected for 1–3 weeks before they were isolated and analyzed for their expression of α1 or α2 integrin. The cell surface expression levels of the integrins were checked by flow cytometry using antibodies against the integrin α subunit (SR-84 antibody for α1 integrin, a gift from Dr. W. Rettig (Boehringer Ingelheim) and 12F1 antibody for α2 integrin, a gift from Dr. W. Voss (University of California, San Diego) (25).

Cell Spreading Assays—Universal binding 96-well microtiter plates (Costar) were precoated with either monomeric or fibrillar type I collagen (0.5 mg/ml; Cellon S.A.) by exposing plates to UV light for 3.5 min. Before coating, fibril formation was allowed to proceed for 48 h at 37 °C. After coating, collagen fibril formation was confirmed using the Sigma rodlike collagen fibril formation test. Fibril-containing sera were detached with 0.01% trypsin and 0.02% EDTA for 3 min at 37 °C. Trypsin activity was inhibited by washing the cells either with 0.2% soybean trypsin inhibitor (Sigma) in a serum-free medium or with serum-supplemented medium. Serum was carefully removed with repeated washing steps when necessary, and cells were suspended in serum-free medium containing 50 μM ATP and 50 μM cycloheximide (Sigma) to inhibit de novo protein synthesis. Next, 26,000 CHO cells/cm², 30,000 Saos-2 cells/cm², or 15,000 HGF cells/cm² were added to the collagen-coated microtiter plates. Nonspecific binding sites on all wells were blocked with 0.1% heat-inactivated BSA in PBS for 1 h at 37 °C before the addition of cells. After 2 h of cell spreading at 37 °C, medium containing non-adhered cells was poured out, and cells were fixed with 4% formaldehyde and 5% sucrose for 30 min at room temperature. Cells in 16 representative fields from four replicate 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 having a clearly visible ring of cytoplasm around the nucleus. The morphology of cells was further analyzed, and the percentage of cells that formed more than one long projection was determined. The statistical difference between cell spreading on monomeric or fibrillar type I collagen was analyzed using two-way analysis of variance. Three independent experiments were performed, and statistical significance was set at p < 0.05.

Integrin containing of Integrin β1, Subunits—Fibrillogensis of type I collagen (Vitrogen®, Cohesion) was allowed to proceed for 48 h at 37 °C on the surface of glass coverslips. Human gingival fibroblasts were prepared for the spreading experiment as described above. Following 120 min of cell spreading, cells were fixed with 4% formaldehyde and 5% sucrose in PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂ (PBS +) for 20 min. After washing, cells were permeabilized with 0.5% Triton X-100 in PBS + for 4 min. Cells were then washed three times with PBS, and nonspecific binding sites were blocked with 3% BSA and 1% glycine in PBS + for 30 min. To localize the β₁-containing integrins on spread HGF cells, a polyclonal antibody against the cytoplasmic domain of integrin β₁ subunit (4080; a generous gift from Ken Yamada, National Institutes of Health) was diluted 1:100 with 1% BSA in PBS. After a 1-h incubation at room temperature, samples were washed with PBS, and specifically bound antibodies were localized using Alexa Fluor 594 donkey anti-rabbit IgG (1:100 dilution in 1% BSA in PBS; Molecular Probes, Inc., Eugene, OR). Secondary antibody was incubated with the samples for 1 h at room temperature following repeated PBS washing steps. Samples treated with primary antibody were covered with 50 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and samples were examined with a Zeiss Axioscope 20 fluorescence microscope, and representative cells were photographed with a Zeiss MC 80 microscope.

Analysis of Cell Morphology by Scanning Electron Microscopy—In order to analyze the effect of the different collagen forms on the morphology of cells, samples were coated with gold (16), and collagen fibrils were analyzed as one having a clearly visible ring of cytoplasm around the nucleus. The number of cells attached and the culture medium was added. After 3 days, the wells were photographed, and the surface area of the gels was measured from the prints.

RESULTS

Type I Collagen Forms Tissue-type Fibrils in Vitro—Collagen molecules themselves contain all of the information needed for fibril assembly. Here, fibrillogensis of bovine dermal collagen was initiated by subjecting a cold monomeric collagen solution to physiological conditions. Aggregation of monomers into fibrils was followed by turbidimetric analysis at 313 nm. When the turbidity of the solution showed that the process had reached equilibrium, electron microscopy analysis was used to confirm the quality of formed fibrils. It appeared that by 48 h, the majority of fibrils were similar in diameter and had the characteristic D-periodic banding pattern (Fig. 1A). In addition
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Fig. 1. Type I collagen forms mainly characteristic tissue-type fibrils in vitro. The fibril formation of the bovine skin collagen (0.1 mg/ml) was induced by neutralizing the soluble collagen preparation and increasing the temperature to 37 °C. Formed fibrils were then adsorbed onto Formvar/carbon-coated electron microscopy grids for 1 h. Finally, specimens were embedded in 1.5% methyl cellulose, stained with 0.4% uranyl acetate, and examined by transmission electron microscopy. After 48-h fibrillogenesis, the majority of collagen monomers were assembled into tight, tissue-type fibrils with the characteristic D-periodic banding pattern and a relatively constant diameter, as indicated in the representative transmission electron micrograph (A). However, also loose collagen bundles, which were typically accompanied with thin collagen fibrils, were occasionally observed among tissue-type fibrils (B).

Fig. 2. CHO cells expressing αβ₁ integrin can attach and spread on both fibrillar and monomeric type I collagen. Universal binding 96-well microtitre plates were coated with either monomeric or fibrillar type I collagen (0.5 mg/ml). Control wells were treated with PBS. CHO cell clones expressing either αβ₁, integrin or αβ₂ integrin were allowed to attach to and spread on collagen for 120 min in a serum-free medium containing 50 μM cycloheximide. Finally, formaldehyde-fixed cells in 16 representative fields from four replicate 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. The data shown are the mean values ± S.D. The difference between cell spreading on monomeric and fibrillar collagen appeared to be statistically significant (p < 0.05; two-way analysis of variance).

To the tissue-type fibrils, a minority of collagen monomers were organized into loosely packed bundles (Fig. 1B). Loose collagen bundles were typically accompanied by thin collagen fibrils as shown in Fig. 1B.

Integrin αβ₁ Mediates Cell Adhesion to and Spreading on Fibrillar Collagen, Promoting the Formation of Long Cellular Projections—To investigate the importance of specific integrins as functional receptors for fibrillar type I collagen, cells expressing different integrin patterns were examined. Wild type CHO cells have no endogenous receptors for type I collagen; thus, CHO cells transfected to express either αβ₁ or αβ₂ integrins were used (38). These cell clones were tested in spreading assays both on monomeric and fibrillar type I collagen matrices. Results indicate in agreement with previous experiments (21) that αβ₁ integrin cannot mediate cell spreading as well as αβ₂ integrin on monomeric type I collagen (Fig. 2). Strikingly, the spreading of CHO-αβ₁ cells appeared to be only slightly poorer on fibrillar than on monomeric type I collagen substratum. However, fibrillar collagen could still support cell adhesion and spreading mediated by αβ₂ integrin, whereas only 8% of CHO cells expressing αβ₂ integrin could spread on fibrillar collagen. Thus, αβ₂ integrin appeared to be a functional receptor for both collagen monomers and fibrils, whereas αβ₁ integrin could effectively mediate cell spreading only on monomeric collagen matrix. The difference between both CHO-αβ₁ and CHO-αβ₂ cell spreading on monomeric and fibrillar collagen was statistically significant (p < 0.05) according to two-way analysis of variance.

The ability of integrins to interact with fibrillar form of type I collagen was also studied by observing the spreading of HGFs on fibrillar collagen. In addition to cell spreading, it was observed that the fibrillar form of type I collagen specifically induced cells to form long cellular projections, which are not...
RESULTS indicate that CHO-α2β1 integrin expression levels on the surface of the CHO-gen were further tested in collagen gel contraction assays. Microroller plates were coated either with monomeric or fibrillar type I collagen (0.05 mg/ml) for the assay. Cells were allowed to spread in a serum-free medium in the presence of 50 μM cytochalasin D292N) inside the CHO-gen receptors are not only able to mediate cell spreading on a fibrillar collagen matrix, but β1 integrins are also essential in the formation of cell projections induced by collagen fibrils.

In order to investigate the role of distinct integrins in the formation of cellular projections, cells expressing different combinations of integrins were used. According to the scanning electron microscopy analysis, also CHO-α2β1 cells spread on monomeric collagen matrix appeared to form wide lamellipodia (upper images), whereas fibrillar collagen induced cells to form long cell projections (lower images). The percentage of cells that formed long cell projections was counted in 12 representative fields from three identical wells (histograms). Data shown are mean ± S.D.

Typically seen on cells spread on monomeric type I collagen. A function-blocking antibody against integrin β1 subunit completely prevented the fibril-induced formation of cellular projections (data not shown). Moreover, an indirect immunostaining showed that β1 integrins were localized into several extracellular matrix contacts along cell projections (Fig. 3, arrows). Thus, the data indicate that cellular integrin-type collagen receptors are not only able to mediate cell spreading on a fibrillar collagen matrix, but β1 integrins are also essential in the formation of cell projections induced by collagen fibrils.

The High Avidity Interactions of α2β1 Integrin with Fibrillar Collagen Are Essential for the Contraction of Collagen Gels—The interactions of α2β1 and α2β1 integrins with fibrillar collagen were further tested in collagen gel contraction assays. Similar integrin expression levels on the surface of the CHO cell clones were determined earlier by flow cytometry (25). The results indicate that CHO-α2β1 cells can mediate collagen gel contraction with significantly higher extent than CHO-α2β1 cells (Fig. 6A). Additionally, gel contraction appeared to require high avidity interactions between integrin and collagen fibrils, since α2β1 integrin harboring two point mutations (D219N and D292N) inside the αI domain, was not able to mediate the process (Fig. 6B).

Fig. 5. α2β1 integrin promotes the formation of fibril-induced cell projections. To analyze the significance of β1 integrin in the formation of cell projections, CHO-α2β1 cells (A), Saos-2 cells lacking endogenous α2β1 integrin expression (B), and Saos-2 cells transfected to express α2β1 integrin (C) were used. Microroller plates were coated either with monomeric or fibrillar type I collagen (0.05 mg/ml) for the assay. Cells were allowed to spread in a serum-free medium in the presence of 50 μM cytochalasin D219N. A function-blocking antibody against integrin β1 subunit completely prevented the fibril-induced formation of cellular projections (data not shown). Moreover, an indirect immunostaining showed that β1 integrins were localized into several extracellular matrix contacts along cell projections (Fig. 3, arrows). Thus, the data indicate that cellular integrin-type collagen receptors are not only able to mediate cell spreading on a fibrillar collagen matrix, but β1 integrins are also essential in the formation of cell projections induced by collagen fibrils.

In order to investigate the role of distinct integrins in the formation of cellular projections, cells expressing different combinations of integrins were used. According to the scanning electron microscopy analysis, also CHO-α2β1 cells spread on monomeric collagen matrix appeared to form wide lamellipodia (Fig. 4A), whereas fibrillar collagen induced cells to form long projections (Fig. 4B). Thus, the major integrin type collagen receptor for fibrillar collagen, α2β1 integrin, appeared to be sufficient for the formation of cellular projections alone (Fig. 5A). However, osteosarcoma cells (Saos-2) lacking endogenous α2β1 integrin expression also formed cell projections after 2 h of cell spreading on fibrillar type I collagen (Fig. 5B). Importantly, when Saos-2 cells transfected to express α2β1 integrin were studied, the number of cells that formed projections was significantly higher compared with wild type Saos-2 cells (Fig. 5C). Thus, it appeared that α2β1 integrin is not absolutely required for the formation of cellular projections, but it promotes the process.

High avidity interactions are required for the contraction of floating collagen gels by CHO-α2β1 cells. In addition to wild type α2β1 or α2β1 integrins (A), the ability of a double mutant (D219N/D292N) α2β1 integrin (B) to mediate collagen gel contraction was studied using stable transfected CHO cell clones. CHO cells transfected with empty vector were used as control cells. After the cell-mediated collagen gel contraction of 72 h, samples were photographed, and the surface areas of the gels were measured. Contrary to wild type α2β1 integrin, the double mutant α2β1 integrin did not enable collagen gel contraction by CHO cells. Data shown are mean ± S.D.

Recombinant Integrin αI Domains Bind More Strongly to Monomeric than to Fibrillar Type I Collagen—The collagen binding features of human recombinant αI and αI domain were examined in a solid phase binding assay using microtiter plates coated with either monomeric or fibrillar type I collagen.

Fig. 6. High avidity interactions are required for the contraction of floating collagen gels by CHO-α2β1 cells. In addition to wild type α2β1 or α2β1 integrins (A), the ability of a double mutant (D219N/D292N) α2β1 integrin (B) to mediate collagen gel contraction was studied using stable transfected CHO cell clones. CHO cells transfected with empty vector were used as control cells. After the cell-mediated collagen gel contraction of 72 h, samples were photographed, and the surface areas of the gels were measured. Contrary to wild type α2β1 integrin, the double mutant α2β1 integrin did not enable collagen gel contraction by CHO cells. Data shown are mean ± S.D.

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Fig. 7. Recombinant integrin αI domains show stronger binding to monomeric than to fibrillar type I collagen. Microtiter plates were precoated with monomeric (○) or fibrillar (■) T I collagen (0.5 mg/ml), and Delfia® Diluent II in PBS was used as a background control (■). The GST fusions of αI(A) and αI(B) domains were allowed to bind collagen for 1 h in the presence of 2 mM MgCl₂. Bound αI domains were detected with Eu³⁺-labeled GST antibody, and the signal was measured using time-resolved fluorescence. Data are presented as mean values ± S.E. of triplicate measurements. Approximate Kd values for αI domain binding to collagen were obtained by fitting the binding data for αI domain concentrations in the range of 10–500 nM to a Michaelis-Menten form equation. Both αI domains appeared to bind stronger to monomeric than to fibrillar type I collagen. According to the two-way analysis of variance, the difference is statistically highly significant (p < 0.05).

The results were fitted to a Michaelis-Menten form equation, and approximate Kd values were determined in order to quantify αI domain binding to collagen (Fig. 7). Results indicate that the αI domain binding to type I collagen is weaker than the binding of the αI domain (Fig. 7). Interestingly, the approximate Kd for αI domain binding to fibrillar collagen appears to be up to 10 times higher (~100 nM; Fig. 7B) than αI domain binding to the monomeric form of type I collagen (~10 nM; Fig. 7B). Similarly, αI domain showed stronger binding to monomeric (~30 nM; Fig. 7A) than to fibrillar type I collagen (~250 nM; Fig. 7A). Also according to the two-way analysis of variance, the difference between αI domain binding to the monomeric or to the fibrillar type I collagen appeared to be highly significant (p < 0.05). Moreover, the shapes of the binding curves for αI and αI domains indicate that, at saturation, fewer αI domains were bound to fibrillar than to monomeric type I collagen.

According to the electron microscopy analysis described earlier, type I collagen typically formed classical striped fibrils during in vitro fibrillogenesis. However, occasionally some loosely packed collagen bundles were also seen (Fig. 1). Since it could not be determined by the solid phase binding assay whether αI domains are really able to bind to the tissue-type, tightly packed fibrils, the immunoelectron microscopy method was developed (Fig. 8). Here, the number of recombinant immunolabeled GST-αI domains bound per D-periodic banding pattern (67 nm) on collagen fibrils was counted from electron micrographs. Using different concentrations of αI domain, it was possible to estimate the dissociation constant. The approximate Kd, for αI binding to fibrillar type I collagen (~62 nM), obtained by fitting the binding data to a Michaelis-Menten form equation (Fig. 8D), appeared to be very similar to the value from the solid phase binding assay. Thus, these results not only show that αI domain is able to bind to tissue-type collagen fibrils but also confirm the solid phase binding assay.

Soluble αI Domains Modify Collagen Fibrillogenesis—Since all of the experiments indicate that αIβI and αIβα integrins are able to bind better to the monomeric than to the fibrillar form of type I collagen, we tested whether soluble GST-αI domain fusion proteins affect type I collagen fibril formation in vitro. It appeared that at first, αI domain accelerated fibrillogenesis, but at later time points it inhibited the process (Fig. 9B). In contrast, high concentrations of αI domain (1:1 molar ratio to collagen) could almost completely inhibit collagen fibril formation from the very beginning (Fig. 9A). Interestingly, the activity of αI domain required a high concentration of Mg²⁺ (2 mM) in the buffer, whereas this was not required for the higher affinity αI domain. Obviously, the experiment does not mimic the conditions in tissues, where integrins are typically anchored to the cell membrane. However, the results indicate that αIβI integrin binding to collagen monomers is stronger than the interactions of collagen molecules with each other.
Integrin-mediated Cell Adhesion to Type I Collagen Fibrils

Collagens form a large protein family with 27 different members described to date (39). In addition to maintaining the structure of various tissues, collagens are currently known to dynamically regulate a number of vital cellular functions. Cells adhere to collagen mainly through four members of the integrin family. Here, we show that the major integrin-type collagen receptor, $\alpha_2\beta_1$ integrin, effectively binds tightly packed type I collagen fibrils and might even participate in collagen fibrillogenesis, in a reaction previously thought to be a simple self-assembly process.

Two major collagen receptors, $\alpha_2\beta_1$ and $\alpha_6\beta_1$ integrins, are known to recognize three high affinity sites on type I collagen monomers through specialized ligand binding $\alpha I$ domains in their $\alpha$ subunit (19, 20, 40). Integrin binding sites on collagen are located within the unique triple helical domain and have been identified to contain a GER motif. In particular, a hexapeptide, GFOGER, has been found to mediate high affinity integrin binding to collagen monomers (34, 35). A glutamic acid residue from the collagen motif has been observed to directly coordinate the metal ion bound to the MIDAS in the integrin $\alpha_2$ domain (41, 42). In addition to the required divalent cation, specific conserved residues surrounding the MIDAS motif are predicted to be critical for collagen binding (43–45). However, the periphery of the collagen binding surface of different $\alpha I$ domains is more variable and thus might explain their collagen type preferences. In accordance with previous findings, all of the results presented here stress the essential role of $\alpha_2\beta_1$ integrin in type I collagen binding (21–23, 25, 26). $\alpha_2\beta_1$ integrin is known to bind tightly to fibril-forming collagens, the structurally highly similar $\alpha_6\beta_1$ integrin preferentially adheres to network-forming type IV collagen (21–25).

Immediately after secretion, type I collagen molecules assemble into cross-striated, D-banded fibrils in which each molecule is displaced about one-quarter of its length relative to its nearest neighbor along the axis of the fibril. Although type I collagen exists in fibrillar form in the extracellular matrix, this is the first time integrin $\alpha I$ domain binding to collagen fibrils has been characterized. Here, immunoelectron microscopy analysis demonstrated the ability of $\alpha_2\beta_1$ domain to recognize and bind to classical D-banded collagen fibrils. However, when the $\alpha I$ domain binding was further characterized by a sensitive solid phase binding assay, it appeared that collagen fibril formation seems to lead to weakened integrin $\alpha_1 I$ and $\alpha_2 I$ domain binding. Thus, the results suggest that fibril formation leads to the conformational changes in the integrin recognition sites on collagen, reducing integrin binding affinity. Moreover, the fibril formation may also reduce the number of integrin binding sites on collagen by hiding several GER motifs inside the fibrillar collagen structure.

In accordance with the binding assays, $\alpha_2\beta_1$ integrin was able to mediate cell spreading both on fibrillar and monomeric type I collagen matrices, whereas $\alpha_6\beta_1$ integrin was a functional receptor mostly for the monomeric form of type I collagen. However, Saos-2 cells, which express $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_11\beta_1$ integrins but not $\alpha_6\beta_1$ integrin, could still attach to and spread on collagen fibrils. Thus, in addition to $\alpha_2\beta_1$ integrin, a combination of other integrin-type collagen receptors seems also to be able to mediate cell binding to type I collagen fibrils.

For the present, the binding of recently identified $\alpha_1 I$ domain resembles $\alpha_1 I$ rather than $\alpha_2 I$ domain in its ligand binding specificity (21), suggesting that it may not be a better receptor for fibrillar collagen than $\alpha_2 I$ domain. Integrin signaling generated by cell attachment to monomeric or fibrillar collagen seems also to be different. Here, it was obvious that $\alpha_6\beta_1$ integrin-mediated cell binding to fibrillar collagen induced cells to form long cellular projections, whereas cells spread on monomeric collagen formed wide lamellipodia. Again, Saos-2 cells, which do not endogenously express $\alpha_6\beta_1$ integrin, could form similar projections. The role of $\alpha_2\beta_1$ integrin in the process was, however, confirmed when transfected $\alpha_2\beta_1$ integrin could still promote the formation of the projections by Saos-2 cells. Recently, small GTPases have been implicated in the formation of cellular projections (46). To support the suggestion of the integrin-mediated formation of the cellular projections, these molecules have been shown to be important also in $\alpha_6\beta_1$ integrin-mediated signaling (32).

In addition to wound retraction, the formation of cellular projections is involved in the collagen gel contraction (46). Reorganization of collagen fibrils, seen as a contraction of a floating collagen gel, reflects the ability of cells to interact with the collagen matrix. In accordance with several previous observations, we stress the essential role of $\alpha_2\beta_1$ integrin in this process (31, 47–49). However, it seems that the function of

![Graph A](image1.png)

**Fig. 9.** $\alpha_1 I$ domain can prevent fibril formation in vitro. The fibrillogenesis of the type I collagen in the presence of either $\alpha_1 I$ or $\alpha_1 I$ domain was recorded every minute at 313 nm for 120 min by turbidimetry. Type I collagen was diluted to 330 $\mu M$, and different molar ratios of $\alpha_1 I$ domain (B) or $\alpha_1 I$ domain (A) were examined. In the presence of 2 mM Mg$^{2+}$, 70 $\mu M$ at $\alpha_1 I$ domain appeared at first to accelerate fibrillogenesis and at later time points to inhibit it (B). However, when Mg$^{2+}$ was removed from the solution using EDTA as a chelating agent, $\alpha_1 I$ domain was not able to actuate type I collagen fibril formation (B). Instead, high concentrations of $\alpha_1 I$ domain almost completely inhibited type I collagen fibril formation even without added Mg$^{2+}$ (A).

**DISCUSSION**

In accordance with the binding assays, $\alpha_2\beta_1$ integrin was able to mediate cell spreading both on fibrillar and monomeric type I collagen matrices, whereas $\alpha_6\beta_1$ integrin was a functional receptor mostly for the monomeric form of type I collagen. However, Saos-2 cells, which express $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_11\beta_1$ integrins but not $\alpha_6\beta_1$ integrin, could still attach to and spread on collagen fibrils. Thus, in addition to $\alpha_2\beta_1$ integrin, a combination of other integrin-type collagen receptors seems also to be able to mediate cell binding to type I collagen fibrils.
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