The binding capacity of $\alpha 1\beta 1$ -, $\alpha 2\beta 1$ - and $\alpha 10\beta 1$ -integrins depends on non-collagenous surface macromolecules rather than the collagens in cartilage fibrils

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ABSTRACT

Interactions of cells with supramolecular aggregates of the extracellular Matrix (ECM) are mediated, in part, by cell surface receptors of the integrin family. These are important molecular components of cell surface-suprastructures regulating cellular activities in general. A subfamily of $\beta 1$ -integrins with von Willebrand-factor A-like domains (I-domains) in their α -chains can bind to collagen molecules and, therefore, are considered as important cellular mechano-receptors. Here we show that chondrocytes strongly bind to cartilage collagens in the form of individual triple helical molecules but very weakly to fibrils formed by the same molecules. We also find that chondrocyte integrins $\alpha 1\beta 1$ -, $\alpha 2\beta 1$ - and $\alpha 10\beta 1$ -integrins and their I-domains have the same characteristics. Nevertheless we find integrin binding to mechanically generated cartilage fibril fragments, which also comprise peripheral non-collagenous material. We conclude that cell adhesion results from binding of integrin-containing adhesion suprastructures to the non-collagenous fibril periphery but not to the collagenous fibril cores. The biological importance of the well-investigated recognition of collagen molecules by integrins is unknown. Possible scenarios may include fibrillogenesis, fibril degradation and/or – phagocytosis, recruitment of cells to remodeling sites, or molecular signaling across cytoplasmic membranes. In these circumstances, collagen molecules may lack a fibrillar organization. However, other processes requiring robust biomechanical functions, such as fibril organization in tissues, cell division, adhesion, or migration, do not involve direct integrin-collagen interactions.

KEY WORDS

Cell-matrix-interactions, mechanoreception, suprastructure, adaptor proteins

1. INTRODUCTION

Interactions of cells with their extracellular matrix (ECM) result in important clues for their activities, including energy requirements, division, differentiation, survival, malignant transformation, programmed death, and the metabolism of small and large molecules [1, 2]. Thus, cells and the ECM arguably form a functional continuum rather than separate entities. From this viewpoint, the ECM is a compartment whose distinction from other cellular compartments and organelles is its location outside of the cytoplasmic membrane. Nevertheless, the cytoplasmic membrane constitutes a barrier, which is essential for the structural and functional specialization of tissues and organs of multi-cellular organisms. To achieve functional continuity between cells and their ECM, specialized cell adhesion suprastructures transduce information from inside out or from outside in. These suprastructures comprise regions integrated into the cytoplasmic membrane as well as intra- and extracellular portions, contain a multitude of macromolecules organized into well-defined nanoscale architectures [3-5], and are multifunctional. For example, cell adhesion suprastructures containing integrin clusters as their main membrane-spanning components can mediate mechano-reception. Integrins are members of a family of dimeric membrane glycoproteins, comprising an α - and a β -subunit. The α - β -composition determines the specificity of integrin clusters for extracellular ligands [6, 7] that typically are macromolecular components of ECM-suprastructures, such as collagen fibrils.

A hallmark of all ECM-macromolecules, including collagens, is the formation of aggregates or suprastructures which fulfill biological functions e.g. in transmission of mechanical forces. Naturally occurring ECM-suprastructures have been designated as heterotypic because they contain almost always several molecular species in larger or smaller quantities. Importantly, the macromolecular organization and, hence, the functional properties are critically determined by the composition even if the quantitatively major molecular species is shared by many matrix suprastructures. Likewise, quantitatively minor components can be essential for the overall aggregate even if their mass fractions are very small. For these reasons, we termed natural collagen fibrils as macromolecular alloys. Their properties strongly depend on their composition in terms of, both, collagens and non-collagenous molecules (e.g. COMP, matrilins, decorin, fibromodulin, lumican, perlecan, and collagen VI-containing microfibrils) [8-13]. In cartilage fibrils, for example, the inclusion of collagen XI as a fibrillar nucleus is vital for the incorporation of the main component, collagen II [14, 15]. Cartilage of mice lacking the expression of collagen II contains collagen I in large quantities. However, cartilage of collagen II-deficient mice also lacks normal collagen XI because the cartilage isoform of this protein also comprises an α -1(II) polypeptide (also known as α -3(XI)-chain). Collagen XI powerfully nucleates fibril incorpora-

tion of collagen I as well [16]. As a consequence, collagen I, although abundantly present, cannot form fibrils in cartilage of collagen II-KO-mice [17, 18].

The suprastructural collagen organization also influences the recognition of collagens by integrins (for review, see [19]). Mercier et al. reported [20] that fibroblasts interact more weakly with collagen I organized into fibrils than with collagen I in molecular suspension and that formation of cytoskeletal structures depends on the organization of extracellular collagen. Here, we followed up on this subject by distinguishing the integrin-mediated interactions of chondrocytes with cartilage collagens in molecular vs. fibrillar form. Integrin-mediated cell-matrix-interactions influence cartilage homeodynamics and are disrupted during joint cartilage degeneration [21-24]. Chondrocyte surfaces contain distinct quantities of $\beta 1$ -integrins with $\alpha 1$ -, $\alpha 2$ -, and $\alpha 10$ -chains, respectively. While no obvious cartilage abnormalities are observed in mice lacking $\alpha 1$ - or $\alpha 2$ -integrin chains [25], $\alpha 10\beta 1$ -integrin is required for normal development of cartilaginous growth plates [26]. All of these three integrin types recognize molecular collagens. They have been intensively investigated with respect to their exact recognition motifs on collagen triple helices [27-30]. For these reasons, it is generally surmised that collagen-binding integrins of chondrocytes also recognize cartilage fibrils but this has never been demonstrated directly. Here, we show that neither collagen binding integrins nor their extracellular binding domains have a significant affinity for the purely collagenous phase of cartilage fibrils although we could confirm their well-known affinity for collagen molecules. Likewise, chondrocytes strongly interact with cartilage collagen molecules but not with cartilage fibrils lacking their non-collagenous periphery.

2. RESULTS

2.1 Chondrocytes attach to cartilage collagen fibrils less than to immobilized monomolecular collagens

To characterize integrin binding to collagens, chondrocyte adhesion to cartilage collagens was studied in initial experiments. For reasons of consistency, we used materials of chicken origin. We obtained chondrocytes from sternal cartilage of 17 d-old chick embryos [31] and investigated their adhesion to native individual cartilage collagen monomers, i.e., collagen types II, IX and XI. These proteins were isolated as native mixtures in molar fractions $f_{II,IX,XI} \approx 8:1:1$ from chondrocytes cultured in agarose gels [14, 32] without proteolytic modification. They were coated to non-tissue culture grade, 96-well polystyrene plates under conditions preventing their aggregation into fibrils ([14], see important experimental details in 4.5, methods section). Chondrocytes were allowed to attach to collagen monomers for 45 min and adherent cells were quantified by direct counting of cells in several randomly selected and representative microscopic fields. As shown in Fig. 1 (open bar, monomers), about two thirds of the seeded cells were attached to the mixture of monomeric collagens II, IX, and XI. Similar observations were made when purified collagens II, IX, or XI were used at similar concentrations (not shown).

We have reported earlier [14] that fibrils reconstituted in vitro from mixtures of collagens II, IX, and XI are stable and closely resemble fibrils of sternal cartilage in their collagen composition and in their appearance in the electron microscope. Solutions of cartilage collagens were allowed to form fibrils after appropriate buffer adjustment to allow for minimal quantities of monomeric collagens in the substrate coats (see 4.5, methods section). To verify fibril formation, proteins were scratched from the plastic surfaces and subjected to transmission electron microscopy. Cartilage-like fibrils were observed as previously described (not shown) [14]. Chondrocytes were seeded on these collagen fibrils. In striking contrast to their attachment on monomeric collagen, only about 20% of the cells adhered to the fibrillar substrates although the same number of cells had been plated (Fig.1, open bar, fibrils).

In addition to several integrin types, chondrocyte surfaces also contain other collagen-binding matrix receptors, including discoidin domain receptor type 2 (DDR2 [33-35]). DDR1 is less abundant on mesenchymal cells. To distinguish contributions to chondrocyte adhesion of integrins from those of DDR2, we performed adhesion experiments also in the presence of 10 $\mu\text{g/ml}$ of soluble recombinant DDR2 as a decoy receptor [36]. As shown in Fig. 1, binding of chondrocytes was not reduced by DDR2 when monomeric collagens were used as substrates (solid bars, monomers). However, on fibrillar collagen substrates binding was further reduced to about 10%. This difference was statistically significant ($p < 0.001$, two-tailed Student's T-test) and suggested that residual cell attachment to fibrils was mediated by DDR2.

When fibronectin, a known attachment substrate for chondrocytes, was used as a control strong binding was observed as expected, regardless of the presence of soluble DDR2 (Fig. 1, fibronectin). No binding occurred on uncoated plates (Fig. 1, HEPES).

2.2 Binding of soluble integrins to collagens II, IX, and XI

In the following experiments, binding of integrins to collagen substrates was directly investigated. Collagens II, IX, or XI were purified as described [14] and were attached to formvar/carbon-coated EM-Grids. After blocking of unspecific binding sites, the grids were exposed to PBS, containing 2 mM MgCl_2 and 25 $\mu\text{g/ml}$ of recombinant ectodomains of human integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, or $\alpha 10\beta 1$. Without possessing transmembrane anchorage domains, the integrin ectodomains were soluble and were kept together by a jun/fos-zipper motif [37]. As negative binding controls, MgCl_2 was replaced by 10 mM EDTA. Integrin binding was activated by the antibody 9EG7 [38]. This antibody was additionally used for detection with an anti-immunoglobulin antibody attached to colloidal gold particles. The grids were stained with uranyl acetate and analyzed by transmission electron microscopy. An example showing the binding of $\alpha 2\beta 1$ -integrin to the cartilage collagens is shown in FigS1 (supplementary materials). Gold particles per unit area were counted on several randomly selected EM-observation fields. Binding of the three integrins to respective cartilage collagen types is represented in Fig. 2, black bars. Clear binding was observed, especially for $\alpha 2\beta 1$ -integrin, the major chondrocyte integrin, although background levels in the presence of EDTA were substantial, especially for $\alpha 1\beta 1$ -integrin and collagen XI. As expected, integrin binding to cartilage collagens was dose-dependent and was abrogated by EDTA as exemplified in Fig. 3 for $\alpha 2\beta 1$ -integrin. In conclusion, these results confirm and extend previous observations [24, 39-41] that monomolecular cartilage collagens were good binding substrates for all collagen-binding integrins expressed by chondrocytes.

We then investigated the same integrin binding to mixtures of collagens II, IX, and XI organized into fibrils. Fibrils were formed as described above and were then adsorbed to the EM-grids. Residual collagen monomers were removed by percolating buffer through uncoated EM grids into filter paper under the grids. Thereafter, the grids were exposed to integrins, antibodies, and gold particles as described above. Finally, the grids were coated by carbon layers. After staining with uranyl acetate the specimens were subjected to electron microscopy. As shown in Fig. 4, large amounts of fibrils could be observed that, however, were devoid of gold particles indicating integrin binding below background level. This was the case in fibrils containing collagens II and XI without or with collagen IX, as found in prototypic fibrils formed in immature cartilages.

As a positive control, no explicit effort was undertaken to remove residual monomeric collagen molecules except for normal washing. As shown in Fig. 5A, integrin binding occurred unless prevented by addition of EDTA (Fig. 5B). However, the occurrence of gold particles was mostly limited to locations obviously remote from fibrils, which presumably, was due to binding to residual monomers. We concluded that, in agreement with the cell-binding studies described above, collagen-binding integrins recognize monomeric cartilage collagens, regardless of their identity, but not after their aggregation into fibrils. In addition, monomeric chicken collagen bound to human integrins, i.e. across species borders.

2.3 Binding of soluble integrins to authentic cartilage fibrils

The lack of integrin binding to collagen fibrils reconstituted *in vitro* may be explained by the absence of certain fibril properties, including covalent cross-linking that would be present in native cartilage fibrils. In addition, native fibrils offer the benefit of being structurally complete in that they also contain non-collagenous components at their periphery. A further advantage of natural fibrils is that binding of human integrins can be studied for fibrils from different species. Therefore, mechanically generated fragments of natural fibrils were extracted from human, bovine, or chicken cartilage by established methods [42] and were used as binding substrates for soluble human integrins in experiments conducted analogously to those described above for reconstituted fibrils. In the electron microscope, crude preparations from human osteoarthritic knee cartilage exhibited clearly identifiable fibril fragments in addition to non-fibrillar, diffusely electron-dense material. As shown in Fig. 6, gold particles indicating integrin binding were abundantly located in the latter material, but much less in sections of isolated fibrils without apparent surface material highlighted by arrowheads in Fig. 6B. This was true for $\alpha 1\beta 1$ -, $\alpha 2\beta 1$ -, and $\alpha 10\beta 1$ -integrins. Integrin binding was abolished in the presence of EDTA, as expected (Fig. 6D). A detailed inspection of residual gold particles on fibrils revealed their location at small quantities of non-fibrillar material associated with the fibrils as highlighted by arrows in Fig. 6B. We concluded that natural cartilage fibrils were not substrates for integrins unless coated by peripheral material that, most likely, consisted of proteoglycans or glycoproteins, which occur abundantly in cartilage matrix. Accordingly, antibodies to COMP, matrilin-1, aggrecan, fibronectin, or collagen VI and to $\alpha 2\beta 1$ -integrin detected strong immuno-gold reactions located at the same regions within the extrafibrillar material (Fig. 7). Entirely analogous observations were made with crude preparations of chick or bovine fetal or bovine adult knee cartilage (not shown).

Preparations of the collagen skeletons of fibril fragments from chick embryo cartilage is possible without disruption of the collagen organization after extraction with 6M guanidinium hydrochloride that removes non-collagenous surface-components [42]. As shown in Fig.8, crude preparations of such fragments contained

large sections which were essentially free of peri- or extrafibrillar material and which exhibited no binding of $\alpha 2\beta 1$ -integrin (Fig. 8). In the few cases where gold particles were localized along the fibrils closer inspection at higher magnification revealed again indirect binding of the integrins via non-fibrillar material. A gallery of such gold particles is shown in the inset of Fig. 8.

2.4 Binding of $\alpha 1$ - or $\alpha 2$ -integrin I-domains to authentic cartilage fibrils

Collagen binding of integrins is entirely mediated via the I- (or A-) domain, a von Willebrand factor A-like motif inserted into the integrin α -subunit. Therefore, we produced GST-tagged, recombinant I-domains of $\alpha 1$ - and $\alpha 2$ -subunits of chicken integrins employing the procedures described previously [43]. Crude preparations of fibril fragments extracted from chick embryo sternal cartilage were adsorbed on formvar / carbon-coated EM-grids as described above and were reacted with the GST-tagged I-domains. Binding of I-domains was monitored by indirect immuno-gold labeling using an anti-GST-antibody attached to Ig-Gold particles. As shown in Figs. 9A and C, diffusely electron-dense, non-fibrillar material was heavily labeled by gold particles specific for $\alpha 1$ - or $\alpha 2$ -I-domains, respectively, whereas discretely deposited fibrils invariably remained undecorated. This Mg^{2+} -dependent reaction was strongly inhibited in the presence of EDTA (Figs 9B and D). Very similar observations were made with fibril fragments from bovine knee cartilage and recombinant bovine I-domains (not shown).

Taken together, our findings are entirely consistent with the notion that collagen molecules lose their affinity for integrins after their incorporation into fibrils. However, as has been shown repeatedly, integrins can react with solubilized collagen molecules without obvious supramolecular organization.

3. DISCUSSION

The study described here demonstrates that neither the extracellular regions of collagen-binding integrins nor their substrate-recognizing I-domains can bind to cartilage collagens organized into fibrils. Consistently with this, chondrocytes do not attach directly to the collagen skeletons of cartilage fibrils via their integrins. However, all natural collagen-containing fibrils, including those of cartilage, not only contain several types of collagens in distinct molar fractions but also non-collagenous components specifically incorporated into their surface (for review, see [13]). The resulting molecular diversity is highly likely to endow fibrils with the structural and functional properties required in a given tissue or even a region thereof. In agreement with this notion, we presented evidence here that the mostly non-collagenous components of collagen-containing fibrils in cartilage contain the relevant binding sites for integrins. Molecular candidates include collagen VI and microfibrils containing collagen VI [24], matrilins [11], COMP (thrombospondin 5) [13], and several SLRPs (decorin, fibromodulin, lumican) [44]. Many of these macromolecules have been associated also with a biomechanical role in cartilage (for review see [12]). Mice lacking any of these components usually have mild skeletal phenotypes, which is consistent with the notion of redundant roles as substrates in mechanoreception and chondrocyte adhesion. An exception to this rule is perlecan, usually a basement membrane component in many tissues. Interestingly, cartilage matrix lacks basement membranes and is particularly rich in perlecan in regions near to chondrocyte surfaces. Perlecan-null mice develop a strong cartilage defect [45]. Hence, the proteoglycan qualifies as an important substrate for cell attachment.

In agreement with many reports in the literature, however, we found that single molecules of cartilage collagens II, IX, or XI without fibrillar organization are well recognized by $\alpha 1\beta 1$ -, $\alpha 2\beta 1$ -, and $\alpha 10\beta 1$ -integrins. Studies on the molecular interaction of a collagen-like peptide comprising the binding sequence GFOGER with the integrin $\alpha 2$ A-domain have shown that two of the three chains within a triple helix are in contact with residues of the $\alpha 2$ A-domain. Modeling calculations predicted that the molecular binding geometries of collagen molecules could be maintained also in fibrils [27]. According to these predictions, binding of A-domains as well as entire integrins could occur also to collagen fibrils. However, this notion is difficult to reconcile with the structural model of thin cartilage fibrils as proposed by Holmes and Kadler [46] in which, both, collagen II and XI molecules bearing potential integrin-binding sites are tightly packed into fibrils composed of 10 and 4 microfibrils located at the surface and the core of the fibrils, respectively. Thus, the accessibility of collagen molecules to integrins would be severely limited. Very similar conclusions have been reached by model calculations performed by Orgel et al. [47] predicting restricted accessibility of all major integrin binding sites even in the minority of collagen molecules located at the fibril surface. Our data presented here support the

latter conclusions that collagen molecules incorporated into cartilage fibrils do not expose their binding sites to any integrin ligands.

The integrin binding sites in monomolecular cartilage collagens are in triple helical conformation and include the amino acid sequences GXX'GEX", where X is a hydrophobic residue and X' often is 4-hydroxyproline. X" is often R, but not K. An outstanding example is the sequence GFOGER [27, 30]. The physiological importance of this promiscuous binding of integrins with molecular collagens is unclear. It does not, however, encompass mechano-reception, or events derived thereof, since single collagen molecules do not exhibit the resilience required of collagen-containing fibrils.

The absence of integrin binding to cartilage fibrils comes as a surprise after consideration of the detailed fibrillar suprastructure. The surfaces of chick embryo sternal cartilage fibrils are D-periodically (D=64 nm) studded by triple helical Col3-domains of collagen IX molecules [48]. Therefore, this region of collagen IX should be available for attachment of integrins, especially since it reportedly contains the only binding site for the I-domains of $\alpha 1\beta 1$ - or $\alpha 2\beta 1$ -integrins [49]. However, integrin binding could not be observed in this study even after treatment of the fibrils with guanidinium hydrochloride. This latter treatment is known to contribute to the free projection of COL3-domains from the fibril surfaces and the studded fibril appearance in the electron microscope after rotary shadowing [48]. Thus, the inertness towards collagens in fibrillar organization appears to be a robust property of integrins.

Our study also shows that chondrocytes do exhibit a small direct attachment activity to collagen-containing fibrils that, presumably, is mediated by DDR2, an alternative collagen-binding receptor with tyrosine kinase activity that can modulate integrin-mediated cell attachment [50]. It remains to be determined whether or not DDR2 or other collagen-receptors crucially contribute to robust chondrocyte attachment to cartilage matrix. However, DDRs may regulate cell attachment through signaling cascades comprising phosphorylation via their tyrosine kinase activities [34, 35].

Mercier et al. [20] have shown that dermal fibroblasts attach well to tissue culture substrates of isolated dermal collagen but only weakly to reconstituted collagen I-containing fibrils. Likewise, Jokinen et al. found that the I-domains of $\alpha 1\beta 1$ - or $\alpha 2\beta 1$ -integrins bound with reduced activity to fibrils reconstituted in vitro from molecular suspensions mainly containing collagen I [51]. However, single collagen molecules persisting in reconstitution mixtures after fibrillogenesis cannot easily be removed from fibrils. In addition, no specific effort has been undertaken in any of the above studies to remove residual collagen molecules from the reconstituted fibrils, which could explain the remaining binding of cells or I-domains to fibrils. Therefore, these studies support our main conclusions, which, moreover, may well apply also to tissues other than cartilage.

4. MATERIALS AND METHODS

4.1 Chondrocyte and cartilage preparation

Primary chondrocytes were obtained from sternal cartilage of 17 d-old chick embryos [52]. After removal of the perichondria the dissected sterna were split up into cranial and caudal parts and washed three times in Krebs-Ringer buffer. Chondrocytes were released from their extracellular matrix by over-night treatment with collagenase B (1 mg/ml) in collagenase buffer (1 mM cysteine, 100 U/ml penicillin, 100 µg/ml streptomycin (sterile filtrated) in DME medium (DMEM)) at 37°C and 5% CO₂. Sedimented cells were resuspended and separated from undigested matrix by filtration through a triple layer of nylon membranes (100 µm pore size) in a Swinnex filter. Residual collagenase was removed by two gentle centrifugation steps (600 x g, 7 min) with substitution of the supernatant with fresh Krebs-Ringer buffer. After an additional centrifugation step the cell pellet was resuspended in DMEM with 100 U/ml penicillin and 100 µg/ml streptomycin (sterile filtrated) and quantified in a Neubauer counting chamber.

4.2 Collagen purification

Mixtures of cartilage collagens II, IX and XI in their native molar fractions $f_{II,IX,XI} = 8:1:1$ were isolated from chick embryo chondrocytes cultured in suspension in agarose as described [32]. Separation of the three collagen types occurred by ion-exchange chromatography as described [14].

Authentic fibril fragments were obtained from sternal cartilage of 17 d-old chick embryos or adult chicken, juvenile and adult bovine knee joint cartilage and human osteoarthritic hip cartilage [42]. In brief, cartilage samples were prepared freshly by homogenizing three times on ice for 20 s with a Polytron (PT 3000, Kinematica, Lucerne, Switzerland) in 500 µl of Tris buffer, containing 50 mM Tris-HCL, pH 7.4. After short centrifugation (2500 x g, 3 min) the supernatant was collected and the pellet homogenized again. The supernatants were combined and used immediately.

4.3 Receptors

Soluble recombinant human integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, in which the transmembrane- and intracellular domains were replaced by a jun-/fos-zipper motif, were prepared as described [53, 54]. The cDNA encoding $\alpha 10$ integrin chain [55] within the plasmid pcDNA3 $\alpha 10$ was digested with Acc65I- and AvrII and a fragment of 2900 bp encoding the N-terminal portion of the $\alpha 10$ integrin ectodomain was isolated. The plasmid pcDNA3 $\alpha 10$ was also used as template to amplify an AvrII-AgeI-fragment of 479 bp length encoding the C-terminal portion of the $\alpha 10$ integrin ectodomain using 5'-AGAACCTAGGCTGCTATGTGG-3' (sense, s) and 5'-GTTACCGGTCCTTCCCTCAATTCCTCCGAGGATAGGCCGGGTCT-3' (antisense, as) as primers. The

antisense primer also introduced an AgeI restriction site as well as a factor X-cleavage site at the C-terminal end of the $\alpha 10$ ectodomain. The amplicon, cloned via the AvrII and AgeI sites into the pCRII-TOPO plasmid, was amplified, validated by sequencing, and re-opened by digestion with Acc65I and AvrII upstream of the amplicon, and religated with the 2900 bp Acc65I-AvrII-fragment to obtain the full length $\alpha 10$ ectodomain encoding cDNA within the pTo $\alpha 10$ plasmid. This cDNA was cloned into the pUC-HMT- $\alpha 1$ fos construct by replacing the integrin $\alpha 1$ ectodomain-encoding sequence via the AgeI site as well as the blunted AvrII and Sall sites of the insert and vector, respectively. The plasmid was transfected into Drosophila S2 Schneider cells together with the pUC-HMT- $\beta 1$ jun-construct, and the ectodomain of integrin $\alpha 10\beta 1$ was isolated and purified as described [53, 54].

Recombinant soluble protein comprising the entire DDR2 ectodomain fused to the F_c -sequence of human IgG2 was produced in episomally transfected HEK293-EBNA cells and was purified by affinity chromatography as previously described [36, 56].

4.4 Recombinant I-domains

cDNA sequences corresponding to the chicken $\alpha 1I$ - and $\alpha 2I$ -domains were amplified by RT-PCR using total RNA from 3-day embryos as the template. The RNA was a gift from Olli Lassila's laboratory (University of Turku). The RT reaction was performed using the Invitrogen Super Script II kit (Invitrogen Inc., USA). Specific primers were designed on the basis of the genomic sequences published in the ENSEMBL database. Chicken $\alpha 1I$ - and $\alpha 2I$ -domain cDNA-sequences were amplified using as primers 5'-GCAGGATCC-GCTCCATCCGTACAAGAGTGT-3' (s) and 5'-GCAGAATTCCTGATCTGTTGTTGCTTCAAG-3' (as), and 5'-GCAGGATCCTGTTCTTCCGTCATAGATATT-3' (s) and 5'-GCAGAATTCACCTTGATCTGTACCTTCTAT-3' (as), respectively. Thirty cycles of amplification using the Phusion DNA polymerase (Finnzymes Ltd., Espoo, Finland) were carried out with the following protocol: denaturation: 10 s at 94°C, annealing: 30 s at 45°C, extension: 40 s at 72°C. The annealing temperature was raised to 55°C for the last 20cycles. The products were subjected to electrophoresis on a 1.5 % agarose gel and were visualized by ethidium bromide staining. RT-PCR products of the expected size were isolated from the gels and purified using the NucleoSpin Extract kit (Macherey-Nagel GmbH, Düren, Germany). Purified RT-PCR products were double digested with BamHI and EcoRI and ligated into thepGEX-4T-3 vector (GE Healthcare Europe GmbH, Freiburg, Germany), digested with the same enzymes, using T4-DNA ligase (Promega, Mannheim, Germany). The ligation mixture was transformed into competent E. coli BL21 cells. Clones containing the chicken $\alpha 1I$ and $\alpha 2I$ inserts were

verified by sequencing. Plasmids for the expression of human GST- α 1I and GST- α 2I were prepared as described earlier. Both human and chicken GST- α 1I and GST- α 2I were produced and purified using glutathione affinity chromatography [57, 58].

4.5 Cell adhesion assay

Non-tissue grade, 96-well microtiter plates (Nunc-Immuno™ MaxiSorp™) were employed in cell adhesion assays. Initially, the bottom of each well was marked with a pen as point of reference for microscopic images. In experiments with monomeric collagens, culture dishes were coated overnight at 4°C with 100 μ l/well of solutions of collagens II, IX, and XI at molar fractions $f_{II,IX,XI} = 8:1:1$ (total collagen concentration: 150 μ g/ml) in 0.1 M HEPES, 0.4 M NaCl, pH 7.4. Under these buffer and temperature conditions, the collagens remain in molecular form and fibril formation is prevented. In alternative experiments, fibril formation was deliberately induced in the wells as follows: 50 μ l of monomeric collagens II, IX, and XI dissolved (300 μ g/ml total concentration) in 0.1 M HEPES, 0.4 M NaCl, pH 7.4 were diluted in the wells by 50 μ l of distilled water and incubated for 3 h at 37°C. This treatment leads to a virtually quantitative conversion of the monomeric collagens into fibril suspensions. Coating was then allowed to be completed overnight at 4°C. Fibronectin coating was achieved by addition to the wells of 100 μ l of HEPES, 0.4 M NaCl, pH 7.4, containing 20 μ g/ml of fibronectin and incubation overnight at 4°C. Unbound substrate proteins were removed by washing twice with 0.1% BSA in DMEM and unspecific binding sites were blocked for 60 min with 0.5% (v/w) BSA in DMEM. After a further washing step, a second blocking step was performed for one hour with 10 μ g/ml recombinant DDR2-receptors in 2 mM Tris-HCL buffer, pH 7.4, containing 150 mM NaCl (in control experiments without DDR2). The wells were washed twice and freshly isolated cranial chondrocytes (7.5×10^4 cells/100 μ l) were allowed to attach to the substrates for 45 min. With an inverse microscope (Axiovert 100; phase-contrast (PH1), 10 fold magnification) each well was photographed and, subsequently, unbound cells were removed by gentle agitation and washing. The wells were photographed again and corresponding microscopic pictures were merged in silico. Cells were quantified by direct counting in at least 5 randomly selected representative fields and the fraction of attached cells was calculated. Results from 3 independent experiments were pooled and means and standard deviations were determined with standard algorithms.

4.6 Immunoelectron Microscopy

15 μ l aliquots of authentic fibril fragments, reconstituted fibrils, or monomeric collagens were spotted onto sheets of Parafilm. Nickel grids coated with Formvar/carbon were floated on the drops for 15 min to allow absorption of the aggregates. After adsorption, the grids were washed with 150 mM NaCl, 2 mM sodium

phosphate, pH 7.4 (PBS), and were blocked for 60 min with 2% (w/v) dried skimmed milk in PBS. The grids were then treated with PBS, containing 2 mM MgCl_2 and 375 ng (25 $\mu\text{g/ml}$) of soluble recombinant human integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, or $\alpha 10\beta 1$ with 450 ng (30 $\mu\text{g/ml}$) of the activating antibody 9EG7 (Lenter et al., 1993, BD Biosciences, Heidelberg, Germany) for 60 min. In controls, MgCl_2 was replaced by 10 mM EDTA. After washing with PBS, the grids were incubated for 60 min with a suspension of colloidal gold particles (18 nm) coated with goat antibodies against rat immunoglobulins (Jackson ImmunoResearch, Newmarket, UK) in PBS, containing 0.2% (w/v) dried skim milk. Finally, the grids were washed five times with distilled water and negatively stained with 2% uranyl acetate. Electron micrographs were taken at 60 kV with an EM 410 electron microscope (Phillips).

In addition, binding experiments were performed with solutions of recombinant, GST-tagged chicken $\alpha 1\text{I}$ - and $\alpha 2\text{I}$ -domains (25 $\mu\text{g/ml}$). Bound A-domains were detected with murine monoclonal anti-GST antibodies (Rockland, Limerick PA, USA) and appropriate immunogold antibodies as described above. Gold particles per unit area (150 μm^2) were quantified by counting on several randomly selected EM-observation fields. Means and standard deviations were determined by standard procedures.

Double-labeling experiments for peripheral non-collagenous macromolecules and for $\alpha 2\beta 1$ -integrin were carried out in a two-step procedure, firstly with specific antibodies to aggrecan (polyclonal, rabbit; Biotrend Chemikalien GmbH, Cologne, Germany), fibronectin (monoclonal, mouse; MAB88916-C, Millipore Corporation, Darmstadt, Germany), decorin (rabbit, polyclonal, kind gift of Daniela Seidler, Hannover), COMP (rabbit, polyclonal, kind gift of Frank Zaucke, Cologne), matrilin-1 (polyclonal, rabbit, kind gift of Raimund Wagener, Cologne), or collagen VI (polyclonal, rabbit, Millipore Corporation) and with 12 nm gold particles coated with species-specific monoclonal anti-immunoglobulins (Jackson ImmunoResearch), followed by washing, and secondly with soluble $\alpha 2\beta 1$ -integrin, the antibody 9EG7 (see above), and with 18 nm gold particles coated with goat antibodies against rat immunoglobulins (Jackson ImmunoResearch). All incubation and washing steps were carried out with buffers and reagents as described above.

Reconstituted collagen fibrils as adhesion substrates were formed by adjusting the buffer salinity to 50 mM HEPES, pH 7.4, containing 200 mM NaCl and warming to 37°C as described above. 10 μl of the collagen fibril suspension (150 $\mu\text{g/ml}$) was applied on the surface of uncoated nickel grids. Although fibril formation is essentially complete under these conditions, traces of collagen monomers were removed by percolating the above buffer solution through the uncoated grids into filter paper. This procedure was repeated several times with PBS and, afterwards, grids were blocked, and exposed to integrins and antibodies as described above. Finally, the grids were coated with a layer of carbon generated on mica plates and floated on distilled water.

The samples were finally stained with 2% (v/w) uranyl acetate and analyzed by transmission electron microscopy.

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Figure legends:

Fig. 1: *Chondrocytes attach to collagen molecules, but not fibrils.*

Polystyrene plates were coated with 150 µg/ml of a mixture of chicken collagens II, IX, and XI (molar fraction $f=8:1:1$) in 0.1 M HEPES-buffer, pH 7.4, containing 400 mM NaCl at 4°C overnight to prevent fibril formation (Monomers) or in 50 mM HEPES-buffer, containing 200 mM NaCl at 37°C for 3h, followed by an incubation overnight at 4°C, to advance fibril formation (Fibrils). Chicken chondrocytes were allowed to attach for 45 min and were quantified. For quantification details, see text. Experiments were carried out with (open bars) or without (solid bars) blocking with recombinant extracellular domains of DDR2. Error bars denote standard deviations ($n = 15$ in total, derived from 3 independent experiments) and differences between experiments specified by brackets were considered statistically significant for p-values of < 0.05 . n.s.: not significant.

Fig. 2: *Soluble recombinant integrins bind to cartilage collagens.*

EM-grids were coated at room temperature for 15 min with 500 µg/ml of purified monomeric cartilage collagens II, IX, or XI in 0.1 M HEPES-buffer, pH 7.4, containing 400 mM NaCl and were reacted after washing and blocking with 375 µg/ml of soluble recombinant $\alpha 1\beta 1$ -, $\alpha 2\beta 1$ -, $\alpha 10\beta 1$ -integrins as indicated. The reaction mixtures also contained the activating antibody 9EG7. After washing, the grids were exposed to immuno-gold particles coated with anti-IgG antibodies and stained with uranyl-acetate. Images were taken from 5 randomly selected fields (see Fig. S1 for $\alpha 2\beta 1$ -integrin, supplementary material) and gold particles/field were counted. Black columns: Binding of integrins in the presence of Mg^{2+} . Grey columns: EDTA-controls. White columns: Background controls without addition of integrins. Differences between mean values specified by brackets were considered as statistically significant for p-values < 0.05 . Error bars denote standard deviations of experimental values ($n = 15$)

Fig. 3: *Binding of $\alpha 2\beta 1$ -integrin is dose-dependent*

Experiments were carried out as in Fig. 2 with increasing concentrations of $\alpha 2\beta 1$ -integrin. Solid squares: Experiments with Mg^{2+} . Open squares: EDTA controls.

Fig. 4: *Reconstituted cartilage collagen fibrils lack integrin binding capacity.*

Fibrils were reconstituted from mixtures of collagen II and XI or collagens II, IX, and XI and were applied to EM-grids. Residual non-polymerized collagen molecules were carefully removed. The integrins were allowed to bind to the different substrates were reacted with integrins in the presence of activating antibody 9EG7, followed by immuno-gold particles and staining with uranyl acetate. Note: Only few gold particles were detected (arrows). Substrates and integrin types are indicated on the upper and left margins, respectively. Scale bar: 200 nm

Fig. 5: *Residual monomeric collagens retain integrin-binding capacity.*

A: Experiment as in Fig. 4 with mixture of collagen II, IX, and XI ($f=8:1:1$), except without removal of residual monomeric collagens. B: Negative control with EDTA. Note: Preparations containing also monomeric collagens readily reacted with integrins (arrows). Scale bar: 200 nm

Fig. 6: *Cartilage fibrils retaining peripheral components are integrin substrates.*

Experiments were carried out as in Figs. 4 and 5. Fibril fragments from 17 d-old chick embryo sterna were used as binding substrates. A: $\alpha 1\beta 1$ -, B: $\alpha 2\beta 1$ -, C: $\alpha 10\beta 1$ -integrin, D: $\alpha 2\beta 1$ -integrin, EDTA-control. Non-fibrillar surface components and fibril sections without surface material are highlighted by arrows and arrow-heads, respectively. Scale bar = 200 nm

Fig. 7: *The fibril periphery contains integrin-binding non-collagenous macromolecules.*

Experiments were performed as in Figs. 4 – 6. Fibril fragments from bovine knee joint cartilage served as binding substrates. Immuno-gold labeling with 12 nm gold particles was done with antibodies to A: aggrecan, B: fibronectin, C: decorin, D: COMP, E: matrilin-1, F: collagen VI. After washing the grids were reacted with $\alpha 2\beta 1$ -integrin in the presence of 2 mM $MgCl_2$ and antibody 9EG7 which activates integrins. Immuno-gold labeling of this antibody was with anti-IgG-immunoglobulins attached to 18 nm gold particles. The insets show selected areas of co-distribution of $\alpha 2\beta 1$ -integrin and macromolecules of the fibril periphery indicating integrin binding. Scale bar = 200 nm

Fig. 8: *After extraction of fibril periphery with 6 M guanidinium hydrochloride chick-embryo sternal cartilage fibrils have lost integrin-binding capacity.*

Experiment as in Fig. 6 with soluble $\alpha 2\beta 1$ -integrin, but with fibril fragments mostly devoid of peripheral components. A gallery of gold particles in specified fields is shown at higher magnification on the right margin. Note: Reactivity is observed only in locations highlighted by arrows with residual diffuse material associated with the fibrils. Scale bar: 200 nm

Fig. 9: *Recombinant I-domains of chicken integrin α -subunits only react with peripheral fibrillar material.*

Crude preparations of fibril fragments from sternal cartilage of 17 d-old chick embryos were used as integrin-binding substrates. A: $\alpha 1$ -I- domain, B: $\alpha 1$ -I-domain, EDTA-control, C: $\alpha 2$ -I-domain, D: $\alpha 2$ -I-domain, EDTA-control, Scale bar = 200 nm

Supplementary material

Fig. S1: *Monomolecular cartilage collagens bind to $\alpha 2\beta 1$ -integrin*

EM-grids were coated for 15 min at room temperature with purified collagens II, IX, or XI (300 $\mu\text{g/ml}$ in 0.1 M HEPES-buffer, pH 7.4, containing 400 mM NaCl to prevent fibril formation. Collagens were reacted with 25 $\mu\text{g/ml}$ of soluble $\alpha 2\beta 1$ -integrin, antibody 9EG7 activating integrins, and 2 mM MgCl_2 (upper panels) or 10 mM EDTA (lower panels, negative controls).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. S1