The Fibrill-associated Collagen IX Provides a Novel Mechanism for Cell Adhesion to Cartilaginous Matrix*

Collagen IX is the prototype fibrill-associated collagen with interruptions in triple helix. In human cartilage it covers collagen fibrils, but its putative cellular receptors have been unknown. The reverse transcription-PCR analysis of human fetal tissues suggested that based on their distribution all four collagen receptor integrins, namely α1β1, α2β1, α10β1, and α11β1, are possible receptors for collagen IX. Furthermore primary chondrocytes and chondrosarcoma cells express the four integrins simultaneously. Chondrosarcoma cells, as well as Chinese hamster ovary cells transfected to express α1β1, α2β1, α10β1, or α11β1 integrin as their only collagen receptor, showed fast attachment and spreading on human recombinant collagen IX indicating that it is an effective cell adhesion protein. To further study the recognition of collagen IX we produced recombinant αI domains in Escherichia coli. For each of the four αI domains, collagen IX was among the best collagenous ligands, making collagen IX exceptional compared with all other collagen subtypes tested so far. Rotary shadowing electron microscopy images of both αI- and αI-collagen IX complexes unveiled only one binding site located in the COL3 domain close to the kink between it and the COL2 domain. The recognition of collagen IX by αI was considered to represent a novel mechanism for two reasons. First, collagen IX has no GFOGER motif, and the identified binding region lacks any similar sequences. Second, the αI domain mutations D219R and H258V, which both decreased binding to collagen I and GFOGER, had very different effects on its binding to collagen IX. D219R had no effect, and H258V prevented type I binding. Thus, our results indicate that collagen IX has unique cell adhesion properties when compared with other collagens, and it provides a novel mechanism for cell adhesion to cartilaginous matrix.

* This work was supported by the Academy of Finland, the Graduate School of Informational and Structural Biology, the Sigrid Juselius Foundation, and the Finnish Cancer Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Collagen IX was the first member to be discovered of a subgroup of collagens, now known as the fibrill-associated collagens with interrupted triple helix (FACITs)1. At the present, FACITs also include collagens XII, XIV, XVI, XIX, XX, XXI, and XXII. Collagen IX is composed of three different α chains, termed α1(IX), α2(IX), and α3(IX). Structurally the collagen IX molecule can be divided into three triple helical domains (COL1, COL2, and COL3) separated and flanked by non-triple helical (NC) domains. Collagen IX is expressed in cartilage and in a limited number of other locations, including developing eye. Collagen IX can be covalently cross-linked to collagen II, and it typically it covers the large fibrils formed by collagens II and XI (2–5). There is a kink between COL2 and COL3 domains making the COL3 domain project into the perifibrillar space, whereas the COL1 and COL2 domains are arranged on the surface of the fibril. Many but not all collagen IX molecules are proteoglycans because a glycosaminoglycan (GAG) chain may be attached to NC3 domain via a serine residue in the α2(IX) chain (6). Collagen IX is essential for the normal structure and function of cartilage. Its mutations in man cause multiple epiphyseal dysplasia (7–13) and in mouse cause degenerative changes in articular cartilage (14, 15).

FACITs are proposed to mediate the interaction of collagen fibrils with other matrix components. Their location on the surface of the fibrils also makes them putative ligands for cell adhesion receptors, but very little is known about their ability to interact with cells. The collagen receptor integrins, αIβ1, α2β1, α10β1, and α11β1 heterodimers (16–19), form a structurally and functionally distinct subgroup of integrins. All four α subunits have a special inserted domain (αI domain) that mediates their binding to collagen. Despite their structural similarities, the collagen receptors have different abilities to signal and to recognize various collagen subtypes (20–28). Based on analyses of recombinant αI domains we have recently shown that αI and αI seem to prefer basement membrane collagen IV and beaded filament-forming collagen VI, whereas fibril-forming collagens such as I and II are best ligands for αI and αII, domains (20, 21, 26–29). The recognition of FACITs by integrins has not been reported. Integrin-mediated cell adhe-

1 The abbreviations used are: FACITs, fibrill-associated collagen with interruptions in triple helix; RT, reverse transcription; GAG, glycosaminoglycan; s, sense; as, antisense; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; BSA, bovine serum albumin.
sion to collagen may be an important regulator of cell behavior and may regulate tissue integrity at several levels. For example, integrins may participate in regulation of collagen fibril formation (30–32).

In this report we demonstrate that based on their expression pattern all four collagen receptor integrins are putative receptors for type IX and that they, and their corresponding recombinant αI and βI domains, each bind to human recombinant collagen IX. For every integrin collagen IX was among the best collagenous ligands. This makes collagen IX exceptional among all collagens tested so far and emphasizes the role of FACLITs as cell adhesion proteins. Our results indicate that αI and αI domains recognize only one site in collagen IX that is in COL3 domains very close to the kink formed by NC3 domain. The binding mechanism does not resemble any of the previously described I-domain-collagen interactions.

MATERIALS AND METHODS

Cell Lines and Reagents—Human osteosarcoma cell lines HOS-MNNG and RHOS-240, Chinese hamster ovary (CHO) cells, and human chondrosarcoma cell line HTB-99 were obtained from American Type Culture Collection (ATCC, Manassas, VA). The human primary fetal chondrocyte cultures were isolated as described previously (33). The cell cultures were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin-G, and 100 μg/ml streptomycin.

Generation of Stable Integrin-expressing CHO Cell Line—CHO-αIβI and CHO-αIβI cell lines were created as described previously (27). Full-length αI cDNA corresponding to nucleotides 19–3525 of the published sequence (19) (GenBankTM accession no. AF074015) was generated with the Access RT-PCR kit (Promega) using RNA purified from SAOS-2 cells (ATCC). Primers were designed to introduce BglII and BamHI restriction sites at the 5’ and 3’ ends, respectively. Digested cDNA was ligated to pcDNA3 expression vector (Invitrogen) containing cytomegalovirus promoter and the gene for neomycin resistance. The sequence was verified by sequencing. CHO cells (ATCC) were stably transfected with FuGENE 6 transfection reagent (Roche Applied Science). Isolated clones were selected with a neomycin analog G418 (400 μg/ml, Invitrogen) for 3 weeks. Clones were analyzed for the expression of the αI integrin with RT-PCR using the Gene Amp PCR kit (PerkinElmer Life Sciences) and immunoprecipitation.

Immunoprecipitation—Polyclonal rabbit antisera against human βI (34), αI (35), αI (a kind gift from Dr. Evy Lundgren-Akerlund, Lund, Sweden) (36), and αI (a kind gift from Dr. Donald Gullberg, Uppsala, Sweden) (37) were used in immunoprecipitation assays. Cell cultures were metabolically labeled with 50 μCi/ml [35S]methionine (Trans35S-label, ICN Biomedicals Inc., Irvine, CA) for 18 h in methionine-free minimum essential medium (Sigma). Cell monolayers were rinsed on ice with a solution containing 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 25 mM Tris-HCl (pH 7.4) and then detached by scraping. Cell pellets obtained by centrifugation at 500 × g for 5 min were solubilized in 200 μl of the same buffer containing 100 mM n-octyl-β-D-glucopyranoside (Sigma) on ice with occasional vortexing. In soluble material was removed by centrifugation at 10,000 × g for 5 min at 4°C. Radioactivity in cell lysates was counted, and an equal amount of radioactivity was used in immunoprecipitation assays. Triton X-100 (0.5%, v/v), and bovine serum albumin (0.5 mg/ml) were added to the supernatants, which were then precleared by incubation with 50 μl of packed protein A-Sepharose® (Amersham Biosciences). Supernatants were incubated with anti-integrin antibodies for 2 h at 4°C. Immune complexes were recovered by binding to protein A-Sepharose and washing the beads four times with 25 mM Tris-buffered isotonic saline (pH 7.4) containing 0.5% Triton X-100 and 1 mg/ml bovine serum albumin and twice with 0.5 mM NaCl and 25 mM Tris-HCl (pH 7.4). The immunoprecipitated samples were electrophoresed on sodium dodecyl sulfate-containing 6% polyacrylamide gels under nonreducing conditions followed by autoradiography.

Detection of mRNA for Specific Integrin Subunits by Reverse Transcription-Polymerase Chain Reaction—Total cellular RNA from cell cultures was isolated using the RNeasy minikit (Qiagen). For extraction and purification of total RNA from human fetal tissues, the samples were immediately frozen and pulverized in liquid nitrogen, homogenized in guanidinium isothiocyanate, and sedimented through 5.7 M CsCl (38). RT-PCR was done using the Gene Amp PCR kit (PerkinElmer Life Sciences). All oligonucleotides were designed to recognize a unique sequence exclusive for each cDNA. Integrins were amplified using the following primers: αI 5′-CAGGGATCCTTCTCATCGCTTATG-3′ and as 5′-GAGCTTCTGTGAGTTCCAGGC-3′; αI 5′-GAGCTTCTGTGAGTTCCAGGC-3′; αI 5′-CAGGGATCCTTCTCATCGCTTATG-3′ and as 5′-GAGCTTCTGTGAGTTCCAGGC-3′; αI 5′-CAGGGATCCTTCTCATCGCTTATG-3′ and as 5′-GAGCTTCTGTGAGTTCCAGGC-3′; αI 5′-GAGCTTCTGTGAGTTCCAGGC-3′; and as 5′-GAGCTTCTGTGAGTTCCAGGC-3′.

Cloning of Human Integrin αI Domain—cDNAs encoding αI and αI domains were generated by PCR as described earlier (26, 28) using human integrin αI and αI cDNAs as templates. Vectors pGEX-4T-3 and pGEX-2T (Amersham Biosciences) were used to generate recombinant glutathione S-transferase (GST) fusion proteins of human αI and αI domains, respectively. The αI domain cDNA was generated by RT-PCR from RNA isolated from RHOS-240 cells (human chondrosarcoma). Total cellular RNA was isolated using the RNasey minikit (Qiagen). RT-PCR was done using the Gene Amp PCR kit (PerkinElmer Life Sciences). Details for the cloning were described earlier (28). The amplified αI domain cDNA was digested along with pGEX-2T expression vector (Amersham Biosciences) using the BamHI and EcoRI restriction enzymes (Promega). To the pGEX-2T vector the 5′-end was ligated with the SureClone linker (Amersham Biosciences). The construct was transformed into the Escherichia coli BL21 strain for production. The DNA construct was sequenced and compared with the published αI sequence (19). Human integrin αI (or αI domain (37) was used as a template when αI domain was generated by PCR. The PCR product, having BamHI and EcoRI sites, was cloned into pGEX-RT, and the DNA sequence was checked by sequencing the vector insert (29).

Expression and Purification of αI Domains—Competent E. coli BL21 cells were transformed with the plasmids for protein production. 500 ml of LB medium (Biokar) containing 100 μg/ml ampicillin was inoculated with 50 ml of overnight culture of wild-type or mutant BL21/pGEX2I domain was 223 amino acids in length corresponding to amino acids 161–384 of the mature protein. The carboxyl terminus of the 2I domain was 223 amino acids in length corresponding to amino acids 161–384 of the mature protein.

Expression and Purification of αI Domains—Competent E. coli BL21 cells were transformed with the plasmids for protein production. 500 ml of LB medium (Biokar) containing 100 μg/ml ampicillin was inoculated with 50 ml of overnight culture of wild-type or mutant BL21/pGEX2I domain was 223 amino acids in length corresponding to amino acids 161–384 of the mature protein.
extra amino acids. Recombinant α1,1 domain contains some GST as an impurity due to the endogenous protease activity during expression and purification (29). Recombinant α1 domains were used as GST fusion proteins for collagen binding experiments.

Site-directed Mutagenesis—Site-directed mutation of the α1 domain cDNA in a pGEX-2T or pGEX-4T-3 vector was carried out using PCR according to Stratagene’s QuickChange mutagenesis kit instructions. The presence of mutations was checked by DNA sequencing. Mutant constructs were then transformed into E. coli strain BL21 for production of recombinant protein (26, 28).

Recombinant Collagen IX and Other Collagens—Rat (rat tail) collagen I and mouse (basement membrane of Engelbreth-Holm-Swarm mouse sarcoma) IV collagen were purchased from Sigma. Collagens II (bovine), III (human), IV (human), and V (human) were obtained from Chemicon. Collagen VI (human) was purchased from Biodiogen International.

Recombinant human collagen IX was produced as described previously (16-18). Briefly Trichoplusia ni insect cells (High Five, Invitrogen) grown in suspension at 27 °C were seeded at 1.0–1.5 × 10⁹ cells/ml in SF900 II SFM medium (Invitrogen) and supplemented with 5% fetal bovine serum. The cells were co-infected with three viruses coding for the α(1)IX, α2(IX), and α3(IX) chains and a double promoter virus pP4HAVI coding for the α- and β-subunits of human prolyl 4-hydroxylase (42) with multiplicities of infection of 2:2:2:2, respectively. Ascorbate (80 μM) was added daily to the culture medium. After 72 h of infection, the cells were harvested from the culture medium by centrifugation. Proteins were precipitated from the culture medium by addition of solid ammonium sulfate to 25% saturation and placing the mixture on ice for 1 h with mixing. The precipitate was collected by centrifugation at 15,000 × g for 1 h at 4 °C, and the pellet was dissolved in 0.1 M Tris, 10 mM EDTA, 0.4 M NaCl, 2 M urea, pH 7 buffer with protein inhibitors (Roche Applied Science) overnight. The dissolved recombinant protein was applied to Superdex 200 gel filtration column (Amersham Biosciences) in the same buffer and subsequently purified with Resource S or HiPrep CM (Amersham Biosciences) cation exchange chromatography in 50 mM PIPES, 20 mM NaCl, 2 M urea, pH 6.5 buffer, eluting with an increasing salt concentration (0.02–1.0 M NaCl). Purified collagen IX was analyzed by SDS-PAGE and subjected to fluorometry (Victor2 multilabel counter, Wallac). At least three parallel assays were performed. The inhibition assay protocol is described above, and the inhibition assay was tested using amino binding plates (Costar DNA-Bind, Corning Inc.). Wells were coated with peptides at 1 μg/ml for 1 h at room temperature in PBS, pH 8.5. Then wells were blocked with a 1:1 solution of 0.1 M of Delfia Diluent II (Wallac) and PBS, pH 8.5. After this assay, the solid-phase binding assay for α1 domains was performed as described above.

Synthesis of Peptides—Peptides were synthesized as described earlier (44, 45). All peptides were found to be the correct theoretical mass by mass spectrometry.

Rotary Shadow Transmission Electron Microscopy—Type IX collagen was mixed with I domains (50 μg of collagen/100–200 μg of I domain) and dialyzed against PBS with 2 mM MgCl₂ at 4 °C. Aliquots were incubated at room temperature for 2–4 h, brought to 70% glycerol, and spayed onto freshly cleaved mica, and rotary shadowing was done after air-drying (46, 47). Complexes were examined and photographed at 80 kV using a Hitachi 7000 transmission electron microscope (Hitachi, Inc.). Photographs were take at 30,000× or 40,000× and printed at 120,000× for analyses. The magnifications were calibrated using a line grating.

RESULTS

Integrins α₁β₁, α₂β₁, α₁β₂, and α₁β₁β₁ Are Expressed in Cartilage-derived Cells—Here we started the search for cellular receptors of collagen IX by analyzing the expression of all collagen-binding integrin subunits, α₁, α₂, α₁₀, and α₁₁, at the same time in different tissues of 20–24-week-old human fetus by RT-PCR. The specificity of the PCR was confirmed by DNA sequencing of the PCR products (not shown). Integrin α₁₀ was expressed in epiphyseal cartilages of knee and shoulder joints of 24-week-old human fetus (Fig. 1). The highest level of α₁₀ mRNA was observed in growth plate of 20-week-old human fetus. The mRNAs for α₁₀ were not seen in meniscus where other collagen-binding integrin subunits α₁, α₂, α₁₀, and α₁₁ were expressed. α₁₀ mRNA was not detected in skin or muscle, but it was present in eye. Integrin α₁₁ had a broader expression pattern; its mRNA was detected in all tissues studied with highest levels in growth plate, bone, meniscus, and calvaria. mRNA for α₁₁, unlike α₁₀ mRNA, was detected in skin. Integrin α₁ was expressed at mRNA level in skin, growth plate, and whole eye. The mRNA levels for integrin α₁ and α₂ subunits were relatively low in epiphyseal cartilage tissues except in...
meniscus. Integrin α2 mRNA expression levels were highest in skin, growth plate, and eye (Fig. 1). The expression of COL9A1 was tested in the same samples, and it was present in cartilage and eye and at a low level in bone (Fig. 1). Thus, based on their expression patterns, all four collagen receptor integrins may act as receptors for collagen IX.

Concomitant Expression of Integrins α1β1, α2β1, α10β1, and α11β1 in Cultured Human Primary Fetal Chondrocytes—RT-PCR was performed to study the expression of α10 and α11 subunits in human chondrosarcoma cells (HTB-99) and human primary fetal chondrocytes. Integrins α1, α2, α10, and α11 were expressed at mRNA level in both cell types (Fig. 2A). To confirm the presence of the corresponding proteins we performed metabolic labeling experiments and immunoprecipitations with antisera against integrin β1, α2, and α11 subunits. Antiserum against β1 subunit precipitated a typical pattern of protein bands (Fig. 2B) previously identified as precursor β1 (about 100 kDa), mature β1 (about 110 kDa), multiple β1-associated α subunits (about 140 kDa), and β1-associated α1 subunit (about 190 kDa) (34). The presence of α2 and α11 subunits was confirmed by specific antisera. Integrin α11 subunit migrated consistently slower than α2 suggesting that it might be more glycosylated (Fig. 2B). Our results suggest that all four collagen receptor integrins may be simultaneously expressed in a single cartilage-derived cell.

Collagen IX Is a Cell Adhesion Protein—Since primary chondrocytes and chondrosarcoma cells had a similar expression pattern of collagen receptor integrins, HTB-99 cells were the first choice to test the ability of cells to attach and spread on human recombinant collagen IX. Their adhesion to fibril-forming collagen I and network-forming collagen IV was tested at the same time (Fig. 3A). The spreading of HTB-99 cells on collagen IX was comparable to spreading on collagens I and IV, and there was practically no spreading on BSA, which was acting as a negative control in the experiment. Thus, collagen IX is a good cell adhesion protein for cartilage cells.

CHO cells do not express any collagen-binding integrins on the their surface and do not spread on collagen I (27). We have previously created stable CHO cell lines that express α1β1 (CHO-α1β1) or α2β1 (CHO-α2β1) as their only collagen receptor (27). Here their ability to spread on collagen IX was tested. CHO-α1β1 cells spread very fast on collagen IV as well as on collagen IX, whereas their spreading on collagen I was remarkably slower (Fig. 3B). CHO-α2β1 spread fastest on collagen I, and collagen IX was almost as good a ligand as collagen I. Spreading on collagen IV was slower. There was no spreading on BSA (Fig. 3B). Since the expression of α10β1 integrin is more restricted to cartilage than the expression of any other collagen receptor, it was important to create CHO cells expressing α10β1 integrin. Fig. 4A shows RT-PCR data from different α10β1 clones indicating the presence of α10 subunit mRNA in transfected CHO cells, and Fig. 4B shows immunoprecipitation of α10β1 in the same cells. Since no previous data have been available about the ligand binding of cells that express α10β1 as their only collagen receptor we tested a larger set of collagen subtypes (collagens I, II, III, IV, V, VI, and IX). CHO-α10β1 cells spread fast on all collagens. The fastest spreading was observed on collagens IV and VI (Fig. 4C). The results indicated that α1β1, α2β1, and α10β1 integrins are receptors for collagen IX. Importantly for every integrin collagen IX was among the best collagenous ligands.

**Fig. 2.** The expression of integrins α1, α2, α10, and α11 in cultured human fetal chondrocytes, human chondrosarcoma cells (HTB-99), and human osteosarcoma cells (HOS-MNNG). A, total RNA was isolated from cells, and RT-PCR was performed with 1 μg of total RNA/reaction with specific primers for integrins α1, α2, α10, α11, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Glyceraldehyde-3-phosphate dehydrogenase was used as a positive control. B, the human chondrocytes were incubated for 18 h in the presence of [35S]methionine. Cellular integrins were immunoprecipitated with specific antibodies and analyzed by electrophoresis and fluorography. ~C is a negative control for RT-PCR. I.P., immunoprecipitation.
Recombinant α1 Domains of All Four Collagen Receptor Integrins Bind to Collagen IX—Recombinant α1-GST (marked as α1) fusion proteins were used to investigate the binding mechanism of collagen receptor integrins to collagen IX. Four collagen receptor α1 domains were tested in a solid-phase binding assay (Fig. 5). α1A, α1B, α1C, and α1D domains bound tightly to collagen IX. Binding of all α1 domains was metal-dependent (data not shown). Binding affinities were estimated using a simple Michaelis-Menten type equation and assuming that the possible multiple binding sites were identical. The number of binding sites could not be estimated from the data. Approximated $K_v$ values obtained were 30 ± 5 nM for α1A, 55 ± 13 nM for α1B (Fig. 5A), 294 ± 25 nM for α1C (Fig. 5B), and 53 ± 5 nM for α1D (Fig. 5C). Our previous reports (28, 29) contain approximated $K_v$ values for α1 domain binding to other collagen subtypes tested so far.

Integrin α1 Domain Has One Binding Site in Collagen IX in COL3 Domain—The present knowledge about α1 domain binding to collagen is based on the detailed structural analysis of α1 domain binding to GFOGER motif in collagen I (48). Therefore α1A was selected for further analysis of collagen IX recognition by integrins. First, rotary shadowing electron microscopy was used to image the α1A-collagen IX complex (Fig. 6). Electron micrographs pointed out one clear binding site close to the amino terminus of collagen IX (Fig. 6). The site was located in triple helical COL3 domain very close to the region where the kink characteristic to collagen IX is situated (Fig. 6, A and B). This area in collagen IX contains no previously proposed integrin binding motifs like GFOGER or GLOGER. The exact binding site remains to be shown. When side chains of arginine residues were chemically inactivated as described in Ref. 43, collagen IX could no longer interact with α1A domain (data not shown). Similar treatment has been described to inhibit α1B binding to collagen IV (43).

We also tested three linear peptides based on the amino acid sequence of NC3 domain in collagen IX: DGDPLCPNACCPP (α1), QGLEGSAFLCPTNC (α2), and PEGATDLQCPSCPP (α3). None of the peptides showed any direct binding to α1A or had any inhibitory effect at 0.5 mg/ml in the binding of α1A to collagen IX or collagen I (data not shown), confirming the fact that NC3 domain does not participate in the integrin binding. α1A Domain Binds to Collagen IX with a Novel Mechanism—Based on the crystal structure of collagen I mimicking the triple helical GFOGER peptide in complex with α1A domain the amino acids Asp-219 and His-258 are known to interact with the GFOGER motif. One strand in GFOGER peptide forms a salt bridge with Asp-219 and hydrogen bond with His-258 (48). Here both amino acids were mutated (Asp-219 to Arg and His-258 to Val), and the consequences of the mutations were studied by binding assays (Fig. 7). Both mutations decreased the binding to collagen I, and typically D219R-α1 had a lower avidity to collagen IX than H258V-α1 (Fig. 7A). Binding of D219R-α1 and H258V-α1 to collagen IV followed the pattern seen in collagen I binding except that typically D219R-α1 bound better than H258V-α1 (Fig. 7B). In accordance with the idea that in collagen I GFOGER represents the major binding site for α1A domain, both mutations had similar effects on the
ability of α2I domain to recognize GFOGER and other related peptides (Fig. 7C). When the binding of the mutant α2I domains to collagen IX was tested, mutation D219R had no effect on the binding, whereas mutation H258V caused a remarkable decrease in the affinity to collagen IX (Fig. 7D). The data indicated that binding of α2I domain to collagen IX represents a novel mechanism of collagen recognition by integrins.

Integrin α2I Domain Binds to the Same Site in Recombinant Collagen IX as α1I Domain in a Mechanism Dependent on Residue Arg-218—Rotary shadowing electron microscopy with α2I-collagen IX complex indicated that there is only one binding site for α2I in collagen IX, and it is located in the COL3 domain close to the NC3 domain (Fig. 8). The α1 binding site was suggested to be the same as for α2I. The binding mechanism of α2I domain to collagen I is considered to be similar to α1I domain (49). However, collagen IV is a much better ligand for α1/β1 integrin than collagen I (20, 27). Residue Arg-218 in α2I domain (the corresponding residue in α1I is Asp-219) has been shown to be an important amino acid for its selective binding to collagenous ligands (28). We have shown previously that integrin α2I domain typically prefers network-forming collagen IV, but mutation R218D leads to an α2I domain that prefers collagen I and binds to collagen IV poorly (28). The binding of R218D-α2I was studied on collagens I, IV, and IX (Fig. 8). The binding of R218D-α2I to collagen I was not affected at all or very little depending on the protein preparation, but the binding to collagens IV and IX was almost diminished to non-existence. In addition to Arg-218 in α2I being important for collagen IV binding, the residue also seems to be important for the recognition of collagen IX.

**DISCUSSION**

FACITs are supposed to mediate the interaction of collagen fibrils with other matrix components. Their location on the surface of the fibrils also makes them putative ligands for cell adhesion receptors, but very little is known about their ability to interact with cells. Here we have revealed the cellular receptors of collagen IX, the best characterized FACIT. Collagen receptor integrins were obvious candidates for receptors that could interact with fibrils via FACITs. However, these integrins cannot bind the collagenous triple helix as such (50), but they need specific recognition sequences inside the triple helix (45). Furthermore the four αI domain-containing collagen binding integrins have differences in their ability to bind to different collagen subtypes despite the fact that they are structurally quite similar. The best characterized difference between α1/β1...
and $\alpha_2\beta_1$ is that $\alpha_1\beta_1$ prefers basement membrane collagen IV over fibril-forming collagens, whereas the preference of $\alpha_2\beta_1$ integrin is the reverse (20, 27). In addition, $\alpha_2\beta_1$ is a receptor for beaded filaments forming collagen VI (51) and transmembrane collagen XIII (27). These phenomena can also be seen when the corresponding $\alpha_1$ domains are analyzed (27, 28). The collagen binding pattern of $\alpha_{10}I$ domain is similar to $\alpha_{11}I$ domain, but differences between the different collagen subtypes are smaller (28). The ligand binding pattern of $\alpha_{11}I$ domain resembles that of the $\alpha_{12}I$ domain (29). All collagen subtypes may not be ligands for the collagen receptor integrins since the largest collagenous domain (COL15) of transmembrane collagen XVII is not recognized by them (52).

The tissue distribution of the collagen receptors has been analyzed in adult and fetal human and mouse tissues by immunostaining and in situ hybridization (18, 36, 53, 54). Integrins $\alpha_{1}I$, $\alpha_{2}I$, and $\alpha_{10}I$, have been reported to be located in cartilage, and $\alpha_{11}I$ is expressed at the site of cartilage development, although highest expression levels are usually seen in mesenchymal cells around the cartilage (18). Here we wanted to analyze the expression pattern of all four integrins at the same time in the same samples of human fetal tissues. Our results indicate that in fetal cartilage the four receptors and in developing eye at least $\alpha_{2}I$, $\alpha_{2}I$, and $\alpha_{11}I$ integrins are co-expressed with collagen IX at mRNA level. Despite their wide distribution the collagen receptor integrins may have evolved later in the evolution than the RGD-binding and laminin receptor integrins (55). It is an interesting possibility that the generation of this subgroup of integrins is related to the evolution of cartilage and bone. Indeed cartilage has several unique collagen subtypes, also suggesting the importance of chondrocyte collagen receptors. The development of bone and cartilage is normal in both $\alpha_{1}$ and $\alpha_{2}$-deficient mice (56–58), but it has been recently shown that the proliferation of mesenchymal stem cells is affected in the $\alpha_{1}$-deficient mouse (59, 60).

We collected several lines of evidence that $\alpha_{1}I$, $\alpha_{2}I$, $\alpha_{10}I$, and $\alpha_{11}I$ can bind to collagen IX. We assayed the ability of cartilage-derived cells and transfected CHO cells to attach and spread on collagen IX. Furthermore we produced recombinant $\alpha_{1}I$ domains and analyzed them in solid-phase binding assays. The fact that all four collagen receptors could bind to collagen IX was not surprising. However, collagen IX turned out to be an exceptional ligand for the collagen receptors because it was among the best ligands for every $\alpha_{1}I$ domain as well as for all integrins tested. We have previously used the same recombinant proteins and the same assay to test integrin binding to other collagen subtypes (28, 29). Since the affinity is dependent on the length of the construct (26) different $\alpha_{1}I$ domains should not be compared with each other, whereas their ability to bind to different collagens can be compared. The following is a list of typical approximations of $K_d$ values for collagens I, IV, and IX based on data in this study or in previous reports (28, 29): $\alpha_{1}I$ domain: collagen I, 160 nM; collagen IV, 60 nM; collagen IX, 30 nM; $\alpha_{2}I$ domain: collagen I, 20 nM; collagen IV, 140 nM; collagen

![FIG. 6. Rotary shadowing electron microscopy of $\alpha_{1}I$ domain binding to recombinant collagen IX.](image)
IX, 55 nM; α1(I) domain: collagen I, 350 nM; collagen IV, 300 nM; collagen IX, 294 nM; α1(II) domain: collagen I, 400 nM; collagen IV, 400 nM; collagen IX, 53 nM. Thus, collagen IX has evolved to mediate high affinity binding of all collagen receptor integrins to cartilaginous matrix. This observation also indicates that the FACITs can have an important function as mediators of cell adhesion to collagen fibrils.

Rotary shadowing and electron microscopy revealed only one binding site for both α1(I) domain and α1(II) domain in collagen IX. This binding site seems to be at the same location for both α1 domains, and it is located in the triple helical COL3 region very close to the NC3 domain. The COL3 domain resides in the region in collagen IX that protrudes outward from the fibril that consists of collagens II, XI, and IX. Thus, this site can be easily reached by the cell surface receptors. Interestingly this site is very near to the GAG binding site described in the NC3 domain. It is tempting to speculate that the GAG chain may disrupt the binding to the cell surface and act as negative regulator of cell adhesion. Collagen IX has a heavy GAG chain especially when expressed in chicken vitreous body (61). Holden et al. (8) have recently shown that cartilage oligomeric matrix protein can interact with collagen IX. All NC domains of collagen IX can bind one cartilage oligomeric matrix protein molecule, suggesting that also cartilage oligomeric matrix protein, when binding to NC3 domain, may compete with collagen receptors.

The best described integrin binding motif in collagen is GFOGER (44, 45), which as a helical tripeptide can interact with α1(I), α2(I), and α11(I) domains (29, 44, 45, 49). The information about α1(II) domain binding to it has not been published, but most probably GFOGER represents a general high affinity binding site for all collagen receptors. Certain variations about GFOGER, such as GLOGER or GFOGEK, can also be recognized by integrins (29, 49). GFOGER binds close to the metal ion-dependent adhesion site in the “top” portion of α2(I) domain. The binding induces a conformational change in α2(I) domain that may contribute to the subsequent integrin outside-in signaling (48). In collagen IX none of the three α(IX) chains contains a GFOGER sequence. More importantly, in the binding region there is no critical GER or GEK sequence in any of the α(IX) chains (Fig. 9). GAPGER sequence in α3(IX) chain within the proposed binding area is not functional based on GAOGER peptide binding data (Fig. 7C). Very little is known about other integrin recognition sequences in collagens, and it is quite possible that the critical residues are not dominantly organized
in one α chain as they are in GFOGER. It has been shown previously that for α1β1 integrin binding to collagen IV three important amino acids, one arginine and two aspartic acids, are located in at least two different α chains (43, 62). Sacca et al. (63) have studied this Arg, Asp, Asp motif in more detail using synthetic peptides mimicking collagen IV. A similar motif cannot be found in the region of collagen IX where we believe α1I/α2I domain binds. However, inside the proposed binding site there are plenty of arginines and aspartates available (amino acids 380–390 corresponding to the α1 chain of collagen IX, Fig. 9). Chemical inactivation of arginine side chains has demonstrated the importance of arginine residue(s) for binding.

To further study the binding mechanism of α1I and α2I domains to collagen IX we used a mutagenesis approach. Residues Asp-219 and His-258 in α2I domain are known to interact with the synthetic triple helical peptide (GFOGER) crystal structure (48), and they have been shown to be important for the binding to collagen I (26, 64). Residues Arg-218 of α1I and Asp-219 of α2I have been shown to have a significant role in ligand specificity (28). The analysis of mutated αI domains indicated that the binding of collagen IX most probably takes place close to the metal ion-dependent adhesion site in α1I and α2I domains. Thus the binding mechanism is different when compared with the collagen recognition by von Willebrand factor A3 domain (65). This domain is structurally closely related to integrin αI domains but does not have a fully formed metal ion-dependent adhesion site and is metal-independent. NMR studies revealed that collagen binds on the side of von Willebrand factor A3 domain in its hydrophobic "front" and not in the "top" portion of the domain (65).

Mutated α1I domains D219R-α1I and H258V-α1I showed significantly different behavior when binding to collagen I or collagen IX. Both mutations decreased the binding of α1I domain to collagen I as well as to GFOGER peptide. The binding to collagen IX was not affected by D219R mutation, whereas H258V mutation markedly decreased the binding.

Integrin α1I domain seems to bind to the same sites in collagen I as α1I domain, suggesting that they both recognize a GFOGER-related sequence (49, 66). Here mutation R218D in α1I domain is very important for collagen IX binding unlike the corresponding residue Asp-219 in α2I domain. At the moment there is no crystal structure for α1I domain complexed with any ligand or peptide making the mutagenesis data harder to interpret. The binding mechanism of α1I domain to collagen IX seems to be
very different when compared with its binding to collagen I, whereas the binding mechanism of α1I to collagen IV and collagen IX might have similarities, but based on the suggested binding site in collagen it cannot be identical.

Our data based on the location of the integrin binding site, sequence analysis of collagen IX α chains, and integrin mutagenesis indicate that integrin binding to collagen IX represents a novel, GE/R/K-independent mechanism. Furthermore inside the discovered binding site none of the α chains has a unique sequence that alone could explain the binding. Thus, we propose that the binding site is formed by two or three α chains together. In αI domain the site seems to be recognized by the surface of the metal ion-dependent adhesion site; however, the binding mechanism to the integrin recognition site in collagen IX is different when compared with binding to GFOGER. An important question remaining to be answered is whether αI domain binding to this novel site in collagen IX triggers a different conformational change than GFOGER.

REFERENCES
1. Olsen, B. R. (1997) Int. J. Biochem. Cell Biol. 29, 555–558
2. Eyre, D. R., Apon, S., Wu, J. J., Ericsson, L. H., and Walsh, K. A. (1987) FEBS Lett. 220, 337–341
3. van der Rest, M., and Mayne, R. (1988) J. Biol. Chem. 263, 1615–1618
4. Wu, J. J., Woods, P. E., and Eyre, D. R. (1992) J. Biol. Chem. 267, 23007–23014
5. Diab, M., Wu, J. J., and Eyre, D. (1996) Biochem. J. 314, 327–332
6. Bruckner, P., Vaughan, L., and Winterhalter, K. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 2608–2612
7. Muragaki, Y., Martiman, E. C., van Beersum, S. E., Peralia, M., van Mourik, J. B., Warman, M. L., Olsen, B. R., and Hamel, B. C. (1990) Nat. Genet. 12, 103–105
8. Holden P., Canty, E. G., Mortier, G. R., Zabel, B., Spranger, J., Carr, A., Grant, M. E., Loughlin, J. A., and Briggs, M. D. (1999) Am. J. Hum. Genet. 65, 31–38
9. Saya, E. C., Joshi, A. P., Wilcox, W. R., Briggs, M., Cohn, D. H., and Olsen, B. R. (2000) Matrix Biol. 19, 121–128
10. Paasila, P., Lohiniva, J., Annnunen, S., Bonaventure, J., Le Merrer, M., Pai, L., and Alas-Kokko, L. (1999) Am. J. Hum. Genet. 64, 1036–1044
11. Bonneman, C. G., Fox, G. F., Shapiro, F., Wu, J.-J., Feener, C. A., Thompson, T. G., Anthony, D. C., Eyre, D. R., Darras, B. T., and Kunkel, L. M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1212–1217
12. Lohiniva, J., Paasila, P., Seppanen, U., Vierimaa, O., Kivirikko, S., and Alas-Kokko, L. (2000) J. Med. Genet. 91, 216–222
13. Czerny-Rataficzak, M., Lohiniva, J., Rogala, P., Kozlowski, K., Peralia, M., Carter, L., Spector, T. D., Koledziej, L., Seppanen, U., Glazar, R., Królewski, J., Lato-Sieliwka, A., and Alas-Kokko, L. (2001) Am. J. Hum. Genet. 69, 969–980
14. Nakata, K., Ono, K., Miyazaki, J., Olsen, B. R., Muragaki, Y., Adachi, E., Yamamura, K., and Kimura, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 2870–2874
15. Fassler, R., Schneegger, P. N. J., Dausman, J., Muragaki, Y., Shinya, T., McCarthy, M. T., Olsen, B. R., and Jaenisch, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5070–5074
16. Briesewitz, R., Epstein, M. R., and Marcantonio, E. E. (1993) J. Biol. Chem. 268, 2899–2996
17. Takada, Y., and Hemler, M. E. (1989) J. Cell Biol. 109, 397–407
18. Tiger, C. F., Fougereau, F., Grundström, G., Velling, T., and Gullberg, D. (2001) Dev. Biol. 237, 116–129
19. Camper, L., Hellman, U., and Lundgren-Åkerlund, E. (1998) J. Biol. Chem. 273, 20383–20389
20. Kern, A., Ehle, J., Golub, E., and Kuhn, K. (1993) Eur. J. Biochem. 215, 151–159
21. Kern, A., Briesewitz, R., Bank, I., and Marcantonio, E. E. (1994) J. Biol. Chem. 269, 22911–22916
22. Langholz, O., Rockel, D., Mauch, C., Kozlowski, E., Bank, I., Krieg, T., and Ecker, E. (1995) J. Cell Biol. 129, 1903–1915
23. Riikonen, T., Westermark, J., Kivistö, L., Broberg, A., Kahari, V. M., and Heino, J. (1995) J. Biol. Chem. 270, 13548–13552
24. Ivaska, J., Reunanen, H., Westermark, J., Kivistö, L., Kahari, V. M., and Heino, J. (1999) J. Cell Biol. 147, 401–416
25. Ravnani, L., Heino, J., Lopez-Otin, C., and Kahari, V. M. (1999) J. Biol. Chem. 274, 2446–2455
26. Käpylä, J., Ivaska, J., Riikonen, R., Nykvist, P., Pentikainen, O., Johnson, M., and Heino, J. (2000) J. Biol. Chem. 275, 3348–3354
27. Nykvist, P., Tu, H., Ivaska, J., Käpylä, J., Pihlajaaniemi, T., and Heino, J. (2000) J. Biol. Chem. 275, 8255–8261
28. Tullia, M., Pentikainen, O. T., Vistadals, T., Käpylä, J., Impola, U., Nykvist, P., Nissinen, L., Johnson, M. S., and Heino, J. (2001) J. Biol. Chem. 276, 48206–48212
29. Zhang, W.-M., Käpylä, J., Puranen, S., Knight, C. G., Tiger, C.-F., Pentikainen, O. T., Johnson, M. S., Fardala, R. W., Heino, J., and Gullberg, D. (2003) J. Biol. Chem. 278, 7270–7277
30. Velling, T., Risteli, J., Wenneberg, K., Mosher, D. P., and Johansson, S. (2002) J. Biol. Chem. 277, 37377–37381
31. Li, S., Van Den Diepstraten, C., D’Souza, S. J., Chan, B. M., and Pickering, J. B., Warman, M. L., Olsen, B. R., and Hamel, B. C. (1996) Int. J. Biochem. Cell Biol. 28, 969–980
32. Takada, Y., and Hemler, M. E. (1989) J. Cell Biol. 109, 397–407
33. Tiger, C. F., Fougereau, F., Grundström, G., Velling, T., and Gullberg, D. (2001) Dev. Biol. 237, 116–129
34. Camper, L., Hellman, U., and Lundgren-Åkerlund, E. (1998) J. Biol. Chem. 273, 20383–20389
35. Kern, A., Ehle, J., Golub, E., and Kuhn, K. (1993) Eur. J. Biochem. 215, 151–159
36. Kern, A., Briesewitz, R., Bank, I., and Marcantonio, E. E. (1994) J. Biol. Chem. 269, 22911–22916
