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

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Collagen IX is the prototype fibril-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 $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$, 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 $\alpha_1\beta_1$, $\alpha_2\beta_1$, or $\alpha_{10}\beta_1$ 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 α_1I- and α_2I-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 α_2I 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 α_2I 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 IX 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.

Collagen IX was the first member to be discovered of a subgroup of collagens, now known as the fibril-associated collagens with interrupted triple helix (FACITs)[1] (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 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, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$ 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 α_1I and α_{10}I 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 α_2I and α_{11}I domains (20, 21, 26–29). The recognition of FACITs by integrins has not been reported. Integrin-mediated cell adhe-

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[1] The abbreviations used are: FACITs, fibril-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 collagens 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 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 FACITs as cell adhesion proteins. Our results indicate that α_1I and α_2I domains recognize only one site in collagen IX that is in COL3 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 KHOS-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 initiated 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 IU/ml penicillin-G, and 100 μg/ml streptomycin.

Generation of Stable Integrin-expressing CHO Cell Line—CHO-$\alpha_1\beta_1$ and CHO-$\alpha_2\beta_1$ cell lines were created as described previously (27). Full-length α_{10} cDNA corresponding to nucleotides 19–3525 of the published sequence (19) (GenBank™ 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, correspondingly. 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 cell clones were selected with a neomycin analog G418 (400 μg/ml, Invitrogen) for 3 weeks. Clones were analyzed for the expression of the α_{10} integrin with RT-PCR using the Gene Amp PCR kit (PerkinElmer Life Sciences) and immunoprecipitation.

Immunoprecipitation—Polyclonal rabbit antisera against human β_1 (34), α_2 (35), α_{10} (a kind gift from Dr. Evy Lundgren-Åkerlund, Lund, Sweden) (36), and α_{11} (a kind gift from Dr. Donald Gullberg, Uppsala, Sweden) (37) integrin subunits were used in immunoprecipitation assays. Cell cultures were metabolically labeled with 50 μCi/ml [^{35}S]methionine (Tran^{35}S-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 $CaCl_2$, 1 mM $MgCl_2$, 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. Insoluble 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 immunoprecipitated with anti-integrin antibodies for 12 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 M NaCl and 25 mM Tris-HCl (pH 7.4). The immunoprecipitates were analyzed by electrophoresis 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: α_{10}: sense (s) 5′-CAGGGATCCCCAACATACATGGATGTTGTC-3′ and antisense (as) 5′-GGCTGAATTCCCCTTCAAGGCCAAAAATCCG-3′; α_{11}: s 5′-CAGACCTACATGGACATCG-3′ and as 5′-CATCTCCAGCCCAAAGGAG-3′; α_1: s 5′-CACAGGGATCCGTCAGCCCCACATTT-3′ and as 5′-GTGGCTGTCGACAGCTGTGGCTTCCAG-3′; α_2: s 5′-CACAGGGATCCCCTGATTTTCAGCTC-3′ and as 5′-GTGGCTGAATTCAACAGTACCTTCAATG-3′. COL9A1 was amplified with the following primers: s 5′-ACAGCAGGACTCCCTGGA-3′ and as 5′-TGATCACCAGGTGCACCAG-3′. Glyceraldehyde-3-phosphate dehydrogenase (a housekeeping gene used as a control) (39) was amplified with the following primers: s 5′-CCCATGGCAAATTCCATGGCA-3′ and as 5′-TCTAGACGGCAGGTCAGGTC-3′. Forty cycles of PCR amplification were done in 2 mM $MgCl_2$ using the following protocol for α_{10} and glyceraldehyde-3-phosphate dehydrogenase: denaturation for 1 min at 94 °C, annealing for 1 min at 67 °C, and extension for 2 min at 72 °C; for α_{11}: denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, and extension for 2 min at 72 °C; for α_1 and α_2 denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 2 min at 72 °C; and for COL9A1: denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C, and extension for 2 min at 72 °C. The products generated were subjected to electrophoresis on a 1.5% agarose gel and were visualized by ethidium bromide staining.

Cloning of Human Integrin αI Domains—cDNAs encoding α_1I and α_2I domains were generated by PCR as described earlier (26, 28) using human integrin α_1 and α_2 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 α_1I and α_2I domains, respectively. The α_{10}I domain cDNA was generated by RT-PCR from RNA isolated from KHOS-240 cells (human Caucasian osteosarcoma). Total cellular RNA was isolated by using the RNeasy 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 α_{10}I 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 α_{10}I cDNA was ligated with the SureClone ligation kit (Amersham Biosciences). The construct was transformed into the *Escherichia coli* BL21 strain for production. The DNA construct was sequenced and compared with the published α_{10} DNA sequence (19). Human integrin α_{11} cDNA (37) was used as a template when α_{11}I domain was generated by PCR. The PCR product, having BamHI and EcoRI sites, was cloned into pGEX-KT, and the DNA sequence was checked by sequencing the whole 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/pαI and the cultures were grown at 37 °C until the A_{600} of the suspension reached 0.6–1.0. Cells were induced with isopropyl 1-thio-β-D-galactopyranoside and allowed to grow for an additional 4–6 h typically at room temperature before harvesting by centrifugation. Pelleted cells were resuspended in PBS (pH 7.4) and then lysed by sonication followed by addition of Triton X-100 to a final concentration of 2%. After incubation for 30 min on ice, suspensions were centrifuged, and supernatants were pooled. Glutathione-Sepharose 4B (Amersham Biosciences) was added to the lysate, which was incubated at room temperature for 30 min with gentle agitation. The lysate was then centrifuged, the supernatant was removed, and glutathione-Sepharose 4B with bound fusion protein was transferred into disposable chromatography columns (Bio-Rad). The columns were washed with PBS, and fusion proteins were eluted using 30 mM reduced glutathione.

Purified recombinant and glutathione-tagged αI domains were analyzed by SDS and native PAGE. Protein concentrations were measured with the Bradford method (40). The recombinant α_1I domain produced was 227 amino acids in length corresponding to amino acids 123–338 of the whole α_1 integrin, while the α_2I domain was 223 amino acids long corresponding to amino acids 124–339 of the whole α_2 integrin. The carboxyl termini of the α_1I and α_2I domains contained 10 and six non-integrin amino acids, respectively (26, 28). The recombinant α_{10}I domain produced was 197 amino acids in length corresponding to amino acids 141–337 of the whole α_{10} integrin. The amino terminus contained two non-integrin residues, and the carboxyl terminus of α_{10}I contained six non-integrin amino acids (28). The recombinant α_{11}I domain contained 204 amino acids in total corresponding to residues 159–354: in the amino terminus there were two extra residues before α_{11}I, and in the carboxyl terminus there were six

extra amino acids. Recombinant α_{11}I domain contains some GST as an impurity due to the endogenous protease activity during expression and purification (29). Recombinant αI domains were used as GST fusion proteins for collagen binding experiments.

Site-directed Mutagenesis—Site-directed mutation of the αI domain cDNA in a pGEX-2T or pGEX-4T-3 vector was carried out using PCR according to Stratagene's QuikChange 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 collagens were purchased from Sigma. Collagens II (bovine), III (human), IV (human), and V (human) were obtained from Chemicon. Collagen VI (human) was purchased from Biodesign International.

Recombinant human collagen IX was produced as described previously (41). Briefly *Trichoplusia ni* insect cells (High Five, Invitrogen) grown in suspension at 27 °C were seeded at 1.0–1.5 × 10^6 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 α1(IX), α2(IX), and α3(IX) chains and a double promoter virus 4PH$\alpha\beta$ 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 μg/ml) 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 proteinase 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 M NaCl). Purified collagen IX was analyzed by SDS-PAGE and subjected to amino acid analysis in an Applied Biosystems 421 analyzer. Concentrated (Amicon Ultra 30,000 molecular weight cut-off, Millipore) collagen IX was stored in 50 mM acetic acid at −20 °C. In some experiments collagen IX was chemically modified to inactivate side chains in arginine residues as described in Ref. 43. Instead of using *p*-azidophenylglyoxal we used phenylglyoxal hydrate (Sigma).

Cell Spreading Experiment—Experiments were performed essentially as described previously (27). Microtiter plate wells were coated (16.4 μg/ml in PBS) with rat collagen I; bovine collagen II; human collagens III, IV, V, and VI; and recombinant human collagen IX overnight. When collagen I, II, III, or V was coated on the microtiter plates the conditions were set so that collagens were bound in monomeric, triple helical form. Cells (10,000 cells/well) were allowed to attach and spread for 60 min and fixed. BSA was used as background control. The total number of attached and spread cells was counted. The data are mean ± S.D. of three parallel measurements.

Solid-phase Binding Assay for αI Domains—The coating of a 96-well high binding microtiter plate (Nunc) was done by exposure to 0.1 ml of PBS containing 5 μg/cm^2 (16.4 μg/ml) collagens or 20 μg/ml triple helical peptides overnight at +4 °C. When collagen I, II, III, or V was coated on the microtiter plates the conditions were set so that collagens were bound in monomeric, triple helical form. Blank wells were coated with 1:1 solution of 0.1 ml of Delfia® Diluent II (Wallac) and PBS. Residual protein absorption sites on all wells were blocked with a 1:1 solution of 0.1 ml of Delfia Diluent II (Wallac) and PBS. Recombinant proteins (αI-GST) were added to the coated wells at a desired concentration in Delfia Assay Buffer and incubated for 1 h at room temperature. Europium-labeled anti-GST antibody (Wallac) was then added (typically 1:1000), and the mixtures were incubated for 1 h at room temperature. All incubations mentioned above were done in the presence of 2 mM $MgCl_2$. Delfia enhancement solution (Wallac) was added to each well, and the europium signal was measured by time-resolved fluorometry (Victor2 multilabel counter, Wallac). At least three parallel wells were analyzed. In some cases a somewhat modified solid-phase assay was used (28). It uses anti-GST and europium-labeled protein G instead of europium-labeled anti-GST antibody.

Three linear peptides based on the amino acid sequence of NC3 domain in collagen IX, DGDPLCPNACPP (α1), QGLEGSADFLCPTNC (α2), and PEGATDLQCPSICPP (α3), were ordered from Sigma, and both direct binding to α_2I domain and inhibition assays were performed. The inhibition assay protocol is described above, and peptides were added with α_2I domain at 0.5 mg/ml. The direct binding assay was tested using amino binding plates (Costar DNA-Bind, Corning Inc.). Wells were coated with peptides at 1 mg/ml for 1 h at room temperature in PBS, pH 8.5. Then wells were blocked with a 1:1 solution of 0.1 ml of Delfia Diluent II (Wallac) and PBS, pH 8.5. After this assay, the solid-phase binding assay for αI domains was performed as described above.

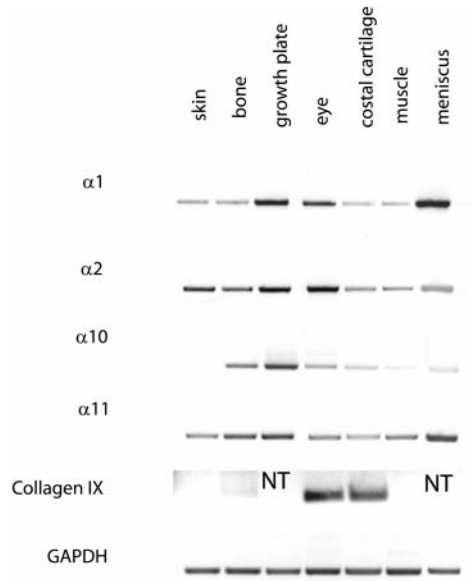

FIG. 1. **The expression of mRNA for collagen IX and integrin subunits α_1, α_2, α_{10}, and α_{11} in human fetal tissues.** RT-PCR was performed with 1 μg of total RNA/reaction with specific primers for α_1, α_2, α_{10}, α_{11}, collagen IX (α1 chain), and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*). Glyceraldehyde-3-phosphate dehydrogenase was used as a positive control mRNA. Osteosarcoma cell line KHOS-240 was a positive control for integrin mRNAs. *NT*, not tested.

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 $MgCl_2$ 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 as previously described (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 $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$ 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, α_1, α_2, α_{10}, and α_{11}, 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 α_{10} was expressed in epiphyseal cartilages of knee and shoulder joints of 24-week-old human fetus (Fig. 1). The highest level of α_{10} mRNA was observed in growth plate of 20-week-old human fetus. The mRNAs for α_{10} were not seen in meniscus where other collagen-binding integrin subunits α_1, α_2, and α_{11} were expressed. α_{10} mRNA was not detected in skin or muscle, but it was present in eye. Integrin α_{11} 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 α_{11}, unlike α_{10} mRNA, was detected in skin. Integrin α_1 was expressed at mRNA level in skin, growth plate, and whole eye. The mRNA levels for integrin α_1 and α_2 subunits were relatively low in epiphyseal cartilage tissues except in

meniscus. Integrin α_2 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 $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$ in Cultured Human Primary Fetal Chondrocytes—RT-PCR was performed to study the expression of α_{10} and α_{11} subunits in human chondrosarcoma cells (HTB-99) and human primary fetal chondrocytes. Integrins α_1, α_2, α_{10}, and α_{11} were expressed at mRNA level in both cell types (Fig. 2*A*). To confirm the presence of the corresponding proteins we performed metabolic labeling experiments and immunoprecipitations with antisera against integrin β_1, α_2, and α_{11} subunits. Antiserum against β_1 subunit precipitated a typical pattern of protein bands (Fig. 2*B*) previously identified as precursor β_1 (about 100 kDa), mature β_1 (about 110 kDa), multiple β_1-associated α subunits (about 140 kDa), and β_1-associated α_1 subunit (about 190 kDa) (34). The presence of α_2 and α_{11} subunits was confirmed by specific antisera. Integrin α_{11} subunit migrated consistently slower than α_2 suggesting that it might be more glycosylated (Fig. 2*B*). Our results suggest that all four collagen receptor integrins may be simultaneously expressed in a single cartilage-derived cell.

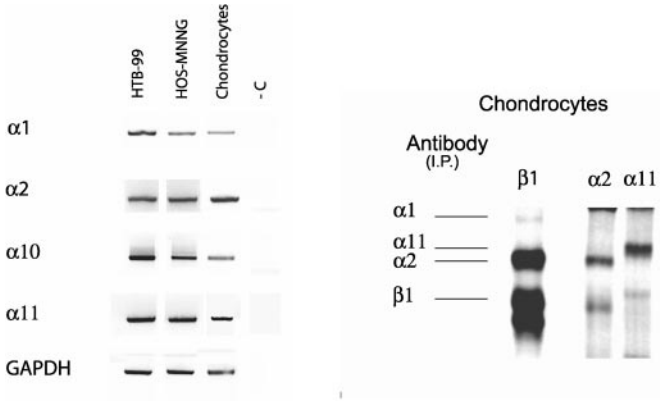

FIG. 2. **The expression of integrins α_1, α_2, α_{10}, and α_{11} in cultured human fetal chondrocytes, human chondrosarcoma cells (HTB-99), and human osteosarcoma cells (HOS-MNNG).** *A*, total RNA was isolated from cells, and RT-PCR was performed with 1 μg of total RNA/reaction with specific primers for integrins α_1, α_2, α_{10}, α_{11}, and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*). Glyceraldehyde-3-phosphate dehydrogenase was used as a positive control. *B*, the human chondrocytes were incubated for 18 h in the presence of [^{35}S]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.

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. 3*A*). 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 $\alpha_1\beta_1$ (CHO-$\alpha_1\beta_1$) or $\alpha_2\beta_1$ (CHO-$\alpha_2\beta_1$) as their only collagen receptor (27). Here their ability to spread on collagen IX was tested. CHO-$\alpha_1\beta_1$ cells spread very fast on collagen IV as well as on collagen IX, whereas their spreading on collagen I was remarkably slower (Fig. 3*B*). CHO-$\alpha_2\beta_1$ 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. 3*B*). Since the expression of $\alpha_{10}\beta_1$ integrin is more restricted to cartilage than the expression of any other collagen receptor, it was important to create CHO cells expressing $\alpha_{10}\beta_1$ integrin. Fig. 4*A* shows RT-PCR data from different $\alpha_{10}\beta_1$ clones indicating the presence of α_{10} subunit mRNA in transfected CHO cells, and Fig. 4*B* shows immunoprecipitation of $\alpha_{10}\beta_1$ in the same cells. Since no previous data have been available about the ligand binding of cells that express $\alpha_{10}\beta_1$ as their only collagen receptor we tested a larger set of collagen subtypes (collagens I, II, III, IV, V, VI, and IX). CHO-$\alpha_{10}\beta_1$ cells spread fast on all collagens. The fastest spreading was observed on collagens IV and VI (Fig. 4*C*). The results indicated that $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_{10}\beta_1$ integrins are receptors for collagen IX. Importantly for every integrin collagen IX was among the best collagenous ligands.

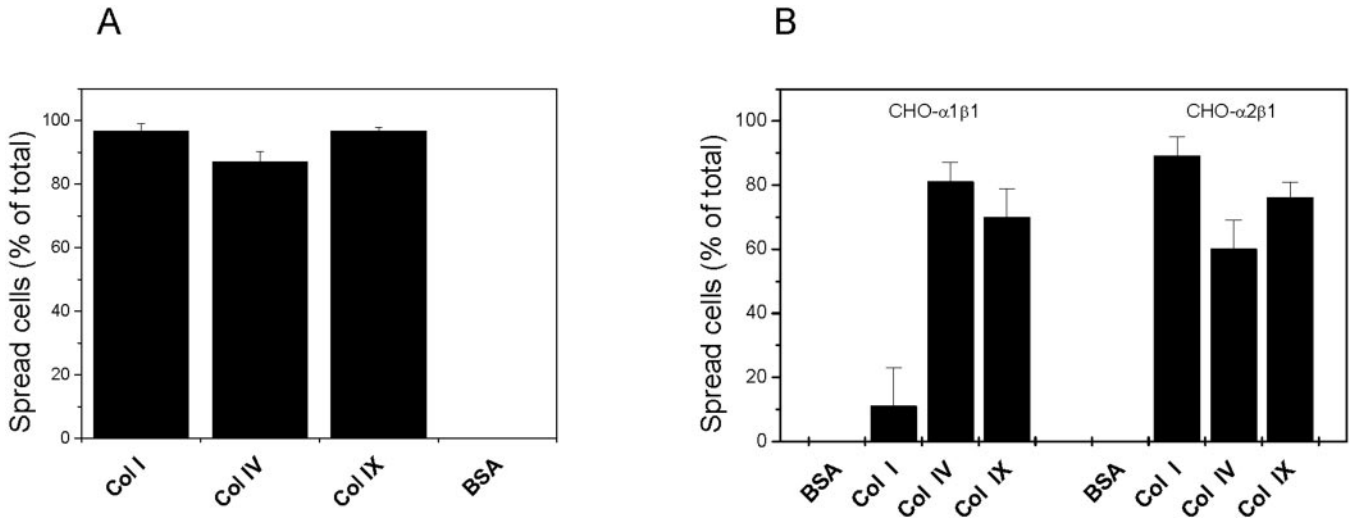

FIG. 3. **CHO-$\alpha_1\beta_1$, CHO-$\alpha_2\beta_1$, and HTB-99 cell spreading on collagens.** *A*, HTB-99 chondrosarcoma cells. *B*, CHO cells expressing $\alpha_1\beta_1$ or $\alpha_2\beta_1$. Microtiter plate wells were coated with rat collagen I, mouse collagen IV, and recombinant human collagen IX overnight. Cells (10,000 cells/well) were allowed to attach and spread for 60 min and fixed. BSA was used as background control. The total number of attached and spread cells was counted. The data are mean ± S.D. of three parallel measurements. *Col*, collagen.

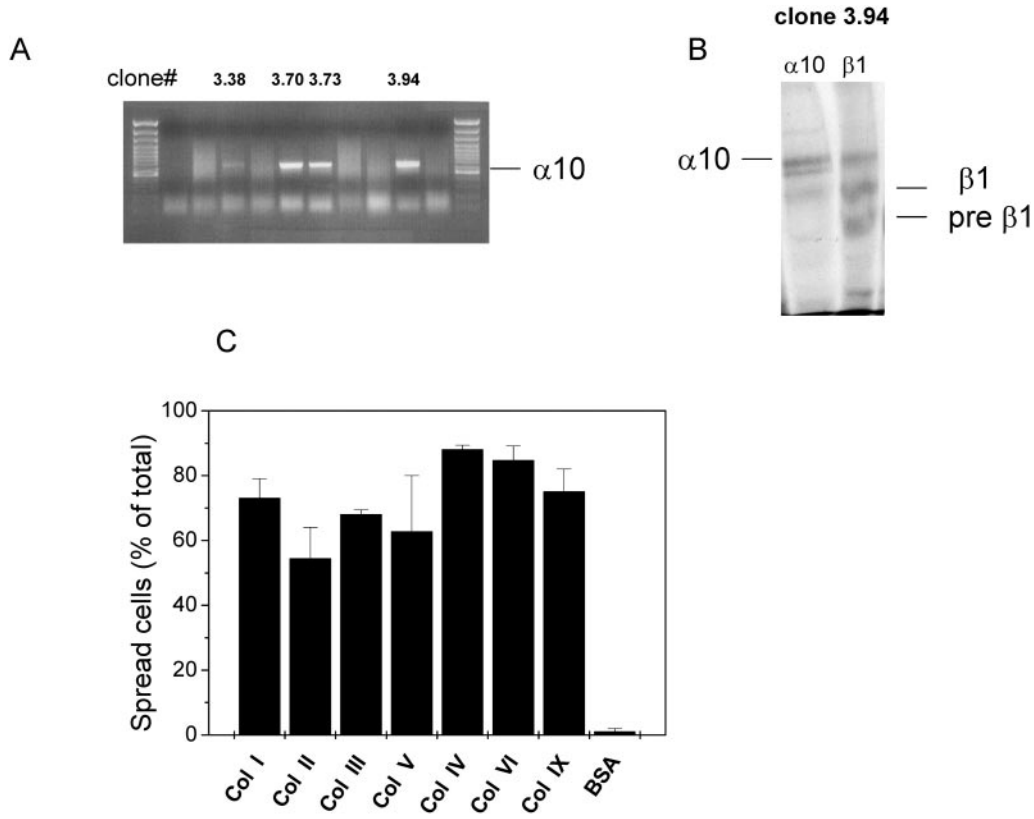

FIG. 4. **CHO-$\alpha_{10}\beta_1$ cell spreading on collagens.** *A*, RT-PCR indicating the expression of α_{10} mRNA in transfected CHO cell clones. Positive clones are marked. Total RNA was extracted from cell clones, and RT-PCR was performed with specific primers for α_{10}. *B*, immunoprecipitation indicating the expression of $\alpha_{10}\beta_1$ integrin in a transfected cell clone. CHO-$\alpha_{10}\beta_1$ cells were incubated for 18 h in the presence of [^{35}S]methionine. Cellular integrins were precipitated with specific antibodies against integrin α_{10} and β_1 subunits and analyzed by electrophoresis and fluorography. *C*, spreading of CHO-$\alpha_{10}\beta_1$ cells on collagens. Microtiter plate wells were coated with rat collagen I; bovine collagen II; human collagens III, IV, V, and VI; and recombinant human collagen IX overnight. Cells (10,000 cells/well) were allowed to attach and spread for 60 min and fixed. BSA was used as background control. The total number of attached and spread cells was counted. The data are mean ± S.D. of three parallel measurements except for collagen IV, which is done only in duplicate. *Col*, collagen.

Recombinant αI Domains of all Four Collagen Receptor Integrins Bind to Collagen IX—Recombinant αI-GST (marked as αI) fusion proteins were used to investigate the binding mechanism of collagen receptor integrins to collagen IX. Four collagen receptor αI domains were tested in a solid-phase binding assay (Fig. 5). α_1I, α_2I, α_{10}I, and α_{11}I domains bound tightly to collagen IX. Binding of all αI 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_d values obtained were 30 ± 5 nM for α_1I, 55 ± 13 nM for α_2I (Fig. 5*A*), 294 ± 25 nM for α_{10}I (Fig. 5*B*), and 53 ± 5 nM for α_{11}I (Fig. 5*C*). Our previous reports (28, 29) contain approximated K_d values for αI domain binding to other collagen subtypes measured by a similar assay. Based on these results it is obvious that for every αI domain collagen IX is among the best collagenous ligands and that makes collagen IX unique when compared with all collagen subtypes tested so far.

Integrin α_2I Domain Has One Binding Site in Collagen IX in COL3 Domain—The present knowledge about αI domain binding to collagen is based on the detailed structural analysis of α_2I domain binding to GFOGER motif in collagen I (48). Therefore α_2I was selected for further analysis of collagen IX recognition by integrins. First, rotary shadowing electron microscopy was used to image the α_2I-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 α_2I domain (data not shown). Similar treatment has been described to inhibit $\alpha_1\beta_1$ binding to collagen IV (43).

We also tested three linear peptides based on the amino acid sequence of NC3 domain in collagen IX: DGDPLCPNACPP (α1), QGLEGSADFLCPTNC (α2), and PEGATDLQCPSICPP (α3). None of the peptides showed any direct binding to α_2I or had any inhibitory effect at 0.5 mg/ml in the binding of α_2I to collagen IX or collagen I (data not shown), confirming the fact that NC3 domain does not participate in the integrin binding.

α_2I 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 α_2I 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-α_2I had a lower avidity to collagen I than H258V-α_2I (Fig. 7*A*). Binding of D219R-α_2I and H258V-α_2I to collagen IV followed the pattern seen in collagen I binding except that typically D219R-α_2I bound better than H258V-α_2I (Fig. 7*B*). In accordance with the idea that in collagen I GFOGER represents the major binding site for α_2I domain, both mutations had similar effects on the

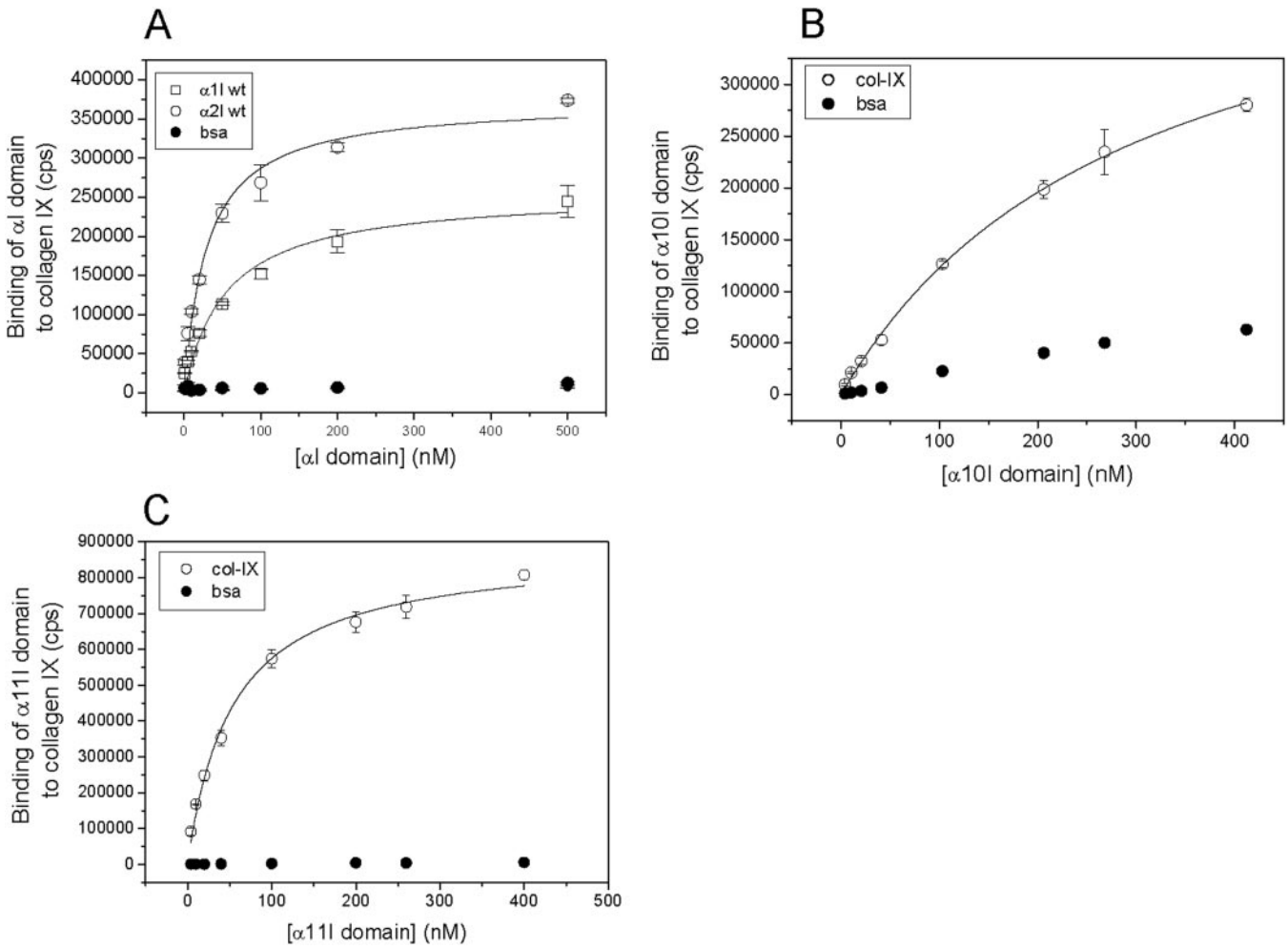

FIG. 5. **The binding of recombinant αI domains to collagen IX.** *A*, binding of α_1I (*squares*) and α_2I (*open circles*) domains. *B* and *C*, binding of α_{10}I domain and α_{11}I domain, respectively. Collagen IX was coated on the microtiter plate wells in PBS overnight (16.4 μg/ml). BSA (*closed circles*) coating was used as a negative control. Wells were blocked with 100 μl of 1:1 PBS + dilution II solution (Wallac, 7.5% BSA) at 37 °C for 1 h. Blocking solution was removed, and I domain was added with assay buffer (Wallac) + 2 mM $MgCl_2$ and incubated at 37 °C for 1 h. Wells were washed twice with 200 μl of PBS + 2 mM $MgCl_2$. 100 μl of anti-GST (1:8000) was added into each well for 1 h at 37 °C. Cells were washed twice with 200 μl of PBS + 2 mM $MgCl_2$. 100 μl of europium-labeled protein G was added into each well and incubated for 1 h at 37 °C. Cells were washed three times with 200 μl of PBS + 2 mM $MgCl_2$, 100 μl of enhancement solution (Wallac) was added into each well, and fluorescence was measured with the Wallac Victor 2. *col*, collagen; *wt*, wild type; *cps*, counts/s.

ability of α_2I domain to recognize GFOGER and other related peptides (Fig. 7*C*). When the binding of the mutant α_2I 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. 7*D*). The data indicated that binding of α_2I domain to collagen IX represents a novel mechanism of collagen recognition by integrins.

Integrin α_1I Domain Binds to the Same Site in Recombinant Collagen IX as α_2I Domain in a Mechanism Dependent on Residue Arg-218—Rotary shadowing electron microscopy with α_1I-collagen IX complex indicated that there is only one binding site for α_1I in collagen IX, and it is located in the COL3 domain close to the NC3 domain (Fig. 8). The α_1I binding site was suggested to be the same as for α_2I. The binding mechanism of α_1I domain to collagen I is considered to be similar to α_2I domain (49). However, collagen IV is a much better ligand for $\alpha_1\beta_1$ integrin than collagen I (20, 27). Residue Arg-218 in α_1I domain (the corresponding residue in α_2I 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 α_1I domain typically prefers network-forming collagen IV, but mutation R218D leads to an α_1I domain that prefers collagen I and binds to collagen IV poorly (28). The binding of R218D-α_1I was studied on collagens I, IV, and IX (Fig. 8). The binding of R218D-α_1I 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 nonexistence. In addition to Arg-218 in α_1I 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 $\alpha_1\beta_1$

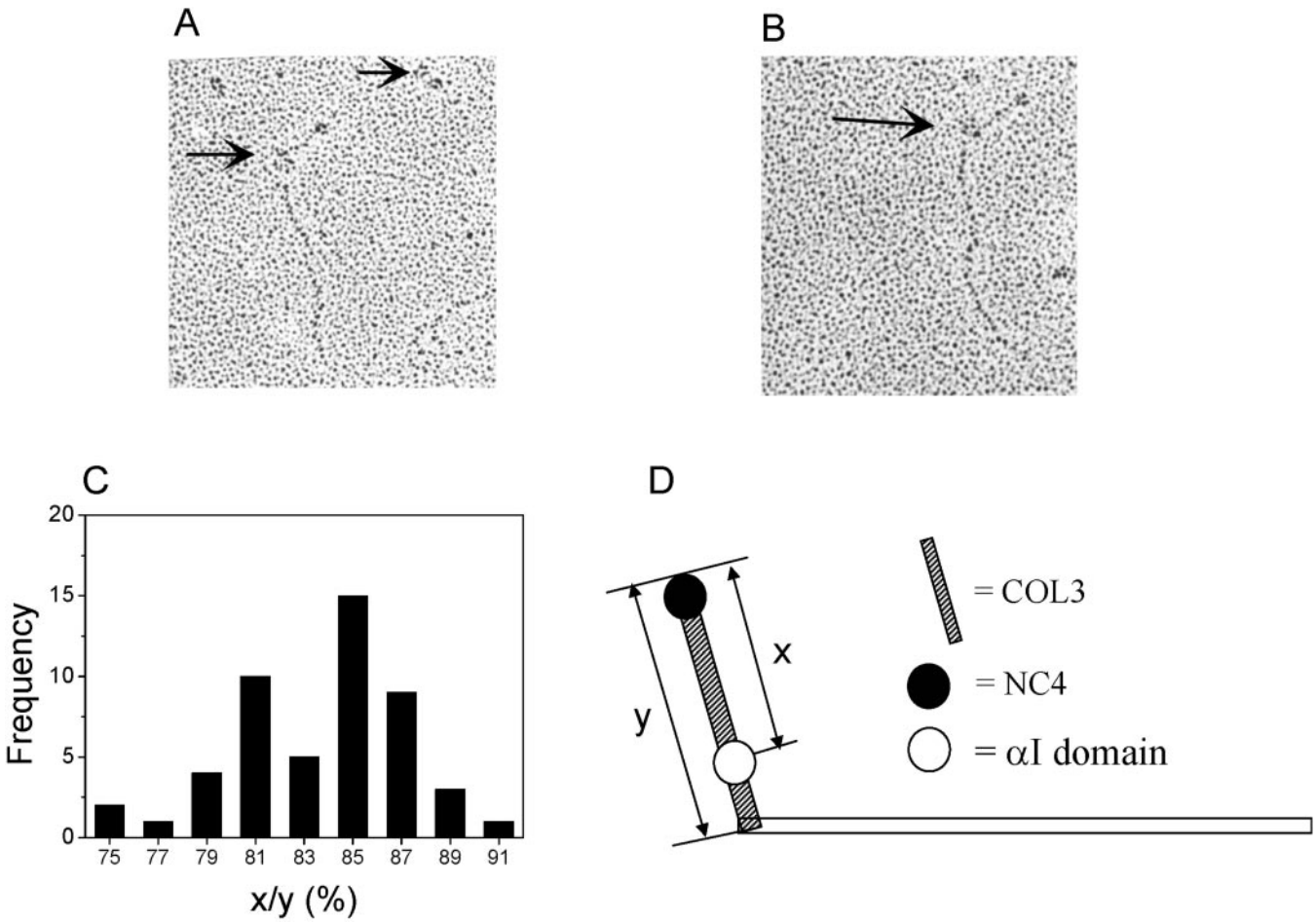

FIG. 6. **Rotary shadowing electron microscopy of α_2I domain binding to recombinant collagen IX.** *A* and *B*, rotary shadowing electron microscopy images of α_2I domain-collagen IX complex. NC4 domain is seen as a bead at the one end of collagen IX; also a kink, typical of collagen IX, is seen in the same region as the bound α_2I. The *arrows* point to collagen-bound and -free α_2I domains. *C*, frequency of the binding events as a function of distance between bound α_2I and the NC4 domain relative to the distance between the kink and NC4 domain (see *D*). *D*, schematic representation of the distance measurements from electron microscopy images.

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, $\alpha_1\beta_1$ is a receptor for beaded filaments forming collagen VI (51) and transmembrane collagen XIII (27). These phenomena can also be seen when the corresponding αI domains are analyzed (27, 28). The collagen binding pattern of α_{10}I domain is similar to α_1I domain, but differences between the different collagen subtypes are smaller (28). The ligand binding pattern of α_{11}I domain resembles that of the α_2I 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 $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_{10}\beta_1$ have been reported to be located in cartilage, and $\alpha_{11}\beta_1$ 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 $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_{11}\beta_1$ 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 α_1- and α_2-deficient mice (56–58), but it has been recently shown that the proliferation of mesenchymal stem cells is affected in the α_1-deficient mouse (59, 60).

We collected several lines of evidence that $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$ 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 αI 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 αI 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 αI 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): α_1I domain: collagen I, 160 nM; collagen IV, 60 nM; collagen IX, 30 nM; α_2I domain: collagen I, 20 nM; collagen IV, 140 nM; collagen

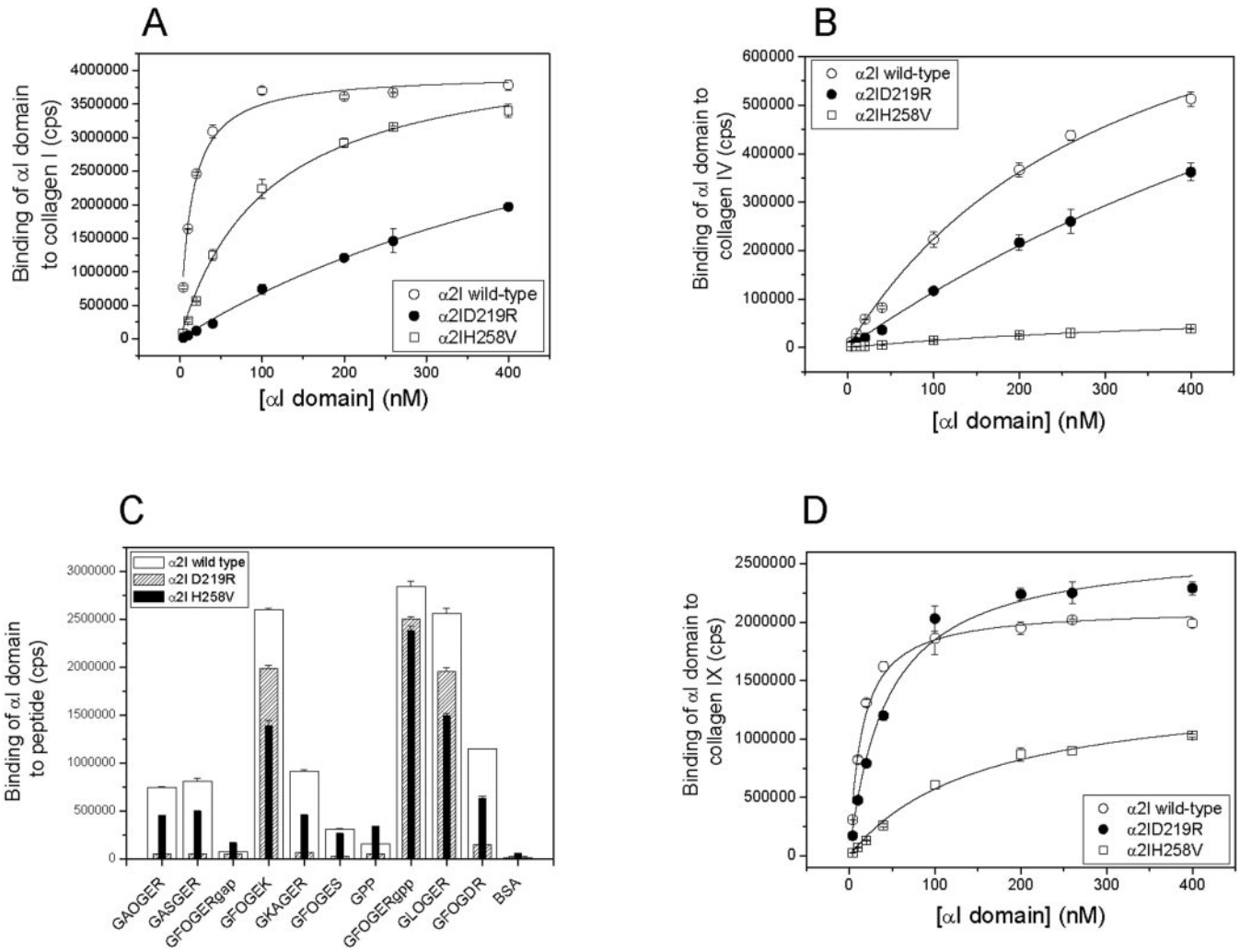

FIG. 7. **The effect of α_2I domain mutations on binding to recombinant collagen IX.** α_2I wild type (*open circles*), D219R-α_2I (*closed circles*), and H258V-α_2I (*squares*) binding to collagen I (*A*), collagen IV (*B*), GFOGER and related peptides (*C*), and collagen IX (*D*) is shown. Microtiter plate wells were coated with collagens (16.4 μg/ml) or peptides (20 μg/ml) in PBS overnight. BSA coating was used as a negative control, but data are not shown for clarity. Wells were blocked with 100 μl of 1:1 PBS + dilution II solution (Wallac, 7.5% BSA) at room temperature for 1 h. Blocking solution was removed, and I domain was added with assay buffer (Wallac) + 2 mM $MgCl_2$ and incubated at room temperature for 1 h. Wells were washed three times with PBS + 2 mM $MgCl_2$. 100 μl of europium-labeled anti-GST (1:1000) was added into each well for 1 h at room temperature. Cells were washed three times with PBS + 2 mM $MgCl_2$, 100 μl of enhancement solution (Wallac) was added into each well, and fluorescence was measured with the Wallac Victor 2. *cps*, counts.

IX, 55 nM; α_{10}I domain: collagen I. 350 nM; collagen IV, 300 nM; collagen IX, 294 nM; α_{11}I 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 α_1I domain and α_2I domain in collagen IX. This binding site seems to be at the same location for both αI 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 α_1I, α_2I, and α_{11}I domains (29, 44, 45, 49). The information about α_{10}I 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 α_2I domain. The binding induces a conformational change in αI 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 chains nearby (Fig. 9). GAPGER sequence in α3(IX) chain within the proposed binding area is not functional based on GAOGER peptide binding data (Fig. 7*C*). Very little is known about other integrin recognition sequences in collagens, and it is quite possible that the critical residues are not dominantly organized

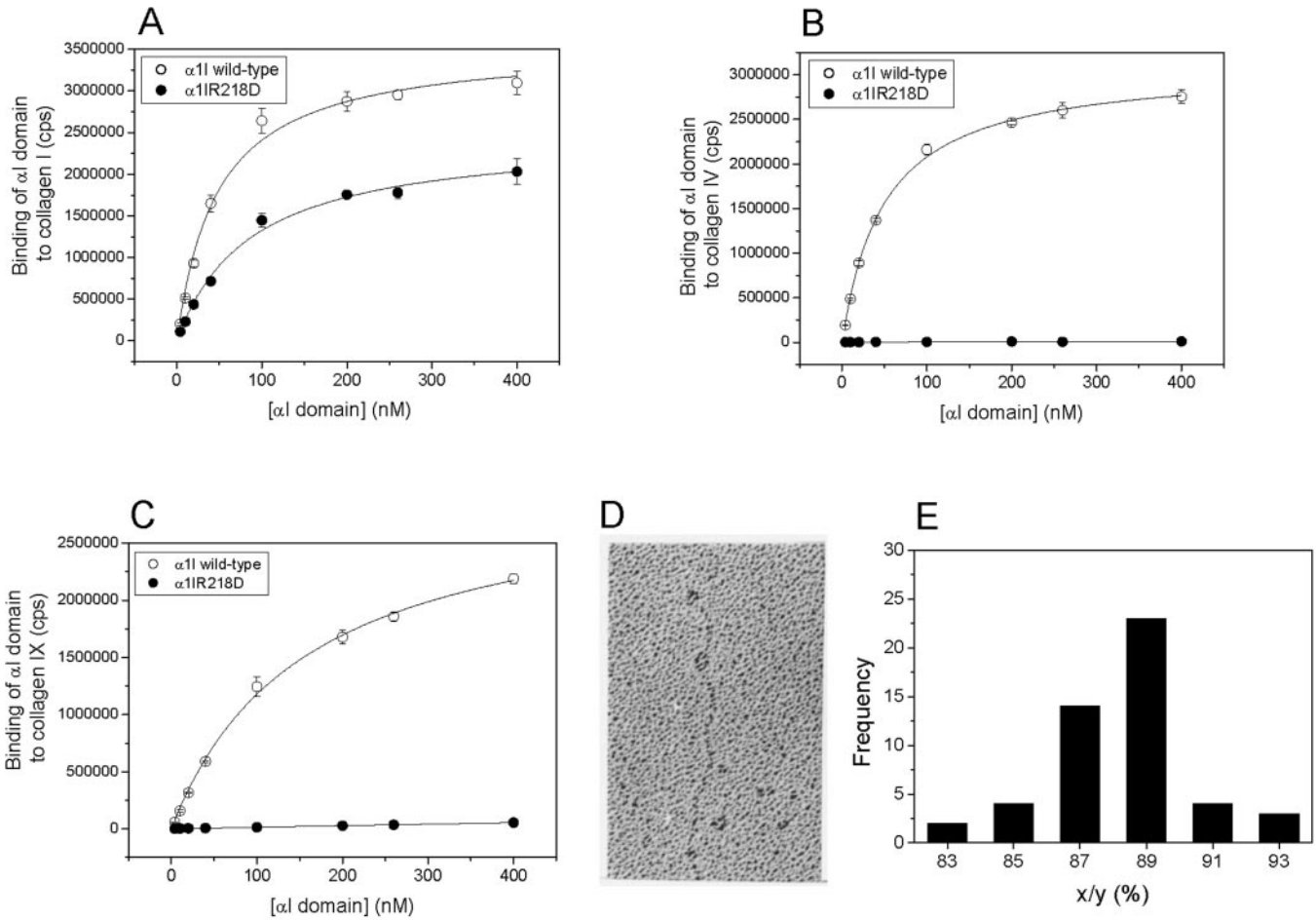

FIG. 8. **The effect of R218D mutation on α_1I domain binding: rotary shadowing electron microscopy of α_1I domain as collagen IX complex.** α_1I wild type (*open circles*) and R218D-α_1I (*closed circles*) binding to collagen I (*A*), collagen IV (*B*), and collagen IX (*C*) is shown. Microtiter plate wells were coated with collagens (16.4 μg/ml) in PBS overnight. BSA coating was used as a negative control, but data are not shown for clarity. Wells were blocked with 100 μl of 1:1 PBS + dilution II solution (Wallac, 7.5% BSA) at room temperature for 1 h. Blocking solution was removed, and I domain was added with assay buffer (Wallac) + 2 mM $MgCl_2$ and incubated at room temperature for 1 h. Cells were washed three times with PBS + 2 mM $MgCl_2$. 100 μl of europium-labeled anti-GST (1:1000) was added into each well for 1 h at room temperature. Cells were washed three times with PBS + 2 mM $MgCl_2$, 100 μl of enhancement solution (Wallac) was added into each well, and fluorescence was measured with the Wallac Victor 2. *D*, rotary shadowing electron microscopy of collagen IX with bound α_1I domain. *E*, frequency of the binding events as a function of distance between bound α_1I and the NC4 domain relative to the distance between the kink and NC4 domain (see Fig. 4*D*). *cps*, counts/s.

in one α chain as they are in GFOGER. It has been shown previously that for $\alpha_1\beta_1$ 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). Saccà *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 α_1I/α_2I 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 α_1I and α_2I domains to collagen IX we used a mutagenesis approach. Residues Asp-219 and His-258 in α_2I 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 α_1I and Asp-219 of α_2I 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 α_1I and α_2I 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 αI domains D219R-α_2I and H258V-α_2I showed significantly different behavior when binding to collagen I or collagen IX. Both mutations decreased the binding of α_2I 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 α_1I domain seems to bind to the same sites in collagen I as α_2I domain, suggesting that they both recognize a GFOGER-related sequence (49, 66). Here mutation R218D in α_1I domain had hardly any effect on binding to collagen I, whereas binding to collagens IV and IX was almost completely prevented. It seems that residue Arg-218 in α_1I domain is very important for collagen IX binding unlike the corresponding residue Asp-219 in α_2I domain. At the moment there is no crystal structure for α_1I domain complexed with any ligand or peptide making the mutagenesis data harder to interpret. The binding mechanism of α_1I domain to collagen IX seems to be

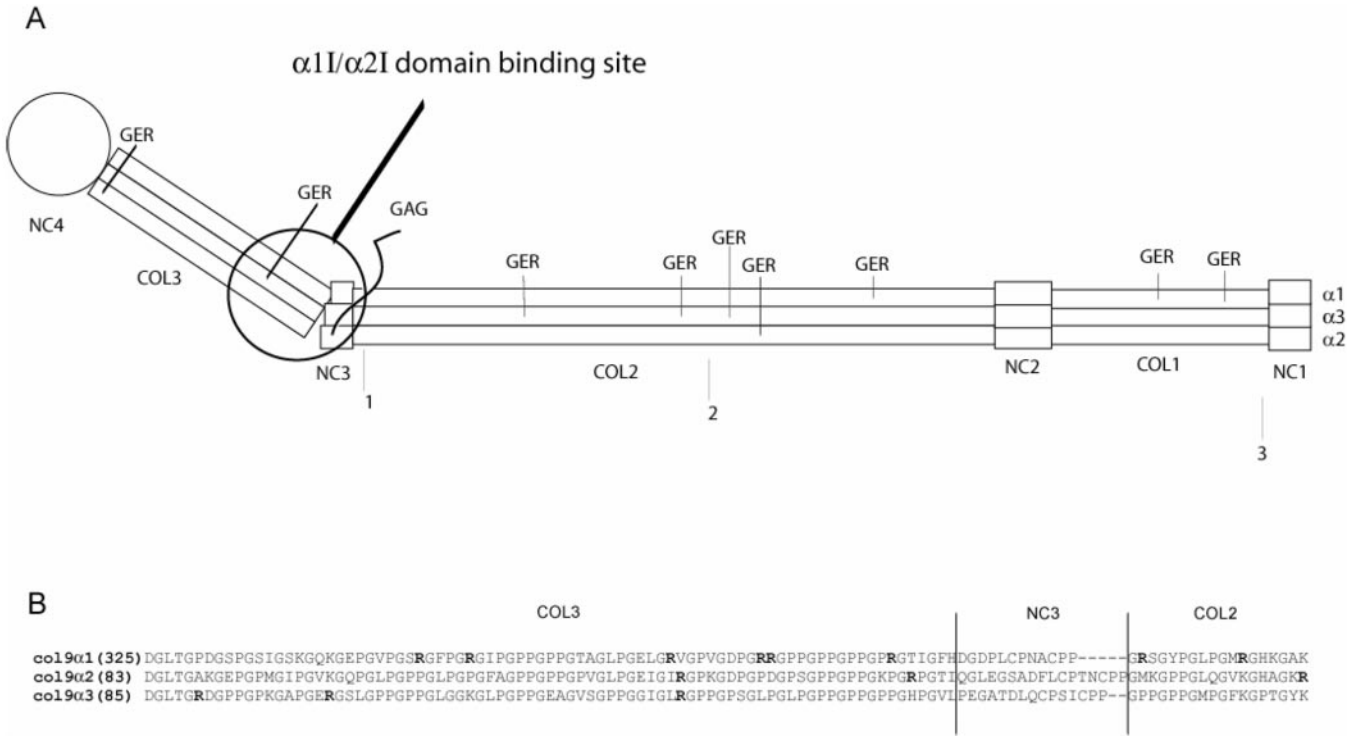

FIG. 9. **Binding sites in recombinant collagen IX molecule.** *A*, schematic representation of recombinant collagen IX molecule. Binding site(s) for α_1I and α_2I domains based on rotary shadowing electron microscopy are marked as a *big circle*. Site *1* indicates binding site to the amino-terminal region of collagen II or to the carboxyl-terminal region of another collagen IX molecule. Site *2* indicates the binding site to carboxyl-terminal region of collagen II (5). Site *3* indicates yet another possible binding site to type II collagen (67). *GAG* indicates the attachment site (serine residue in α2(IX) of NC3 domain) for a glycosaminoglycan side chain. *GER* sites show relative locations of GER sequences in the corresponding α chains of collagen IX molecule. *B*, amino acid sequences of all α chains surrounding the proposed α_1I/α_2I binding site in the collagen IX molecule. (The positively charged amino acid arginine is marked as *bold*.)

very different when compared with its binding to collagen I, whereas the binding mechanism of α_1I 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.

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

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