Integrin $\alpha_2$I Domain Recognizes Type I and Type IV Collagens by Different Mechanisms*

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The collagens are recognized by the $\alpha$I domains of the collagen receptor integrins. A common structural feature in the collagen-binding $\alpha$I domains is the presence of an extra helix, named helix $\alpha$C. However, its participation in collagen binding has not been shown. Here, we have deleted the helix $\alpha$C in the $\alpha$I domain and tested the function of the resultant recombinant protein (Dco$_2$I) by using a real-time biosensor. The Dco$_2$I domain had reduced affinity for type I collagen (430 ± 90 nM) when compared with wild-type $\alpha$I domain (90 ± 30 nM), indicating both the importance of helix $\alpha$C in type I collagen binding and that the collagen binding surface in $\alpha$I domain is located near the metal ion-dependent adhesion site. Previous studies have suggested that the charged amino acid residues, surrounding the metal ion-dependent adhesion site but not interacting with Mg$^{2+}$, may play an important role in the recognition of type I collagen. Direct evidence indicating the participation of these residues in collagen recognition has been missing. To test this idea, we produced a set of recombinant $\alpha$I domains with mutations, namely D219A, D219N, D219R, E256Q, D259N, D292N, and E299Q. Mutations in amino acids Asp$^{219}$, Asp$^{259}$, Asp$^{292}$, and Glu$^{299}$ resulted in weakened affinity for type I collagen. When $\alpha_2$ D219N and D292N mutations were introduced separately into $\alpha_2\beta_1$ integrin expressed on Chinese hamster ovary cells, no alterations in the cell spreading on type I collagen were detected. However, Chinese hamster ovary cells expressing double mutated $\alpha_2$ D219N/D292N integrin showed remarkably slower spreading on type I collagen, while spreading on type IV collagen was not affected. The data indicate that $\alpha$I domain binds to type I collagen with a different mechanism than to type IV collagen.

The integrins are a large family of transmembrane proteins forming $\alpha/\beta$ heterodimers, which act as receptors for connective tissue components and also bind to counter receptors on other cells (1, 2). Integrin $\alpha$ and $\beta$ subunits show some similarity but have evolved independently (3). Integrin $\alpha$ subunits can be divided into two different subgroups depending on the presence of an inserted domain (I domain) in their N-terminal part. In general, the $\alpha$ subunits participating in the formation of extracellular matrix receptors do not contain I domain, whereas it is found in all four $\beta_1$ integrin-associated $\alpha$ subunits involved in cell-cell adhesion of leukocytes. The four collagen-binding integrin $\alpha$ subunits, namely $\alpha_1$, $\alpha_2$, $\alpha_5$, and $\alpha_6$, are an exception; they contain an I domain, and at least $\alpha_1$ and $\alpha_5$ domains can, as recombinant proteins or proteolytic fragments, bind to collagen (4, 5). Thus, the collagen receptor integrins have a unique ligand binding mechanism when compared with the interaction of the other matrix receptor integrins with their ligands. Indeed, whereas many functional motifs in integrin ligands contain aspartic acid in a critical position (6, 7), often situated at a protruding loop (8), these motifs are not recognized by I domains (9). Integrin I domains contain a Mg$^{2+}$ ion that might coordinate the folding of the domain (10). There is some evidence that the Mg$^{2+}$ ion also forms the actual binding site for the ligands (10). One model of collagen binding to $\alpha_1$ I domain suggests that a glutamic acid residue on the surface of the tropocollagen molecule binds to Mg$^{2+}$ in the $\alpha_1$ I domain (11). Some evidence suggests, however, that the metal ion is displaced from the $\alpha_1$ I domain during the process of ligand binding (12). The initial recognition of collagen and also its binding might be a complex process involving multiple steps and interactions. Furthermore, it seems to be evident that both the fibrillar collagens, such as type I collagen (13), and the basement membrane-associated network-forming type IV collagen (5, 14) have multiple individual sites recognized by integrins.

We have recently described a short cyclic peptide that binds to $\alpha_1$ I domain and prevents its interaction with matrix molecules, including type I and type IV collagens (15). Three amino acids, RRK, and the proper cyclic conformation seem to be critical for the function of the peptide (15). The structure of the peptide suggests the importance of charged amino acid residues in collagen recognition. Other studies support this as well. For example, in type IV collagen, three amino acid residues, one arginine and two aspartic acids, all from separate collagen $\alpha$ chains, might be necessary for receptor binding (16). Recently, an $\alpha_2\beta_1$ integrin binding type I collagen-derived triple helical peptide was described (17). The importance of a short sequence, GER, and especially the essential role of the two charged amino acids in it, also suggest that interactions between positively and negatively charged amino acid residues are involved in integrin-collagen binding. However, in previous studies collagen binding could be affected only by mutation of the amino acid residues directly interacting with Mg$^{2+}$ ion (4).

Integrins $\alpha_2\beta_1$ and $\alpha_2\beta_3$ are known to have some differences in their ligand binding specificity. Integrin $\alpha_2\beta_1$ binds with better affinity to type IV collagen than to type I collagen or to other fibrillar collagens, whereas $\alpha_2\beta_3$ is a better receptor for...
fibrillar collagen than for type IV collagen (18). A similar difference can be seen also in the function of the corresponding recombinant αI domains (5) and a chimeric αβ2 integrin containing αιI domain instead of αιI has ligand binding functions similar to αβ2 (19). The determination of the atomic structure of αιI (20–22) and αιI (11) domains has made it possible to estimate their similarities and differences and to study the structural basis for the distinct function of the two collagen receptors. A common feature of the collagen-binding αI domains is the presence of an extra a-helix, helix aC (11, 20–22). It is probably present also in the collagen-binding αιαI and αιαI domains (23, 24) but missing from the other integrin αI domains (10, 25). Here, we have made mutations to the αιI domain and produced the variants as recombinant proteins. Some of the mutations were also introduced into αβ2 integrin expressed on CHO1 cells. Our results indicate the important role of helix aC and several charged amino acids surrounding the Mg2+ binding site in the recognition of type I collagen. Furthermore, αβ2 integrin seems to recognize type IV collagen by a mechanism different from that used in type I collagen binding.

EXPERIMENTAL PROCEDURES

Generation of αI and Mutant I Domains—Recombinant αI domain was produced as described previously (15). Protein concentrations were determined using Bradford’s method (26). Protein purity and folding were checked in native and SDS-polyacrylamide gel electrophoresis (Phast-System; Amersham Pharmacia Biotech). Site-specific mutations in the αiI domain were made using the Stratagene QuickChange mutagenesis kit, essentially following the manufacturer’s instructions. PCR primers having the desired mutation for both DNA-strands were designed, and then PCR was performed using Pfu polymerase (Stratagene), which makes at 68 °C exactly one copy of the whole GEX-2T vector (Amersham Pharmacia Biotech) containing the αI domain sequence. The PCR was digested with DpnI, which cuts only methylated DNA (i.e. only the template is digested). After this, PCR product DNA strands having the desired mutation were paired. The resulting GEX-2T vector having the mutated αI domain was transformed into Escherichia coli strain BL21. The resulting GEX-2T vector having the mutated αI domain was transformed into Escherichia coli strain DH5α, and the construct was checked by sequencing the entire αI domain. For the production of the recombinant protein, the construction was transformed into E. coli strain BL21.

Binding Assay with Europium-labeled I Domains—The I domain binding assay, based on the use of europium labeling, was used as described previously (15). Labeling of I domains with europium was carried out using 1/10 volume of 1 μl NaHCO3 (pH 8.5) with I domains, and the labeling reagent (Wallac) was added in the molar ratio 0.3:2–1 (label:protein) and incubated overnight at 4 °C. Unbound label was removed by gel filtration on a Sephadex G50 column (Amersham Pharmacia Biotech), and the fractions containing the labeled protein were pooled. Typically, coating of 96-well immunoplates (Maxisorp, Nunc) was performed by exposing the plates to 0.1 ml of PBS containing 5 μg/ml of type I collagen (1 mg/ml) or type IV collagen (native, isolated from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma; Sigma) overnight at 4 °C. Alternatively, matrix proteins (5 μg/ml) were coated on 96-well amine binding plates (Costar) according to the manufacturer’s instructions. Residual protein absorption sites on all wells were blocked with the Difco II solution (including 7.5% bovine serum albumin (BSA); Wallac) in PBS for 1–2 h at 37 °C. Europium-labeled αI was added at a concentration of 1 μl/ml in PBS, 2 mg MgCl2, to the coated wells and incubated for 3 h at 37 °C. Wells were then washed three times with PBS, 2 mg MgCl2. Finally, 0.1 ml of Delfia enhancement solution (Wallac) was added to each well, and the fluorometric signal was measured by fluorometry (Victor 2 or model 1232 Delfia; Wallac).

Cell Lines and Construction of the αI Integrin Expression Plasmid—CHO cells obtained from the American Type Culture Collection (Manassas, VA) were used as hosts for expression of integrin αι subunits. Integrin αι cDNA corresponding to nucleotides 1–4559 in the published sequence (27) was kindly provided by Dr. M. Hember (Dana-Farber Cancer Institute, Boston, MA). cDNA was ligated into the pAWneo2 expression vector (Ref. 28; a kind gift from Dr. A. Weiss; University of California, San Francisco, CA), which carries the spleen focus-forming virus long terminal repeat promoter and a neomycin resistance gene. Stable transfections were carried out using lipofectin reagent (Life Technologies Inc.) according to the manufacturer’s recommendations. Neomycin analogue G418 (400 μg/ml) was added to the culture medium. After 2–3 weeks of selection, the nontransfected control cells were dead, and G418-resistant clones were isolated and analyzed for their expression of αι integrin by flow cytometry as follows. Cells were grown to confluence and detached with trypsin-EDTA, and trypsin activity was inhibited by medium supplemented with serum. Cells were washed with PBS (pH 7.4) and then incubated with PBS containing 10 ng/ml BSA, 1 mg/ml glycine, and 0.02% NaN3 for 20 min at 4 °C. Cells were collected by centrifugation, exposed to a saturating concentration of monoclonal antibody against αι integrin (12F1) in BSA/PBS (BSA concentration 1 mg/ml) containing NaN3, for 30 min at 4 °C and stained with rabbit anti-mouse IgG coupled to fluorescein (1:20 dilution; Dakopatts, Denmark) for 30 min at 4 °C. Cells were washed twice with PBS containing NaN3 and suspended in the same buffer. In order to measure the amount of αι integrin on the cell surfaces, the fluorescent excitation spectra were analyzed by using a FACSkan apparatus (Becton Dickinson). Control samples were prepared by treating the cells with a primary antibody.

Cell Spreading Experiments—96-well maxisorp plates (Nunc) or high binding microtiteration plates (Nunc) were coated by exposing them to 0.1 ml of PBS containing 1 or 5 μg/cm2 (3 or 15 μg/ml) type I or type IV collagen for 12 h at 4 °C. Residual protein absorption sites on all wells were blocked with 0.1% heat-inactivated bovine serum albumin in PBS for 1 h at 37 °C. CHO cell clones expressing αβ2 integrins were used in spreading studies. Cells (10,000 cells/well) were allowed to spread in serum-free Dulbecco’s modified Eagle’s medium. Cells were fixed with 4% formaldehyde (Merck) and 5% sucrose (BDH) for 30 min, and at least three parallel wells (three parallel fields each) were examined and photographed by using a phase-contrast microscope. The total number of cells attached to collagen and the percentage of spread cells were counted. A spread cell was characterized as one having a clearly visible ring of cytoplasm around the nucleus.

IASYS Experiments—IASYS experiments were performed on the IASYS Auto plus apparatus (Affinity Sensors, Ltd.). A carboxymethyl-dextran cuvette was coated with type I collagen according to the manufacturer’s instructions. The coupling buffer was sodium acetate, pH 5.5, and in the coupling process the type I collagen concentration was 10 mg/ml. Type I collagen was coupled to the cuvette up to the saturation level (based on the reading of the apparatus). A concentration series from 10 to 300 μg/ml in PBS, 2 mg MgCl2 for wild-type and mutant αI domains was measured. Regeneration of the cuvette was performed with 10 mg or 100 mg EDTA in PBS, and the task was not always easy; for the tightest bound I domain, several successive regeneration steps were needed to remove most of the bound I domain from collagen. Results were analyzed with Fastfit software from IASYS.

Molecular Modeling of ΔαCαI—A three-dimensional model of the structure of the ΔαCαI domain was built using MALIGNE (29, 30) in the BODIL modeling packagea and MODELLER 4.0 (31). The model was constructed almost entirely from the x-ray structure of the αI domain (11), but the αII (10) and αLI (25) domains were used to build the structure in the vicinity of the deleted helix. This was done because the number of residues in that area in ΔαCαI is exactly the same as in αM and αL. This makes the modeling more reliable in that region. The x-ray structures were obtained from the Protein Data Bank (32).

RESULTS

Deletion of Helix αC in the αI Domain Inhibits Binding to Type I Collagen—The helix αC is a unique structural feature shared by the collagen-binding αI domains. Furthermore, one of the major differences between the putative collagen binding surfaces of αI and αII domains (the αI domain is shown in Fig. 1) is in the structure of helix αC. In the αI, the groove situated on the metal ion-dependent adhesion site (MIDAS) face of the I domain is wider than in the αII, because αII residues Ser284 and Glu288 oriented toward the groove in the
helix αC, are replaced in αI by the bulky residues tyrosine and asparagine (20–22). To study the role of the helix αC for the collagen binding function of αI domain, a deletion was produced; residues 284–288 (GYLNR) were deleted from the αI domain by using PCR with specifically designed primers. We used molecular modeling to estimate the effects of helix αC deletion on the αI domain (Fig. 1). The model predicted that the surface of mutant αI domain would become more negatively charged than the wild type and more flattened. In the x-ray structure of the αI domain, there is an arginine (Arg288) at the end of helix αC. This arginine forms a salt bridge with glutamate (Glu318) between strand βF and helix αC. As a result of the deletion of helix αC in αI, Glu318 and the adjacent aspartate (Asp317) are placed in the vicinity of the MIDAS.

The recombinant αI domain lacking the helix αC (named as ΔαCaI) was produced in E. coli as a GST fusion protein. To measure the effect of the helix αC deletion on the affinity of type I collagen binding, we used IASYS technology. For this, the ΔαCaI domain was tested as a fusion protein (GST/αI domain) to achieve greater mass and easier detection by IASYS. Collegen (100 μg/ml) was attached chemically to the cuvette according to the manufacturer’s instructions, and different concentrations of I domain (10–300 μg/ml) were added into the cuvette. The binding event was monitored in real time, and examples are shown in Fig. 2A. The ΔαCaI domain binds to collagen type I (Fig. 1) and the mutant ΔαCaI GST fusion protein to type I collagen. Fusion protein concentration was 100 μg/ml, and buffer was PBS, 2 mM MgCl2. Temperature was 25 °C (A). Shown are Kd (binding to type I collagen) determination of the wild-type αI (○) and the ΔαCaI GST fusion protein (○). Binding at equilibrium was determined as a function of fusion protein concentration, and results were fitted to a Michaelis-Menten form equation (solid line) to obtain Kd. Running buffer was PBS, 2 mM MgCl2, and temperature was 25 °C (B).

After GST was removed, the resultant αI domain was labeled with europium. Binding of the mutant and wild-type αI domains to collagen types I and IV was tested. Wells were coated with different concentrations of collagen type I or IV (1–15 μg/cm2), incubated with europium-labeled I domain (1 μg/ml), and bound I domain was measured using a fluorescence spectrophotometer. These experiments suggested that the recognition of collagen subtypes by ΔαCaI may be different when compared with the wild-type αI domain. The mutant preferred type IV collagen over type I collagen (Fig. 3), indicating differences in type IV collagen binding mechanism when compared with type I collagen binding. Interestingly, the collagen binding pattern of ΔαCaI domain resembled the one previously described for αI domain (5), suggesting that the differences in αC structure might partially explain the functional differences between αI and αI domains. In collagen-binding α domains, there is one extra amino acid residue in the loop between helices α and αC. However, in αI and αI domains, the extra amino acid has an opposite charge (Asp219 in αI domain, Arg219 in αI domain), suggesting another possible mechanism for the different functions of the two integrins. To test this possibility, we produced a mutant αI domain, D219R. This mutation did not, however, have a clear effect on the ratio of
type I collagen/type IV collagen binding (Fig. 3).

Negatively Charged Amino Acids Surrounding the Mg$^{2+}$ Binding Site in αI Domain Are Involved in Type I Collagen Binding—Five negatively charged amino acid residues in the αI domain were mutated, and the effects on ligand binding were tested. The αI domain variants were prepared by using PCR with primers having the desired mutation, and the entire DNA of the mutated I domain was sequenced by using IASYS technology. The mutant αI domain was expressed as GST fusion proteins, and they were purified as both "plain" I domains and as fusion proteins (GST/I domain). Mutant αI domain D259A could not be expressed in E. coli BL21, and it was not studied any further. Expression levels of αI domain D259N were also quite low. All expressed proteins were routinely checked by SDS and native gel electrophoresis for purity and quality of protein. In native gel electrophoresis, every αI domain protein sample had a small extra band having larger mass than the true I domain band (not shown). The extra band disappeared with a reducing agent (dithiothreitol). This extra band is likely to be a small proportion of misfolded I domain that was able to refold back to correct conformation when dithiothreitol was added. A second, shorter version of wild-type αI domain was prepared, where the amino terminus of the I domain was deleted at the position of the first cysteine (Cys$^{140}$). This protein, named as wild type (ΔC140), did not show an extra band in native gel electrophoresis (not shown) under nonreducing conditions, but unfortunately the collagen binding ability of wild type (ΔC140) decreased significantly. All tested mutant αI domains were based on the first wild-type version.

Since mutations in the αI domain were made close to the MIDAS, it was necessary to check that none of the mutations affected significantly the metal dependence of the I domain binding. The binding of mutant αI domains to type I collagen was measured at four different magnesium concentrations (1, 2, 5, or 10 mM) and with 10 mM EDTA, which was used as "0 mM." Eu-labeled I domain (1 µg/ml) was incubated in collagen-coated (5 µg/cm$^2$) wells, and bound I domain was measured. The results are shown in Fig. 4. The data indicate that the produced mutations, including ΔCaα2I, have no effects on metal binding site, and the concentration of 2 mM Mg$^{2+}$, conditions where all αI domains were tested, represent saturating conditions.

The effects of the mutations on collagen binding were tested by using IASYS technology. The mutant αI domains were produced as GST fusion proteins. The binding event was monitored in real time, and examples are shown in Fig. 5. Concentration series were measured for each αI domain, and the results representing saturating conditions (300 µg/ml) are listed in Table I. The wild type (ΔC140) showed only about 20–40% binding when compared with wild-type αI domain, indicating the importance of the amino terminus of the I domain. αI mutants D219A, D219N, D219R, D259N, D292N, and E299Q bound collagen at 5–60% of the wild-type levels (Table I). While the other mutants gave satisfactory reproducible results, the binding levels of the E256Q αI domain mutant were more variable, and the measured binding to type I collagen could be at wild-type level (35–100% compared with the wild-type binding levels). The participation of Glu$^{256}$, as well as Asp$^{219}$, in collagen binding was therefore studied further by first removing the GST tag and then analyzing its binding by Biacore technology and solid phase binding assays (not shown). In these experiments, D219N showed constantly lowered binding to type I collagen, as was seen already by using IASYS, whereas the mutation E256Q had no effect. The results indicate that at least four negatively charged amino acids in αI, namely Asp$^{219}$, Asp$^{256}$, Asp$^{292}$, and Glu$^{299}$, have a significant role in collagen I binding.

Double Mutation D219N/D292N in the α2 Subunit of α2β1 Integrin Affects CHO Cell Spreading on Type I Collagen but Not on Type IV Collagen—Amino acids Asp$^{219}$ and Asp$^{292}$ were found to be essential for αI domain binding to collagen. To test the importance of these residues for the function in the complete receptor, mutations D219N and D292N were introduced into full-length αc DNA before it was transfected into CHO cells. Wild-type CHO cells have no endogenous collagen receptors and cannot bind to collagens. They produce, however, the β1 integrin subunit, and therefore αc β1 integrin is expressed on the cell surface after αc cDNA transfection (not shown). The expression levels of αc β1 integrin on the cell surface were analyzed by flow cytometry. Two αc transfected cell clones (clone 9 had about 10% of αc β1 integrin expression when compared with clone 12) were used in all assays. Cell clones harboring the D219N or D292N mutations or the double mutation D219N/D292N had αc β1 expression levels comparable with the

![Collagen Recognition by α2β1 Integrin](http://www.jbc.org/)

**Fig. 3.** Helix oC deletion (ΔCaα2I) changes the type I/type IV collagen binding profile. Shown is a solid phase collagen binding assay of wild-type αI and mutants ΔCaα2I and D219R to type I (○) and type IV (□) collagens. Wells were coated with proper collagen concentrations and were blocked with BSA. Eu-labeled αI (1 ng/µl) in PBS, 2 mM MgCl$_2$ was added into the wells for 3 h at 37°C. Wells were washed three times, and bound αI was measured with time-resolved fluorescence. BSA coating was used as control, and its signal was subtracted from the signal obtained from the samples. The data shown are the mean values ± S.D. of a representative experiment done in triplicate.

**Fig. 4.** Eu-labeled I domain (1 µg/ml) was incubated in collagen-coated (5 µg/cm$^2$) wells, and bound I domain was measured.

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CHO-α2 clone 12 (Table II). To test the function of α2β1 integrin, we measured the spreading of cells plated on type I collagen in a 2-h spreading assay. Neither of the cell clones harboring a single point mutation showed any significant alterations in the spreading rate (Table II). However, CHO cells expressing doubly mutated α2 integrin spread on type I collagen remarkably more slowly than the CHO-α2 cells (Table II, Fig. 6). Their spreading was comparable with the clone 9 expressing 10-fold less α2β1 integrin (not shown). The fact that one mutation alone is not enough to cause significant changes in this integrin function supports the idea that several charged amino acids in the α2I domain participate at the same time in type I collagen binding. Interestingly, no differences in cell spreading were seen on type IV collagen (Fig. 6; Table II), indicating that the α2I domain binds to type I collagen by using a different mechanism than is used to recognize type IV collagen.

### DISCUSSION

The development of collagen-like cell adhesion proteins and their cellular integrin-type receptors has been suggested to be one of the steps critical for the evolution of metazoan organisms (33). In modern vertebrates, collagens are recognized by at least four I domain-containing integrins, namely α1β1, α2β1, α10β1, and α11β1. Other matrix receptor integrin α subunits do not have the αI domain, and, somewhat surprisingly, the collagen-binding α integrins seem to belong to the same evolutionary branch of the integrin family tree as the leukocyte cell-cell adhesion integrin α subunits (3). One cell type, for example chondrocytes, can express three collagen receptors at the same time (23, 34). This raises the question of whether the three receptors have, despite their structural similarities, their own specific function. Indeed, α1β1 and α2β1 integrins seem to differ at least in their signaling function (35). Furthermore, α2β1 is a better receptor for type IV collagen than α1β1, whereas α2β1 binds type I collagen with better affinity (5, 18, 19). The distinct signaling functions may be due to differences in intracellular (35) and transmembrane domains, while the ligand binding specificity is dictated by the structural properties of the αI domains.

The three-dimensional structures of the α1 and α2 domains have been solved recently by x-ray crystallography (11, 20–22), but the mechanism of collagen recognition and binding is mostly unknown. One possibility is that Mg2⁺, present in all αI domains at the MIDAS site (10, 11, 20–22, 25), directly mediates collagen binding (11). Although the exact role of the metal is still under discussion, most models have located the

### TABLE I

| Mutation        | Binding compared with wild type (%) |
|-----------------|-------------------------------------|
| Wild type       | 100                                 |
| D219A           | 20–35                               |
| D219N           | <10                                 |
| D219R           | 5–35                                |
| E256Q           | 35–100                              |
| D259N           | 35–60                               |
| D292N           | 35–60                               |
| D292A           | 5–35                                |
| E299Q           | 10–30                               |
| Wild type (ΔC140) | 20–40                             |

CHO-α2 clone 12 (Table II). To test the function of α2β1 integrin, we measured the spreading of cells plated on type I collagen in a 2-h spreading assay. Neither of the cell clones harboring a single point mutation showed any significant alterations in the spreading rate (Table II). However, CHO cells expressing doubly mutated α2 integrin spread on type I collagen remarkably more slowly than the CHO-α2 cells (Table II, Fig. 6). Their spreading was comparable with the clone 9 expressing 10-fold less α2β1 integrin (not shown). The fact that one mutation alone is not enough to cause significant changes in this integrin function supports the idea that several charged amino acids in the α2I domain participate at the same time in type I collagen binding. Interestingly, no differences in cell spreading were seen on type IV collagen (Fig. 6; Table II), indicating that the α2I domain binds to type I collagen by using a different mechanism than is used to recognize type IV collagen.

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### FIG. 4

The effect of magnesium concentration on α2I binding to type I collagen. Solid phase binding assay of the wild-type α2I, Δα2α2I, D219R, and D292N to type I collagen as a function of magnesium concentration. Wells were coated with 5 μg/cm² type I collagen and were blocked with BSA. Eu-labeled α2I (1 ng/μl) in PBS buffer with a proper magnesium concentration was added into wells for 3 h at 37 °C. Wells were washed three times, and bound α2I (●) was measured with time-resolved fluorescence. BSA coating was used as a control (○). The data shown are the mean values ± S.D. of a representative experiment performed in triplicate.

### FIG. 5

IASYS assays. IASYS binding curves are shown for the wild-type α2I, D292N, and D292N GST fusion protein to type I collagen. Fusion protein concentration was 300 μg/ml. Buffer was PBS, 2 mM MgCl₂, and temperature was 25 °C.
Collagen Recognition by α2β1 Integrin

96-well plates were coated with type I or IV collagen. CHO cell clones expressing wild-type α2 integrin or α2 with mutation D292N, D219N, or double mutation D219N/D292N (two clones: number 2 and 4) were used. 10,000 cells/well were allowed to spread in serum-free Dulbecco’s modified Eagle’s medium for 2 h. They were fixed, and at least three parallel wells were examined by using a phase-contrast microscope. The ratio of spread cells:total number of cells attached was counted. A spread cell was characterized as one having a clearly visible ring of cytoplasm around the nucleus. The level of α2β1 integrin on cell surface was measured by flow cytometry (mean fluorescence value). In experiment (Expt.) 4, D219N clone had some α2 negative cells, and the result was confirmed by staining with anti-α2 antibodies and counting the spread cells under a fluorescence microscope.

| Cell clone | Expression level of α2β1 | Ligand μg/cm² | Cell spreading |
|------------|--------------------------|---------------|----------------|
|            |                          | Type I collagen | Type IV collagen |
| Expt. 1    |                           |               |                 |
| α2 wild type | 60.0                     | 3             | 0.83 ± 0.01     | 0.54 ± 0.09 |
| D219N/D292N #2 | 86.6                   | 3             | 0.40 ± 0.10     | 0.47 ± 0.14 |
| D219N/D292N #4 | 72.8                   | 3             | 0.47 ± 0.03     | 0.74 ± 0.04 |
| D292N       | 51.1                     | 3             | 0.72 ± 0.08     | 0.55 ± 0.2  |
| Expt. 2    |                           |               |                 |
| α2 wild type | 60.0                     | 0.5           | 0.84 ± 0.04     |                 |
| D219N/D292N #2 | 86.6                   | 0.5           | 0.51 ± 0.05     |                 |
| D219N/D292N #4 | 72.8                   | 0.5           | 0.62 ± 0.11     |                 |
| D292N       | 51.1                     | 0.5           | 0.86 ± 0.07     |                 |
| Expt. 3    |                           |               |                 |
| α2 wild type | 60.0                     | 3             | 0.77 ± 0.03     | 0.77 ± 0.05 |
| D219N/D292N #2 | 86.6                   | 3             | 0.42 ± 0.12     | 0.78 ± 0.04 |
| D219N/D292N #4 | 72.8                   | 3             | 0.28 ± 0.02     | 0.70 ± 0.05 |
| D219N       | 64.6                     | 3             | 0.67 ± 0.05     | 0.91 ± 0.03 |
| Expt. 4    |                           |               |                 |
| α2 wild type | 60.0                     | 3             | 0.61 ± 0.07     | 0.82 ± 0.05 |

Collagen binding site close to the MIDAS. On the putative collagen binding surface of α1 and α2 domains, and probably on α10 and α11 based on the DNA sequence, helix αC helps to form a suitable groove for the collagen molecule to bind to (11, 20–22). However, in previous reports, the mutation of single amino acid residues on that surface, D219A, E256A, or D229A, did not affect CHO cell attachment on collagen (4), unlike mutations to residues directly participating in Mg2+ binding (36).

We have tested the binding of recombinant mutant α2 domains to type I collagen by using solid phase binding assays and IASYS technology. IASYS biosensor measures the mass changes during the binding process indirectly. With this technique, the kinetics of binding can be monitored in real time. The experiments indicated that residues Asp219, Asp292, and Glu299 are important for type I collagen binding by α2. However, none of the mutations could completely prevent collagen binding, suggesting that several amino acid residues on the binding surface may simultaneously contribute to the phenomenon. The mutations in amino acids Asp219 and Asp292 were also introduced to full-length α2 cDNA, which was then expressed in CHO cells. In place of the cell attachment assay, we used a cell spreading assay that, according to our experience, is more sensitive. In agreement with a previous report (4), we were not able to see differences when compared with CHO cells expressing wild-type α2 integrin. However, the importance of Asp219 and Asp292 for collagen binding could be confirmed in experiments in which both amino acids had been mutated at the same time. The possibility that Asp219 may participate in collagen binding has been suggested previously based on molecular modeling (11). We have recently published indirect evidence that Asp259 and Asp292 may participate in collagen binding by suggesting their interaction with RKK peptides inhibiting collagen binding (37). Here we show the first direct evidence that amino acid residues next to the MIDAS site, but not essential for Mg2+ binding, can participate in collagen recognition. We also suggest that several residues make concomitant and more or less equal contribution to the phenomenon.

Another interesting observation made with the CHO cells harboring the double mutant α2 D219N/D292N was that, despite the fact that their spreading on type I collagen was affected, they could spread on type IV collagen with similar efficiency as the wild-type α2 integrin-expressing cells. This finding clearly indicates that type I and type IV collagens are recognized by distinct mechanisms. It is also tempting to speculate that by using the same approach it is possible to reveal the structural reasons for proposed differences in ligand binding by α1 and α2 domains (5). Neither Asp219 nor Asp292 is conserved in α1 domain, but the corresponding amino acid residues are arginine and serine, respectively. The idea that the fact that α1 binds better to type IV collagen than α2 could be due to one single amino acid residue was tested by making the mutation D219R. However, the mutant α1 domain did not favor type IV collagen over type I collagen, indicating that the hypothesis was wrong.

A major structural difference between the putative ligand binding surfaces of α1 and α2 domains is the bulky helix αC in α2, which limits the width of the neighboring groove. Despite the fact that helix αC is found only in collagen-binding α1 domains, its participation in collagen recognition has never been shown. Here, the importance of helix αC was tested by deleting it from α2. No drastic structural changes were expected, since helix αC is a separate “loop” on the surface of the α2 domain. It was encouraging to observe that there was no change in metal dependence of ligand binding. IASYS experiments showed that helix αC is important for collagen type I binding. Kd of wild-type α2 integrin to type I collagen was close to values reported earlier and measured with Biacore technology in the presence of manganese (5). The Kd of ΔαCα1 integrin was about 5-fold higher than the Kd of the wild type. The curious kinetics of ΔαCα1 integrin can be explained so that after the deletion more space is available for the binding of type I collagen to the surface of the I domain. In the absence of the helix αC, the specificity of the binding is diminished, and the binding and release of the ligand occurs more easily. The experiments measuring recombinant ΔαCα1 integrin binding to type I and IV collagens showed that the binding profile may be different when compared with the profile of the wild-type α2. The ΔαCα1 seemed to bind better to type IV collagen than to type I collagen, and type IV collagen binding was saturating at higher coating concentrations than type I collagen binding. Based on previous
and may actually disturb the binding of type IV collagen, which contains interrupted sequences between triple helices and is therefore less compact than type I collagen.

To conclude, our results strongly support the earlier suggestions that the collagen binding surface in integrin αI domain is located around the metal ion-binding MIDAS site. Furthermore, we have shown the important role of helix cαC and amino acid residues Asp^{215} and Asp^{292} in type I collagen binding. They may not, however, participate in type IV collagen binding, suggesting that the two collagen mechanisms are recognized by different mechanisms.

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Fig. 6. The spreading of α2 integrin-transfected CHO cells on type I and type IV collagen. CHO cells were transfected with the wild-type or the variant α2 integrin. 96-well plates were coated by exposing them to type I (A) or type IV collagen (B). Residual protein absorption sites on all wells were blocked with heat-inactivated bovine serum albumin. CHO cell clones expressing the wild-type α2 integrin or α2 integrin with double mutation D219N/D292N (A; two cell clones, numbers 2 and 4) were used in spreading studies. Cells (10,000 cells/well) were allowed to spread in serum-free Dulbecco's modified Eagle's medium for 1, 2, or 4 h. They were fixed, and at least three parallel wells (one per well) were allowed to spread in serum-free Dulbecco's modified Eagle's medium for 1, 2, or 4 h. They were fixed, and at least three parallel wells (three parallel fields each) were examined and photographed by using a phase-contrast microscope. The number of spread cells was counted and divided by the total number of cells attached to collagen. A spread cell was characterized as one having a clearly visible ring of cytoplasm around the nucleus.

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