Identification of Cell-binding Sites on the Laminin α5 N-terminal Domain by Site-directed Mutagenesis*

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The newly discovered laminin α5 chain is a multidomain, extracellular matrix protein implicated in various biological functions such as the development of blood vessels and nerves. The N-terminal globular domain of the laminin α chains has an important role for biological activities through interactions with cell surface receptors. In this study, we identified residues that are critical for cell binding within the laminin α5 N-terminal globular domain VI (270 residues) using site-directed mutagenesis and synthetic peptides. A recombinant protein of domain VI and the first four epidermal growth factor-like repeats of domain V, generated in a mammalian expression system, was highly active for HT-1080 cell binding, while a recombinant protein consisting of only the epidermal growth factor-like repeats showed no cell binding. By competition analysis with synthetic peptides for cell binding, we identified two sequences: S2, 125GQVFHVAYVLK135 and S6, 225RDFTKATNIRL-RFLR239, within domain VI that inhibited cell binding to domain VI. Alanine substitution mutagenesis indicated that Arg239 of S6 was critical for both heparin and cell binding functions for cell surface receptor and for matrix proteins, including integrin α5β1, α5β2, and α5β1 integrins (6, 7). Laminin-11 has been shown to inhibit neurite outgrowth in vitro, while other laminin isoforms promote neurite outgrowth, suggesting a unique role of the α5 chain in nerve regeneration (11).

The N-terminal globular domain (domain VI, 250–270 residues) is specific for laminin and is the most conserved (60% sequence identity) among the laminin domains (12). Studies with function-blocking antibodies and cell binding assays have indicated that domain VI of both the laminin α1 and α2 chains contains binding sites for the α5β1 and α5β2 integrins (13–15). Furthermore, domain VI is also capable of binding heparin and heparan sulfate chains of perlecan (13–15). In addition to binding functions for cell surface receptor and for matrix proteins, domain VI is also essential for the self-assembly of laminins (13, 16, 18). Previous studies showed that the synthetic peptide RQVFQVAYIIKA (A-13), derived from domain VI of the laminin α1 chain, binds β1 subunit-containing integrins (19). The active core sequence (VAYI) of this peptide is conserved in the α5 chain, but the functional importance of this site within domain VI has not yet been examined.

In the present work, we studied cell binding functions of mouse laminin α5 domain VI using site-directed mutagenesis and synthetic peptides. We found that two sites, spaced by ~90 amino acids, are required for cell binding. We also identified four residues within the two sites that are essential for the binding. In addition, we demonstrated that an arginine residue of one of the sites is critical for both heparin and cell binding. Our findings suggest that the protein conformation surrounding these sites is important for cell binding through integrin and heparan sulfate-containing cell surface receptors.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors and Site-directed Mutagenesis—**Mouse kidney cDNA was used as a template in polymerase chain reaction to amplify sequences encoding domain VI and the first four EGFlike repeats of domain V of the laminin α1 and α2. Polymerase chain reaction was performed with PfTurbo DNA polymerase (Stratagene, La Jolla, CA) using the following primers: 1, GAGGAGAAGCTTCGCACTCCCGGGGGCGATGGC; 2, GAGGACTGAGAGTCCCGAGCC

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1 The abbreviations used are: EGF, epidermal growth factor; PBS, phosphate-buffered saline.
GGCAGCTAGGCCCGTGGAATTC; 3, GAGAGAAAGCTTCAGAGAGAGGAG- GCCTGTTTCTGTC; 4, GAGAGCTGAGAGGAGAGCCCTCTGGA-GTTTCC. Primers 1 and 2 were used for laminin α5 domain VI (Arg101, His152), and primers 3 and 4 for the laminin α5 domain VI (Gln161, Ser240).

For polymerase chain reaction amplification of the EGF-like repeats of laminin α5 domain V (Arg141–His152), primers 2 and 5, GAGAGAGCTCCTGCTCCTGCTCAGCCACG, were used. In addition to the coding sequences, these primers contained either a HindIII or a XhoI restriction site. The polymerase chain reaction products were digested with HindIII and XhoI and ligated into the expression vector pSecTag2/I and ligated into a Superose 12 HR 10/30 column on an ÄKTA. Samples were eluted with 50 mM Tris-HCl, 0.3 M NaCl, 250 mM imidazole, pH 6.0, were added to concentrations of 1 and 10 mM, respectively. Nickel-charged agarose resin (Probond, Invitrogen) was formed in 96-well round-bottom microtiter plates (Immulon-2HB, Dynex Technologies, Inc., Chantilly, VA). Wells were coated for 1 h at room temperature with 50 μg/ml recombinant proteins or laminin-10/11 (U. S. Food and Drug Administration). All the peptides were prepared with a C-terminal amide group. The peptides were purified by reverse-phase high performance liquid chromatography and characterized by mass spectrometry.

**Table I**

| Name    | Sequence | Residues |
|---------|----------|----------|
| S1      | EVNVTLDLQQYFH | 115–127  |
| S2      | GQVFHVAYVLKE  | 123–135  |
| S3      | QPWQFFASSKRD  | 157–168  |
| S4      | LERGPGRTLERI | 170–181  |
| S5      | LRDFTKATNIKL | 224–235  |
| S6      | RDFKATRNLRFRLR| 225–239  |
| S7      | NLRRLFRLTLL  | 232–243  |
| S8      | GKLKRDPVTLRR | 249–260  |
| S9      | TVTRRYYYSIKD | 256–267  |
| S10     | HPVSNADGTER  | 89–100   |
| S11     | PRDPLWLEISTDFGHTY | 139–156 |
| S12     | STDFQGQPWQF   | 147–162  |
| S13     | PTLKIRQDDDV   | 175–188  |
| S14     | TTEYSRIVPLEGEI| 190–204  |
| S15     | PPLSRGLYEVEYN| 105–116  |

**Fig. 1. Expression of recombinant laminin α5 domain VI.** Schematic representation of the laminin α5 chain constructs used for mammalian expression. The N-terminal globular domain VI and the first four EGF-like repeats of domain V are indicated by VI and E1–4, respectively. The positions of two of the sites (S1, S2) collected 7 days after transfection. The medium was cleared of detached cells modified Eagle’s medium containing 1% fetal calf serum) was used. For recombinant production, conditioned medium (Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Indianapolis, IN). The cells were maintained at 37 °C in a humid atmosphere with 5% CO2 in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine.

**Discussion**

For polymerase chain reaction amplification of the EGF-like repeats of laminin α5 domain V (Arg141–His152), primers 2 and 5, GAGAGAGCTCCTGCTCCTGCTCAGCCACG, were used. In addition to the coding sequences, these primers contained either a HindIII or a XhoI restriction site. The polymerase chain reaction products were digested with HindIII and XhoI and ligated into the expression vector pSecTag2/I and ligated into a Superose 12 HR 10/30 column on an ÄKTA. Samples were eluted with 50 mM Tris-HCl, 0.3 M NaCl, 250 mM imidazole, pH 6.0, were added to concentrations of 1 and 10 mM, respectively. Nickel-charged agarose resin (Probond, Invitrogen) was formed in 96-well round-bottom microtiter plates (Immulon-2HB, Dynex Technologies, Inc., Chantilly, VA). Wells were coated for 1 h at room temperature with 50 μg/ml recombinant proteins or laminin-10/11 (U. S. Food and Drug Administration). All the peptides were prepared with a C-terminal amide group. The peptides were purified by reverse-phase high performance liquid chromatography and characterized by mass spectrometry.

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**Real-time Heparin BindingKinetics of Recombinant Proteins Measured by Surface Plasmon Resonance—Biotinylated heparin (Celsius Laboratories, Inc., Cincinnati, OH) at 40 μg/ml in running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, including 0.005% surfactant P20) was immobilized on a streptavidin-coated sensor chip (Sensor Chip SA, BIAcore, Inc., Piscataway, NJ) at 10 μl/min for 4 min to an immobilization level of 300 resonance units. In the affinity measurements, recombinant proteins at different concentrations (50–200 nM) were injected on the heparin-coated surface at 30 μl/min in the running buffer at 25 °C, and the binding and dissociation were registered (2 min each) in a BIAcore™ 1000 instrument (BIAcore, Inc.). The streptavidin-heparin surface was regenerated at the end of each run by two successive injections of 30 μl of 20 mM NaOH containing 1 mM NaCl. In control experiments with the same concentrations of recombinant proteins, but with a blank streptavidin sensor chip, no binding was seen. The sensorgrams obtained were analyzed by nonlinear least square curve fitting using BIAevaluation 2.1 software assuming single-site association and dissociation models.**

**Cell Binding Assays—HT-1080 fibroblasts and 293 cells (CCL-121, ATCC) were detached with 0.05% (w/v) trypsin, 0.02% (w/v) EDTA in PBS, washed with Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin, resuspended to a concentration of 3 × 10⁶ cells/ml, and incubated at 37 °C for 30 min. For evaluation of the effects of synthetic peptides or monoclonal antibodies against integrin subunits, cells were incubated with peptides or antibodies at 37 °C for 30 min.**

**Function-blocking monoclonal antibodies against integrin α1 (FD12), α2 (P1D6), α3 (P1B5), α5 (ASC-1), α6 (P1H4), αβ (P1D6), αβ (NKI-GoH3), αβ (P3G6), and β1 (ASC-3) were purchased from Chemicon International, Inc., Temecula, CA. Anti-integrin α5 (A2-HIE10) was from Upstate Biotechnology, Lake Placid, NY, and anti-integrin β1 (mAb13) was a gift from Dr. K. Yamada, National Institutes of Health. Assays were performed in 96-well round-bottom microtiter plates (Immulon-2HB, Dynex Technologies, Inc., Chantilly, VA). Wells were coated for 1 h at room temperature with 50 μl of recombinant proteins or laminin-10/11 (U. S. Food and Drug Administration). All the peptides were prepared with a C-terminal amide group. The peptides were purified by reverse-phase high performance liquid chromatography and characterized by mass spectrometry.**
Expression and Cell Binding Activity of Recombinant Laminin α5 N-terminal Globular Domain VI— Constructs were generated for expression of mouse laminin α5 domain VI and the first four EGF-like repeats of domain V in mammalian cells (Fig. 1). In addition, a similar construct was also generated for laminin α1 domain VI, which corresponds to that described previously (15). The secreted recombinant proteins were purified by Ni-agarose chromatography from the conditioned medium of transfected COS-7 cells with a yield of 1–2 μg/ml medium. The purity of the protein preparations was more than 95% as judged from Coomassie Blue-stained gels after SDS-polyacrylamide gel electrophoresis (data not shown). Recombinant fragments containing domains VI through IV of the laminin α5 and α2 chains have previously been shown to promote binding of HT-1080 fibrosarcoma cells (13, 14). This cell line was therefore used to analyze the adhesive properties of the recombinant laminin α5 domain VI. HT-1080 cells showed strong binding to domain VI, while the recombinant domain V, consisting of the EGF-like repeats, showed no cell binding, demonstrating that the cell binding activities reside in domain VI (Fig. 1).

Effect of Synthetic Peptides on Cell Binding to Laminin α5 Domain VI—To localize potential cell-binding sites on laminin α5 domain VI, we screened synthetic peptides for their inhibitory effect on HT-1080 cell binding to domain VI. We synthesized 15 peptides that corresponded to possible integrin recognition sequences (for review, see Ref. 20) within laminin α5 domain VI and to active sequences previously identified in laminin α1 domain VI (Table I). Four peptides (S1, S2, S6, and S7) were found to inhibit HT-1080 cell binding to domain VI (Fig. 2), whereas the other peptides showed no or only small effects on the cell-domain interaction. A GRGDS peptide, which is reported to block the function of various integrins, had no effect on cell binding. The sequences of peptides S1 and S2 overlap with four residues, but only S2 showed strong activity when tested in a direct cell binding assay (data not shown). The S6 and S7 peptides overlap by eight residues and showed no activity when tested for direct cell binding (data not shown).

None of the peptides inhibited cell binding to laminin-10/11, suggesting that other cell-binding sites are available on the intact molecule (data not shown). Taken together, these results suggest that two sequences: S2, 125RGQVFQVAY135 and S6, 225RDFTKATNIIRLFLR239, separated by ~90 residues, directly interact with cell surface receptors within domain VI.

Cell Binding Activity of Laminin α5 Domain VI Mutants—We next examined the functional role of these two sites within domain VI by alanine substitution mutagenesis. Our laboratory has previously identified several synthetic peptides active for cell binding within domain VI of the mouse laminin α1 chain (19). The S2 peptide corresponds to the highly active peptide A-13 (QQVFQVAYHIKA) of laminin α1 domain VI. Deletion analysis revealed that the VAYI sequence within A-13 is critical for high cell binding activity (19). The active core sequence VAYI is conserved in the laminin α5 domain VI and to active sequences previously identified in laminin α1 domain VI (Table I). Four peptides (S1, S2, S6, and S7) were found to inhibit HT-1080 cell binding to domain VI (Fig. 2), whereas the other peptides showed no or only small effects on the cell-domain interaction. A GRGDS peptide, which is reported to block the function of various integrins, had no effect on cell binding. The sequences of peptides S1 and S2 overlap with four residues, but only S2 showed strong activity when tested in a direct cell binding assay (data not shown). The S6 and S7 peptides overlap by eight residues and showed no activity when tested for direct cell binding (data not shown). None of the peptides inhibited cell binding to laminin-10/11, suggesting that other cell-binding sites are available on the intact molecule (data not shown). Taken together, these results suggest that two sequences: S2, 125RGQVFQVAY135 and S6, 225RDFTKATNIIRLFLR239, separated by ~90 residues, directly interact with cell surface receptors within domain VI.

### RESULTS

**Expression and Cell Binding Activity of Recombinant Laminin α5 N-terminal Globular Domain VI**— Constructs were generated for expression of mouse laminin α5 domain VI and the first four EGF-like repeats of domain V in mammalian cells (Fig. 1). In addition, a similar construct was also generated for laminin α1 domain VI, which corresponds to that described previously (15). The secreted recombinant proteins were purified by Ni-agarose chromatography from the conditioned medium of transfected COS-7 cells with a yield of 1–2 μg/ml medium. The purity of the protein preparations was more than 95% as judged from Coomassie Blue-stained gels after SDS-polyacrylamide gel electrophoresis (data not shown). Recombinant fragments containing domains VI through IV of the laminin α5 and α2 chains have previously been shown to promote binding of HT-1080 fibrosarcoma cells (13, 14). This cell line was therefore used to analyze the adhesive properties of the recombinant laminin α5 domain VI. HT-1080 cells showed strong binding to domain VI, while the recombinant domain V, consisting of the EGF-like repeats, showed no cell binding, demonstrating that the cell binding activities reside in domain VI (Fig. 1).

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cell binding, as the mutant domain VI with Y130A (A5VI-Y130A) reduced cell binding to ~25% of that of wild-type domain VI (Fig. 4).

The S6 peptide overlaps with two previously reported active synthetic peptides (A-24 and A-25) derived from the laminin α1 chain (19). The S6 sequence is characterized by five basic residues (four Arg and one Lys). Notable, three of the four Arg residues are conserved in domain VI of all the laminin α chains (Fig. 3). To analyze the contribution of the individual Arg/Lys residues within the S6 site to cell binding, we generated five single substitution mutants by replacing Arg/Lys residues with Ala (R225A, K229A, R234A, R236A, and R239A). All the mutants were expressed at a level similar to wild-type domain VI, suggesting that the mutations did not cause unstable synthesis of domain VI due to unfolding of the proteins (data not shown).

Cell binding to A5VI-K229A was very poor, while neither A5VI-R234A nor A5VI-R236A had a significant effect on cell binding (Fig. 4). R225A and R239A also showed a significant reduction in cell binding, by ~60% of the wild type (Fig. 4). These data indicate that four residues, Tyr, Arg, Lys, and Arg, within these two regions are crucial for cell binding.

**Kinetic Analysis of Interactions between Heparin and Laminin α5 Domain VI Mutants using Surface Plasmon Resonance—**

Since heparin binding activity has been localized to domain VI of the laminin α1 and α2 chains (13–15), cell binding to recombinant laminin α5 domain VI was examined in the presence of heparin and other heparin-like glycosaminoglycans. Heparin and heparan sulfate inhibited HT-1080 cell binding to domain VI, whereas keratan sulfate was much less inhibitory (Fig. 5). These results indicate that the heparin-binding site overlaps with the cell-binding sites.

To examine the relationships between the heparin and cell-binding site, the binding kinetics between immobilized biotinylated heparin and the laminin α5 domain VI mutants were measured directly by real-time biomolecular interaction analysis using surface plasmon resonance on a BIAcoreTM system. Similar equilibrium dissociation constants ($K_d \approx 9–16$ nM) were obtained for the interactions of wild-type domain VI of the laminin α5 chain and five of the mutants with heparin (Table II). Less than a 2-fold difference was observed for the kinetic binding constants between wild-type domain VI and five of the mutants including Y130A, R225A, K229A, R234A, and R236A. Statistically significant differences in kinetic constants derived from BIAcore experiments are generally considered to be at least 5–10-fold. This demonstrates that the heparin binding activity was unchanged, indicating that the structural integrity of domain VI was maintained in these mutants and that these positions were not part of the heparin-binding site. In contrast, no binding of the R239A mutant to heparin was observed even at a high protein concentration (200 nM). Mutations

![Cell binding to laminin α5 domain VI mutants.](http://www.jbc.org/)

**Fig. 4.** Cell binding to laminin α5 domain VI mutants. Wells were coated with laminin α5 domain VI mutants (10 μg/ml). HT-1080 cells were added, and crystal violet staining was used to assess the number of attached cells after 1 h. Data are given as percentage of cell binding to the wild-type laminin α5 domain VI (A5VI). Each value represents the mean of three separate determinations ± S.D. Duplicate experiments gave similar results.

![Effects of heparin/heparan sulfate and EDTA on cell binding to laminin α5 domain VI.](http://www.jbc.org/)

**Fig. 5.** Effects of heparin/heparan sulfate and EDTA on cell binding to laminin α5 domain VI. HT-1080 binding assays were performed on control without inhibitors (C), or in the presence of heparin (HP), heparan sulfate (HS), keratan sulfate (KS), and 5 mM EDTA. Each value represents the mean of three separate determinations ± S.D. Duplicate experiments gave similar results.

![Table II](http://www.jbc.org/)

**Table II**

| Mutants  | $k_d$         | $k_d$        | $K_d$       |
|----------|---------------|--------------|-------------|
| A5VI     | $7.9 \times 10^{-4}$ | $7.1 \times 10^{-4}$ | 9 ± 3       |
| A5VI-Y130A | $2.6 \times 10^{-4}$ | $4.2 \times 10^{-4}$ | 16 ± 4     |
| A5VI-R225A | $3.3 \times 10^{-4}$ | $4.4 \times 10^{-4}$ | 13 ± 3     |
| A5VI-R234A | $3.1 \times 10^{-4}$ | $3.8 \times 10^{-4}$ | 8 ± 2      |
| A5VI-R236A | $7.7 \times 10^{-4}$ | $4.5 \times 10^{-4}$ | 5 ± 2      |
| A5VI-R239A | $9.0 \times 10^{-4}$ | $7.8 \times 10^{-4}$ | 9 ± 4      |
| A1VI     | $10.4 \times 10^{-4}$ | $47.1 \times 10^{-4}$ | 45 ± 6     |
| A5V      | No binding    | No binding   | No binding  |

**a** No uptake of resonance units was detected.
tion of Arg^{239} was also found to be critical for cell binding, reducing activity by ~60% compared with wild-type domain VI (see above). Consistent with the cell binding results, no heparin binding was observed for a recombinant protein consisting of the first four EGF-like repeats of domain V. These results demonstrate that the heparin-binding site is located within domain VI. Interestingly, the binding affinity of domain VI of the laminin α1 chain to heparin was about 4-fold weaker ($K_d = 45 \text{ nM}$), demonstrating differences in affinity for heparin between the laminin domain VI isomers. Taken together, these results indicate that Arg^{239} within the S6 site is critical for both heparin and cell binding.

**Effect of Integron Monoclonal Antibodies on Cell Binding to Laminin α5 Domain VI**—Cell binding to laminin α5 and α1 domain VI and to laminins containing either the α5 chain (laminin-10/11) or the α1 chain (laminin-1) was examined in the presence of different anti-integrin antibodies to identify integrin receptors involved in domain VI binding. The β1 integrin antibody demonstrated partial inhibition of HT-1080 cell binding to laminin-10/11 but strong inhibition of cell binding to laminin-1 (Fig. 6). The monoclonal antibody against integrin α6 inhibited cell binding to laminin-10/11 but not laminin-10/11, while a monoclonal antibody against integrin α6 showed a weak inhibitory effect on cell binding to laminin-10/11. Other antibodies against integrin subunits, including β1, α1, α2, α3, α5, and αv, had no effect on cell binding. Cell binding to both laminin-1 and laminin-10/11 using the different α1 integrin receptors mediated HT-1080 cell binding to laminin-10/11 and that the α5β1 integrin binds laminin-10/11, agreeing with previous reports (6, 7).

The monoclonal antibody against the β1 integrin subunit inhibited HT-1080 binding to recombinant domain VI of both laminin α5 and α1 (Fig. 7, A and B). These results show that β1 subunit containing integrins is critical for cell binding to domain VI of both chains. Furthermore, 5 mM EDTA was also found to inhibit cell binding, supporting cation and integrin dependence of the interaction (Fig. 5). We then tested several monoclonal antibodies against integrin α subunits to identify partners for the β1 integrins. Anti-integrin α5 antibodies strongly inhibited HT-1080 cell binding to laminin α5 and α1 domain VI (Fig. 7, A and B). Monoclonal antibodies against α1, α5, and αv had no or only small effects on cell binding, indicating that these integrins were not major mediators of cell binding to laminin α5 and α1 domain VI (Fig. 7, A and B). HT-1080 cell binding to laminin α5 and α1 domain VI was reduced to about 30–50% by anti-integrin α3 and α4 antibodies. Integrin α6 antibody also weakly reduced cell binding to laminin α5 domain VI but not to laminin α1 domain VI (Fig. 7B). Taken together, these results identify α5β1 integrin as a major receptor to α5 domain VI.

**DISCUSSION**

The newly discovered laminin α5 chain has been implicated in various biological activities, such as angiogenesis and nerve regeneration. Previously, we identified a cell-binding site within the C-terminal G domain of the mouse laminin α5 chain using recombinant proteins and synthetic peptides (21). In the present study, we identified cell-binding sites on the laminin α5 N-terminal globular domain VI.

Recombinant proteins of laminin α5 domain VI and the first four EGF-like repeats of domain V were generated by mammalian expression. The recombinant protein was highly active for HT-1080 fibrosarcoma cell binding, while a recombinant protein consisting of only the EGF-like repeats showed no binding, indicating that domain VI contributed to the cell-binding site. This result is similar to previous reports from studies on recombinant fragments consisting of domains VI through IV, of the laminin α1 and α5 chains (13–15). In addition, recombinant laminin α5 domain VI was also found to bind other cell lines including mouse B16-F10 melanoma and mesangial cells, while weak binding was observed for a human submandibular gland cell line, indicating cell type-specific interaction of this domain (data not shown).

To localize cell-binding sites on laminin α5 domain VI, we screened domain VI-derived synthetic peptides for their effect on HT-1080 cell/domain VI interactions. Four peptides: S1, EVNVTLDLGQVFH; S2, GQVFHYVYLKF; S6,
225RDFTKATNIRLRFLR239; and S7, 232NIRLRFLRTNTL243, inhibited cell binding. These peptides corresponded to sequences in the laminin α1 chain (A-12, A-13, A-24, and A-25) previously reported to be highly active for cell binding (19). The S1 and S2 peptides overlap, as do S6 and S7, thus the results indicate that at least two sequences, 123GQVFHVAYVLIKF135 and 225RDFTKATNIRLRFLR239, spaced by ~90 residues, directly interact with cell surface receptors within domain VI (Fig. 3). Alanine mutagenesis of recombinant domain VI identified four positions within these two sequences as critically involved in cell binding: Tyr130, Arg225, Lys229, and Arg239. A comparison with the sequences of domain VI of the laminin α1, α2, and α3B chains reveals that Tyr130 and Arg239 are conserved, while Arg225 and Lys229 are conserved between the α3 and α5 chains, indicating that the position of the cell-binding sites varies. These differences may be important for regulation and specificity of receptor interactions within domain VI. The site-directed mutagenesis data also demonstrate that the two sites together contribute to a cell binding epitope and imply that these cell-binding sites are highly dependent on the conformation of domain VI. The importance of the three-dimensional structural cell-binding sites has also been shown for other integrin ligands, including fibronectin and vascular cell adhesion molecule-1, where multiple contacts, involving several different ligand peptide segments, are formed between ligand and receptor (22, 23).

Kinetic data obtained here by surface plasmon resonance analysis for six single-site alanine substitution mutants of laminin α5 domain VI demonstrate that one position (Arg239) within the cell-binding site is also critical for heparin interactions. Cell binding to domain VI was sensitive to inhibition by heparin/heparan sulfate, demonstrating overlap of cell and heparin-binding sites. Interestingly, the binding constants for binding to heparin show a 4-fold difference between domain VI of the laminin α5 and α1 chain, with the α5 domain demonstrating highest affinity. Strong heparin binding affinity suggests the potential to bind heparan sulfate containing matrix molecules or cell surface receptors (21, 24, 25); accordingly, these molecules may interact mainly with the basic residues within the S6 site. Our results suggest that heparan sulfate-containing cell surface receptor interaction is required for efficient cell binding to laminin α5 domain VI. This is in accordance with previous studies, which indicate that heparan sulfate-containing cell surface receptors can function as co-receptors for integrins and that these co-receptors are essential for cell binding to some ligands, including the heparin III domain of fibronectin and the angiogenic inducer Cyr61 (17, 26). The heparan sulfate-containing cell surface receptors may include syndecan-1 or α-dystroglycan; the latter has been shown to bind laminin-10/11 and domain VI of the laminin α1 chain (8, 24). The role of these interactions may be important for cell type-specific binding or signaling (17, 26). The heparin-binding site on laminin α5 domain VI may also function as a binding site for other matrix molecules, since it has been reported that laminin α1
domain VI binds to heparan sulfate chains of perlecain (24). Accordingly, binding through the heparin-binding site may be a mechanism for the regulation of interactions with cells and matrix assembly.

Several integrins have previously been implicated as receptors for laminin-10/11, including α3β1, α3β1, and α6β1 (7). In this study, we demonstrate that domain VI of the laminin α5 chain is a binding site for integrins α6β1, α5β1, α6β1, and α5β1. HT-1080 cell binding was completely blocked by anti-integrin α3 or β1 antibodies, while function-blocking antibodies against the α2, α4, and α6 integrins showed a weaker effect, suggesting that the α5β1 integrin is a major mediator of cell binding. Small or no effects were observed with antibodies against α1, α5, and αv integrins. Comparison with a recombinant protein of laminin α1 domain VI showed similar integrin specificity except for α6, where no effect was observed for laminin α1 domain VI. The inhibition results are in agreement with the reported integrin specificity of recombinant fragments of domains VI through IV of the laminin α1 and α2 chains (13, 14). The previous studies used the same cell line as here, but only results using anti-integrin α1 and α2 antibodies were reported. Our results using various monoclonal antibodies suggest that several integrins bind domain VI of the laminin α1 and α1 chains and that these receptors bind similar recognition sites within domain VI.

In conclusion, the present data represent the first mapping of sites within the N-terminal globular domain VI of the mouse laminin α5 chain responsible for cell binding. Our results suggest that heparan sulfate-containing receptors and integrins recognize domain VI. We found that two sequences, spaced by ~90 residues within laminin α5 domain VI, are critical for cell surface receptor binding and that at least two residues within these two regions together form a binding site(s) critical for receptor binding.

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