Molecular Basis of the Recognition of Nephronectin by Integrin α8β1

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Integrin α8β1 interacts with a variety of Arg-Gly-Asp (RGD)-containing ligands in the extracellular matrix. Here, we examined the binding activities of α8β1 integrin toward a panel of RGD-containing ligands. Integrin α8β1 bound specifically to nephronectin with an apparent dissociation constant of 0.28 ± 0.01 nM, but showed only marginal affinities for fibronectin and other RGD-containing ligands. The high-affinity binding to α8β1 integrin was fully reproduced with a recombinant nephronectin fragment derived from the RGD-containing central “linker” segment. A series of deletion mutants of the recombinant fragment identified the LFEIFEIER sequence on the C-terminal side of the RGD motif as an auxiliary site required for high-affinity binding to α8β1 integrin. Alanine scanning mutagenesis within the LFEIFEIER sequence defined the EIE sequence as a critical motif ensuring the high-affinity integrin-ligand interaction. Although a synthetic LFEIFEIER peptide failed to inhibit the binding of α8β1 integrin to nephronectin, a longer peptide containing both the RGD motif and the LFEIFEIER sequence was strongly inhibitory, and was ∼2,000-fold more potent than a peptide containing only the RGD motif. Furthermore, trans-complementation assays using recombinant fragments containing either the RGD motif or LFEIFEIER sequence revealed a clear synergism in the binding to α8β1 integrin. Taken together, these results indicate that the specific high-affinity binding of nephronectin to α8β1 integrin is achieved by bipartite interaction of the integrin with the RGD motif and LFEIFEIER sequence, with the latter serving as a synergy site that greatly potentiates the RGD-driven integrin-ligand interaction but has only marginal activity to secure the interaction by itself.

Integrins are a family of adhesion receptors that interact with a variety of extracellular ligands, typically cell-adhesive proteins in the extracellular matrix (ECM). They play mandatory roles in embryonic development and the maintenance of tissue architectures by providing essential links between cells and the ECM. Integrins are composed of two non-covalently associated subunits, termed α and β. In mammals, 18 α and 8 β subunits have been identified, and combinations of these subunits give rise to at least 24 distinct integrin heterodimers. Based on their ligand-binding specificities, ECM-binding integrins are classified into three groups, namely laminin-, collagen- and RGD-binding integrins (2, 3), of which the RGD-binding integrins have been most extensively investigated. The RGD-binding integrins include α5β1, α8β1, α1β3, and αv-containing integrins, and have been shown to interact with a variety of ECM ligands, such as fibronectin and vitronectin, with distinct binding specificities.

The α8 integrin subunit was originally identified in chick nerves (4). Integrin α8β1 is expressed in the metanephric mesenchyme and plays a crucial role in epithelial-mesenchymal interactions during the early stages of kidney morphogenesis. Disruption of the α8 gene in mice was found to be associated with severe defects in kidney morphogenesis (5) and stereocilia development (6). To date, α8β1 integrin has been shown to bind to fibronectin, vitronectin, osteopontin, latency-associated peptide of transforming growth factor-β1, tenascin-W, and nephronectin (also named POEM) (7–13), among which nephronectin is believed to be an α8β1 integrin ligand involved in kidney development (10).

Nephronectin is one of the basement membrane proteins whose expression and localization patterns are restricted in a tissue-specific and developmentally regulated manner (10, 11). Nephronectin consists of five epidermal growth factor-like repeats, a linker segment containing the RGD cell-adhesive motif (designated RGD-linker) and a meprin-A5 protein-receptor protein-tyrosine phosphatase μ (MAM) domain (see Fig. 3A). Although the physiological functions of nephronectin remain only poorly understood, it is thought to play a role in epithelial-mesenchymal interactions through binding to α8β1 integrin, thereby transmitting signals from the epithelium to the mesenchyme across the basement membrane (10). Recently, mice deficient in nephronectin expression were produced by homologous recombination (14). These nephronectin-deficient mice frequently displayed kidney agenesis, a phenotype reminiscent of α8 integrin knock-out mice (14), despite the fact that other RGD-containing ligands, including fibronectin and osteopontin, were expressed in the embryonic kidneys (9, 15). The failure of the other RGD-containing ligands to compensate for the deficiency of nephronectin in the developing kidneys suggests that nephronectin is an indispensable α8β1...
ligand that plays a mandatory role in epithelial-mesenchymal interactions during kidney development.

Although ligand recognition by RGD-binding integrins is primarily determined by the RGD motif in the ligands, it is the residues outside the RGD motif that define the binding specificities and affinities toward individual integrins (16, 17). For example, αβ1 integrin specifically binds to fibronectin among the many RGD-containing ligands, and requires not only the RGD motif in the 10th type III repeat but also the so-called “synergy site” within the preceding 9th type III repeat for fibronectin recognition (18). Recently, DiCara et al. (19) demonstrated that the high-affinity binding of αVβ6 integrin to its natural ligands, e.g. foot-and-mouth disease virus, requires the RGD motif immediately followed by a Leu-Xaa-Xaa-Leu/Ile sequence, which forms a helix to align the two conserved hydrophobic residues along the length of the helix. Given the presence of many naturally occurring RGD-containing ligands, it is conceivable that the specificities of the RGD-binding integrins are dictated by the sequences flanking the RGD motif or those in neighboring domains that come into close proximity with the RGD motif in the intact ligand proteins. However, the preferences of αβ1 integrin for RGD-containing ligands and how it secures its high-affinity binding toward its preferred ligands remain unknown.

In the present study, we investigated the binding specificities of αβ1 integrin toward a panel of RGD-containing cell-adhesive proteins. Our data reveal that nephronectin is a preferred ligand for αβ1 integrin, and that a LFEIFEIER sequence on the C-terminal side of its RGD motif serves as a synergy site to ensure the specific high-affinity binding of nephronectin to αβ1 integrin.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—HT1080 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HT1080 cells were transfected with an expression vector for human full-length α8 integrin (described below). The cells were passaged at 24 h after transfection and maintained in medium containing 0.8 mg/ml G418 to select stable transfectants. The transfectants were subjected to selection by cell adhesion to a nephronectin-coated substratum as follows. The cells were seeded onto 6-well plates (Falcon) that had been coated with 10 nm nephronectin and blocked with phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA), and allowed to adhere for 30 min at 37 °C. After removal of non-adherent cells using serum-free medium, the remaining cells were detached with PBS containing 0.025% trypsin and 1 mM EDTA, plated on 6-well plates and grown to confluence. The resulting cells (designated HT1080-A8 cells) were cloned by limiting dilution and used for cell adhesion assays. K562 human erythroleukemic cells stably transfected with a chicken α8 integrin cDNA (K8A cells) were kindly provided by Dr. Louis F. Reichardt (University of California, San Francisco, CA) and maintained as described previously (7).

Human plasma fibronectin and vitronectin were purified from outdated human plasma by gelatin and heparin affinity chromatography, respectively, as described previously (20, 21). Human fibrinogen was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). A polyclonal antibody against actin and a horseradish peroxidase-conjugated monoclonal antibody (mAb) against FLAG tag were obtained from Sigma. An horseradish peroxidase-conjugated polyclonal antibody against glutathione S-transferase (GST) was purchased from GE Healthcare. Horseradish peroxidase-conjugated peanut agglutinin (PNA) was purchased from Seikagaku Co. (Tokyo, Japan). mAbs against the human integrin α8 subunit were produced by fusion of Sp2 mouse myeloma cells with spleen cells from mice immunized with recombinant soluble integrin αβ1, and selected by both positive reactivity for recombinant integrin αβ1 and negative reactivity for recombinant integrin αβ5. mAb 10A8 was capable of binding to the denatured integrin α8 chain on immunoblots under reducing and non-reducing conditions. mAb 7A5 was capable of binding to the non-denatured integrin α8 chain, and was used for flow cytometry. Synthetic peptides were purchased from Biologica Co. (Nagoya, Japan) and dissolved into 100% dimethyl sulfoxide.

**Expression Vectors**—A mouse nephronectin cDNA was obtained from Dr. Yoshihide Hayashizaki (Riken, Yokohama, Japan). The cDNA was subcloned into the pFLAG-CMV vector (Invitrogen) in-frame with a FLAG tag at the 3' end. For expression of a panel of nephronectin deletion mutants in mammalian cells, a cDNA fragment encoding a mouse nephronectin 5'-non-translated region and the signal peptide (nucleotides −61 to 69) was amplified by PCR with the primer set 5'-GAATTC-TTGGAGATCCCGGGACGC-3' (forward) and 5'-GGGC-CCGTCAAAGTTGGGCACGC-3' (reverse) using the full-length nephronectin cDNA as a template. This fragment was digested with EcoRI and Apal, and fused in-frame with cDNAs encoding the RGD-linker segment or the MAM domain. The cDNAs encoding the RGD-linker and the MAM domain were amplified by PCR with primer sets 5'-GGGCCCCAAGTCTGATTGTAAC-3' (forward) and 5'-GTCGACGCTGCTTCTATTACTTTCTC-3' (reverse), and 5'-GGGCCCCGTTCTCTCATGACACGC-3' (forward) and 5'-GTCGACGCTGCTTCTACTTCAAGCTT-3' (reverse), respectively. After verification by DNA sequencing, each PCR-amplified cDNA was digested with Apal and Sall, and inserted into the EcoRI and Sall sites of the pFLAG-CMV vector together with the cDNA encoding the 5'-non-translated region and signal peptide.

An expression vector for the RGD-linker as a GST fusion protein was prepared as follows. A cDNA encoding the RGD-linker was amplified by PCR with the primer set 5'-GTCGACGCTGCTTCTACTCTCTCATGACACGC-3' (forward) and 5'-GTCGACGCTGCTTCTACTTCAAGCTT-3' (reverse) using an E13.5 mouse embryo cDNA as a template. The PCR product was subcloned into the pBluescriptII SK+ vector (Stratagene, La Jolla, CA), digested with EcoRI and Sall, and inserted into the corresponding restriction sites of the pGEX 4T-1 expression vector (GE Healthcare) (designated pGEX-RGD-linker). cDNAs encoding a series of deletion mutants and substitution mutants of the RGD-linker region were amplified by PCR using pGEX-RGD-linker encoding the GST-RGD-linker fusion protein as a template. A list of the primer sequences used for PCR is available upon request. The PCR products were digested with...
Expression and Purification of Recombinant Proteins—For purification of recombinant nephronectin, its mutants and integrins, Freestyle™-293-F cells were transiently transfected with individual plasmids using a Freestyle 293 Expression system (Invitrogen) according to the manufacturer’s instructions. Conditioned media were collected at 48–72 h after transfection and centrifuged to remove cells and debris. For purification of nephronectin and its mutants, EDTA (5 mM), phenylmethylsulfonyl fluoride (1 mM), and sodium azide (0.02%) were added to the conditioned media. The media were then incubated with anti-FLAG M2 affinity beads (Sigma) overnight with gentle agitation at 4 °C. The beads were transferred into an empty column and washed with PBS. Bound proteins were eluted with 100 μg/mL FLAG peptide dissolved in PBS. The purified proteins were dialyzed against 2 mM CAPS buffer (pH 11.4) containing 0.5 mM EDTA, and used in the following assays. For purification of recombinant integrins, phenylmethylsulfonyl fluoride (1 mM) and sodium azide (0.02%) were added to the conditioned media. The media were then incubated with anti-FLAG M2 affinity beads (Sigma) overnight with gentle agitation at 4 °C. The beads were transferred into an empty column and washed with PBS. Bound proteins were eluted with 100 μg/mL FLAG peptide dissolved in PBS. The purified proteins were dialyzed against 2 mM CAPS buffer (pH 11.4) containing 0.5 mM EDTA, and used in the following assays. For purification of recombinant integrins, phenylmethylsulfonyl fluoride (1 mM) and sodium azide (0.02%) were added to the conditioned media. The media were then incubated with nickel-nitrirotiacetic acid-agarose beads (Qiagen, Valencia, CA), followed by washing with Tris-buffered saline (TBS). Bound proteins were eluted with TBS containing 200 mM imidazole. The eluted proteins were applied to anti-FLAG M2 affinity beads, and the bound proteins were eluted with 100 μg/mL FLAG peptide and dialyzed against TBS.

Gel Filtration—Purified nephronectin (500 μL) was loaded on a Superose 6 gel filtration column (10 × 300 mm; GE Healthcare) equilibrated in PBS. Fractions were collected at a flow rate of 0.2 mL/min and monitored for their absorbance at 280 nm. The column was calibrated with the following molecular mass standards: chymotrypsinogen, 25 kDa; ovalbumin, 43 kDa; albumin, 22 kDa; ferritin, 440 kDa; thyroglobulin, 669 kDa.

Integrin Binding Assay—Integrin binding assays were performed as described previously (23). Briefly, microtiter plates were coated with various substrate proteins (10 nM) overnight at 4 °C, and then blocked with 10 mg/mL BSA. In trans-complementation assays, the plates were coated with the first substrate protein, washed with PBS, coated with the second substrate proteins overnight at 4 °C, and then blocked with BSA. The plates were incubated with integrins in the presence of 1 mM MnCl₂ with or without 10 mM EDTA. In some experiments, integrins were incubated on the plates in the presence of 1 mM CaCl₂ and 1 mM MgCl₂ to see the effects of divalent cations or in the presence of synthetic peptides at various concentrations to evaluate their inhibitory activities. The plates were washed with TBS containing 1 mM MnCl₂, 0.1% BSA, and 0.02% Tween 20 with or without 10 mM EDTA, followed by quantification of bound integrins by an enzyme-linked immunosorbent assay using a biotinylated rabbit anti-Velcro (ACID/BASE coiled-coil) antibody and horseradish peroxidase-conjugated streptavidin. Dissociation constants were calculated by saturation binding assays as described previously (27).

Flow Cytometry—Flow cytometric analyses were performed as described previously (28). Suspended cells were incubated with anti-integrin mAbs for 30 min at 4 °C. After washing with PBS, the cells were incubated with an Alexa Fluor® 488-labeled secondary antibody (Molecular Probes, Eugene, OR) for 30 min at 4 °C and subjected to flow cytometric analysis using a FACScan flow cytometer (BD Biosciences).

Cell Adhesion Assays—Microtiter plates were coated overnight at 4 °C with substrate proteins diluted to various concentrations in PBS in a volume of 50 μL/well. The coating efficiencies of GST fusion proteins were determined by their reactivities with an anti-GST polyclonal antibody. The plates were blocked with blocking medium (serum-free Dulbecco’s modified Eagle’s medium containing 10 mg/mL BSA and 10 mM HEPES, pH 7.5) for 1 h at 37 °C. HT1080-A8 cells were suspended in blocking medium and plated at 4.0 × 10⁴ cells/well and incubated for 30 min at 37 °C in a humidified atmosphere of 5% CO₂ in air. For cell adhesion inhibition assays, cells were preincubated with synthetic peptides in blocking medium at room temperature for 10 min prior to plating. After removal of nonadherent cells by washing with blocking medium, the attached cells were fixed with 3.7% formaldehyde, washed three times with PBS, and stained for 10 min with 0.1% toluidine blue in PBS. After lysis in 1% SDS, the attached cells were quantified by their absorption at 595 nm.
RESULTS

α8β1 Integrin Preferentially Binds to Nephronectin—To examine the binding specificities of α8β1 integrin toward nephronectin and other RGD-containing ECM ligands, we expressed and purified recombinant nephronectin using a mammalian expression system. A FLAG tag was added to the C terminus of nephronectin to facilitate affinity purification of the recombinant protein. The authenticity of the purified nephronectin was verified by SDS-PAGE and immunoblotting against the FLAG tag (Fig. 1A). Purified nephronectin gave a broad band at ~120 kDa under reducing conditions, which was significantly larger than the predicted protein size (61 kDa). The apparent discrepancy was probably caused by modification with a large number of O-linked sugar chains, which has been predicted to occur at the mucin-like region within the central linker segment (10, 11). The recombinant nephronectin gave a less prominent band at ~120 kDa upon immunoblotting with an anti-FLAG mAb under non-reducing conditions (Fig. 1A), possibly due to the occurrence of disulfide-bonded multimers that did not enter the resolving gel. Multimer formation by nephronectin was further confirmed by gel filtration chromatography using a Superose 6 column. Nephronectin eluted at a position slightly after the void volume (Fig. 1B). The second major peak that eluted at 21 ml was identified by N-terminal amino acid sequencing as the FLAG peptide used for elution of recombinant nephronectin from the immunoaffinity column (data not shown). Multimer formation by recombinant nephronectin was further confirmed by native PAGE (data not shown).

Purified recombinant nephronectin was subjected to α8β1 integrin binding assays along with other RGD-containing proteins including fibronectin, vitronectin, and fibrinogen. The assays were performed in the presence of 1 mM Mn²⁺ to fully activate integrins. Although α8β1 integrin was previously reported to bind to fibronectin and vitronectin (7, 8), the binding activity of α8β1 integrin toward nephronectin far exceeded those toward fibronectin, vitronectin, and fibrinogen (Fig. 2A). Saturation binding assays revealed that α8β1 integrin bound to nephronectin with a dissociation constant of 0.28 ± 0.01 nM, which was approximately 2 orders of magnitude lower than that for fibronectin (Fig. 2B). Vitronectin and fibrinogen exhibited only marginal binding activities, even at the highest α8β1 integrin concentration examined. The low binding affinities of α8β1 integrin toward fibronectin, vitronectin, and fibrinogen were not due to inactivation of these adhesive proteins because they retained their abilities to bind to their specific integrin receptors, namely α5β1, αVβ3, and αIIbβ3, respectively (data not shown). Taken together, these results demonstrate that nephronectin is a preferred ligand for α8β1 integrin among RGD-containing proteins known to bind to α8β1 integrin.

The RGD-linker Segment of Nephronectin Harbors the α8β1 Binding Activity—Because the RGD motif is situated within the linker segment connecting the N-terminal epidermal growth factor-like repeats and the C-terminal MAM domain, we examined whether the RGD-linker segment could recapitulate the high-affinity binding to α8β1 integrin. To this end, we expressed the RGD-linker in mammalian cells or as a GST fusion protein in bacteria (Fig. 3A). We also expressed the C-terminal MAM domain in mammalian cells and a GST fusion protein with a C-terminal extension of the PRGDV sequence of nephronectin in bacteria as controls. The RGD-linker expressed in 293-F cells gave a broad band migrating at 35–60 kDa upon SDS-PAGE, whereas the RGD-linker expressed as a GST fusion protein in bacteria gave a sharp band at 50 kDa (Fig. 3B). The apparent heterogeneity of the RGD-
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The purified RGD-linkers were assayed for their binding activities toward α8β1 integrin (Fig. 3C). Not only the full-length nephronectin but also the RGD-linkers, irrespective of the cells used for recombinant protein expression, bound strongly to α8β1 integrin, whereas the MAM domain did not show any binding activity. Furthermore, the PRGDV peptide expressed at the C terminus of GST had only marginal, if any, binding activity toward α8β1 integrin, even though it contained the RGD motif. Because the GST-fused PRGDV peptide retained the ability to bind to αVβ3 integrin (see Fig. 4D), these results indicate that the RGD motif is necessary but not sufficient for binding of nephronectin to α8β1 integrin. Saturation binding assays revealed that the titration curves of the RGD-linkers were essentially the same as that of full-length nephronectin (Fig. 3D), yielding dissociation constants of 0.2–0.3 nM (Table 1). We also performed saturation binding assays in the presence of 1 mM Ca²⁺ and Mg²⁺, instead of 1 mM Mn²⁺, and confirmed that the activities of the RGD-linkers to bind to α8β1 integrin were the same as that of full-length nephronectin (data not shown). These results indicate that the linker segment is sufficient for the high-affinity recognition of nephronectin by α8β1 integrin. However, the PRGDV peptide modeled after nephronectin was barely active in binding to α8β1 integrin, even at the highest α8β1 integrin concentration tested, suggesting that in addition to the RGD motif, the linker segment contains a region that potentiates the binding activity of nephronectin to α8β1 integrin.

Identification of the Region within the Linker Segment That Warrants High Affinity Binding to α8β1 Integrin—To explore the region within the linker segment that potentiates the RGD-dependent binding of nephronectin to α8β1 integrin, we produced a series of RGD-linker deletion mutants as GST fusion proteins (Fig. 4A). Because the linker segment expressed as a GST fusion protein was fully competent in high-affinity binding to α8β1 integrin and devoid of the mucin-like sugar chains that have been predicted to attach to the N-terminal two-thirds of the linker segment (based on Net-O-Glyc (29)), we first produced a deletion mutant comprising the C-terminal one-third of the linker segment, designated linker segment (LS)/366–414. LS/366–414 bound avidly to α8β1 integrin with a dissociation constant of 0.32 ± 0.03 nM, comparable with that of the full-length RGD-linker (Fig. 4C and Table 1). Given the retention of the full α8β1 binding activity within LS/366–414, we employed this fragment as a template for the production of a series of deletion mutants (Fig. 4A). The authenticities of the resulting mutant proteins were verified by SDS-PAGE (Fig. 4B).

The purified mutant proteins were assayed for their binding activities toward α8β1 integrin. LS/378–414, a mutant protein lacking the N-terminal 12 amino acid residues of LS/366–414, was capable of binding to α8β1 integrin with a comparable potency to that of LS/366–414 (Fig. 4C and Table 1). On the other hand, LS/366–393, a deletion mutant lacking the C-terminal 21 amino acid residues, was barely active in binding to α8β1 integrin, although the RGD motif remained intact in this mutant. These results indicate that the C-terminal 21 residues (394–414) are required for the high-affinity binding of nephronectin to α8β1 integrin. To further narrow down the residues required for the high-affinity binding to α8β1 integrin.
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![Diagram](image)

**FIGURE 3.** Binding activities of α8β1 integrin to nephronectin and its fragments. **A**, schematic diagrams of full-length nephronectin (NN) and its fragments. RGD-linker and RGD-linker (GST), the central RGD-containing linker segments expressed in mammalian and bacterial expression systems, respectively; PRGDV, a short RGD-containing peptide modeled after nephronectin and expressed as a GST fusion protein (see Fig. 4B for the peptide sequence). The arrowheads indicate the positions of the RGD motif. **B**, purified recombinant proteins were analyzed by SDS-PAGE in 7–15% gradient (left and center panels) and 12% (right panels) gels, followed by Coomassie Brilliant Blue (CBB) staining, immunoblotting with an anti-FLAG mAb, or lectin blotting with PNA. The quantities of proteins loaded were: 0.5 μg (for Coomassie Brilliant Blue staining) and 0.1 μg (for blotting with anti-FLAG and PNA) in the left and center panels; 1 μg in the right panel. **C**, recombinant proteins (10 μm) were coated on microtiter plates and assessed for their binding activities toward N- and C-terminal deletion mutants of LS/378–407, i.e. LS/378–407, LS/378–403, LS/378–401, LS/378–399, and LS/378–393 (Fig. 4A). Among these C-terminal deletion mutants, LS/378–407 and LS/378–403 retained comparable integrin binding activities to that of LS/378–414, whereas LS/378–401, LS/378–399, and LS/378–393 exhibited stepwise reductions in their integrin binding activities, resulting in an almost complete loss of the activity after deletion of residues 394–414 (Fig. 4C and Table 1). We also assessed the binding activities of α8β1 integrin to the C-terminal deletion mutants of the RGD-linker in the presence of 1 mM Ca2+ and Mg2+, instead of 1 mM Mn2+. The stepwise reductions of the binding activities of α8β1 integrin to LS/378–401, LS/378–399, and LS/378–393 were reproduced in the assays performed in the presence of Ca2+ and Mg2+, although the binding activities of the mutants were less pronounced than those attained in the presence of Mn2+ (data not shown). Because LS/378–403 retained almost full activity, these results indicate that residues 394–403 (DLFEIFEIER) are involved in the high-affinity binding to α8β1 integrin.

The RGD motif has been shown to be a prerequisite for the integrin binding activity of nephronectin (10, 11). To confirm the critical role of the RGD motif, we produced a series of N-terminal deletion mutants of LS/378–407, i.e. LS/381–407, LS/391–407, and LS/395–407. Among these mutants, LS/391–407 and LS/395–407, both lacking the RGD motif but retaining the (D)LFEIFEIER sequence, were barely active in binding to α8β1 integrin, whereas LS/381–407, an N-terminal deletion mutant possessing both the RGD motif and the DLFEIFEIER sequence, was fully active in binding to α8β1 integrin (Fig. 4C). Taken together, these results indicate that both the RGD motif and the DLFEIFEIER sequence at 10 residues to the C-terminal side of the RGD motif are required for the high-affinity binding of nephronectin to α8β1 integrin.

In contrast to the clear dependence of α8β1 integrin on both the RGD motif and the DLFEIFEIER sequence for binding to nephronectin, the binding activities of the mutant fragments to αVβ3 integrin, another integrin known to bind to nephronectin (10), remained almost unchanged regardless of the presence or absence of the DLFEIFEIER sequence, as long as the fragments contained the RGD motif (Fig. 4D). Therefore, the DLFEIFEIER sequence is involved in nephronectin recognition by α8β1 integrin but not αVβ3 integrin.

**Identification of Critical Amino Acid Residues in the DLFEIFEIER Sequence**—To further define the amino acid residues in the DLFEIFEIER sequence that are critical for high-affinity binding to α8β1 integrin, we produced a series of alanine substitution mutants of LS/378–403, in which individual amino acid residues of the DLFEIFEIER sequence were substituted.
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A, amino acid sequences of deletion mutants of the RGD-linker segment. The mutant proteins were expressed in bacteria as GST fusion proteins. The RGD motif is indicated in bold. The vector-derived sequences are shown in italics. B, SDS-PAGE profiles of the mutant proteins (1 μg/lane) stained with Coomassie Brilliant Blue. The positions of molecular size markers are shown in the left margin. C and D, binding activities of α8β1 (C) and αVβ3 (D) integrins to the mutant proteins. Microtiter plates were coated with the individual mutant proteins (10 nM) and incubated with the integrins at the indicated concentrations. Bound integrins were quantified as described under “Experimental Procedures.” The results represent the means of duplicate determinations.

**FIGURE 4. Binding activities of α8β1 integrin toward deletion mutants of the RGD-linker segment.**

**TABLE 1**

| Ligands             | K<sub>d</sub> (nM) |
|---------------------|--------------------|
| NN                  | 0.28 ± 0.01        |
| RGD-linker          | 0.23 ± 0.05        |
| MAM                 | ND (--)           |
| RGD-linker (GST)    | 0.22 ± 0.04        |
| PRGDV (GST)         | ND                 |
| LS/366–414          | 0.32 ± 0.03        |
| LS/378–414          | 0.33 ± 0.02        |
| LS/378–393          | ND                 |
| LS/378–407          | 0.33 ± 0.01        |
| LS/378–403          | 0.44 ± 0.04        |
| LS/378–401          | 7.1 ± 0.6          |
| LS/378–399          | 13 ± 1            |
| LS/378–393          | ND                 |
| LS/381–407          | 0.42 ± 0.08        |
| LS/391–407          | ND                 |
| LS/395–407          | ND                 |

**a** Values represent the mean ± S.D. of three independent experiments.  
**b** ND, not determined. The dissociation constant was not determined owing to the absence of significant binding.  
**c** The dissociation constants were not determined owing to only partial saturation at the highest integrin concentration examined.

with alanine (Fig. 5A), except for Asp-394, which is not conserved in nephronectin among different species (supplemental Fig. S1). Although the L395A, E397A, I398A, and R403A substitutions did not cause any detectable decreases in the integrin binding activity of LS/378–403, the E400A, I401A, and E402A mutants exhibited moderate reductions (Fig. 5B), resulting in 2-, 3-, and 5-fold decreases in their integrin binding affinities, respectively, compared with that of LS/378–403 (Table 2). Small, yet reproducible, decreases were also observed for the F396A and F399A mutants.

Given the involvement of two glutamic acid residues (Glu-400 and Glu-402) in nephronectin recognition by α8β1 integrin, we substituted both of these Glu residues with alanine. The resulting double-substitution mutant showed a significant decrease in α8β1 binding activity, although no such additive effects were found when E397A substitution was combined with E400A or E402A substitutions. Therefore, the marked loss of α8β1 binding activity upon the E400A/E402A double substitutions was not simply due to an extra loss of negative charges but due to the involvement of both residues in binding to α8β1 integrin. Despite the substantial impact of the E400A/E402A double mutations on binding to α8β1 integrin, these mutations did not compromise the binding affinity of LS/378–403 toward the αVβ3 integrin (supplemental Fig. S2), underscoring a role of Glu-400/Glu-402 in the high-affinity binding of nephronectin to α8β1 integrin. Given that I401A substitution also caused a moderate decrease in the α8β1 integrin binding activity, these mutations resulted in the possibility that the EIE sequence serves as a bipartite recognition site within nephronectin that directly interacts with α8β1 integrin in concert with the RGD motif.

**Effects of the LFEIFEIER Peptide on the Interaction of α8β1 Integrin with Nephronectin—**To address the role of the LFEIFEIER sequence as one of the bipartite α8β1 integrin recognition sites in nephronectin, we examined whether a synthetic peptide modeled after the sequence was able to inhibit the interaction of nephronectin with α8β1 integrin. We synthesized a 23-mer peptide encompassing the region from the RGD motif to the LFEIFEIER sequence and its mutant forms with RGD → RGE and EIE → AIA substitutions (Fig. 6A), and examined their effects on the binding of nephronectin to α8β1 integrin (Fig. 6B). The 23-mer peptide strongly inhibited the binding of α8β1

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**TABLE 1** Dissociation constants of α8β1 integrin toward nephronectin and its deletion mutants

| Ligands             | K<sub>d</sub> (nM) |
|---------------------|--------------------|
| NN                  | 0.28 ± 0.01        |
| RGD-linker          | 0.23 ± 0.05        |
| MAM                 | ND (--)           |
| RGD-linker (GST)    | 0.22 ± 0.04        |
| PRGDV (GST)         | ND                 |
| LS/366–414          | 0.32 ± 0.03        |
| LS/378–414          | 0.33 ± 0.02        |
| LS/378–393          | ND                 |
| LS/378–407          | 0.33 ± 0.01        |
| LS/378–403          | 0.44 ± 0.04        |
| LS/378–401          | 7.1 ± 0.6          |
| LS/378–399          | 13 ± 1            |
| LS/378–393          | ND                 |
| LS/381–407          | 0.42 ± 0.08        |
| LS/391–407          | ND                 |
| LS/395–407          | ND                 |
integrin to nephronectin with an IC_{50} of ~0.6 nM (Fig. 6B and Table 3). Because this IC_{50} value was comparable with the dissociation constant of αβ1 integrin for nephronectin, the 23-mer peptide appeared to be fully competent in high-affinity binding to αβ1 integrin. Substitution of the EIE motif with AIA resulted in an ~10-fold decrease in the potency of the peptide to inhibit the integrin-nephronectin interaction, whereas substitution of the RGD motif with RGE resulted in an ~1000-fold decrease. These results indicate that, although both RGD and EIE motifs are involved in the binding of αβ1 integrin to nephronectin, the contribution of the RGD motif is much greater than that of the EIE motif in securing the association of the synthetic peptide with αβ1 integrin. We also synthesized two shorter peptides harboring the RGD or EIE motifs (Fig. 6A), and assessed their potencies to inhibit the binding of αβ1 integrin to nephronectin. The PRGDVFIP peptide containing only the RGD motif was moderately inhibitory toward binding with an IC_{50} of 1.2 μM, ~2000-fold less potent than the 23-mer peptide. On the other hand, the LFEIFEIER peptide containing only the EIE motif was barely inhibitory, in good agreement with the dominant contribution of the RGD motif.
over the EIE motif in the association of the 23-mer peptide with α8β1 integrin. The LFEIFEIER peptide did not potentiate the inhibitory activity of the PRGDVFIP peptide when mixed with an equimolar amount of the RGD-containing peptide, suggesting that the RGD and EIE motifs need to be aligned in tandem within the same polypeptide to exert their potency to competitively inhibit the binding of α8β1 integrin to nephronectin.

Trans-complementation of Activity between the RGD and EIE Motifs—To further explore the role of the EIE motif in the high-affinity binding of nephronectin to α8β1 integrin, we employed trans-complementation assays (30) using GST fusion proteins containing only the RGD (LS/378–393) or EIE (LS/395–407) motifs. Plates were coated with LS/378–393, followed by a second coating with increasing concentrations of LS/395–407, and then subjected to integrin binding assays. Plates coated with LS/378–407 or GST were also subjected to a second coating with LS/395–407 and subsequent integrin binding assays as controls. Although LS/378–393 alone was inactive in binding to α8β1 integrin, it became active upon the second coating with increasing concentrations of LS/395–407 (Fig. 7A). A second coating with LS/395–407 at >10 nM also endowed GST with integrin binding activity. However, the activity restored to LS/378–393 was significantly greater than the activity restored to the GST-coated plates, indicating that close juxtaposition of LS/395–407 harboring the EIE motif and LS/378–393 harboring the RGD motif synergistically potentiated their integrin binding activities. Saturation integrin binding assays demonstrated that the affinity of LS/378–393 for α8β1 integrin was significantly enhanced upon a second coating with increasing concentrations of LS/395–407 (Fig. 7B) and reached a dissociation constant of 1.2 nM, which was comparable with that of LS/378–407 containing both the RGD and EIE motifs within the same polypeptide chain. These results support the conclusion that nephronectin has a bipartite α8β1 integrin binding site comprising the RGD and EIE motifs, of which the EIE motif plays an auxiliary role in stabilizing the complex of nephronectin and α8β1 integrin. To further confirm the importance of the EIE motif in high-affinity binding of α8β1 integrin to nephronectin, we performed trans-complementation assays using an LS/395–407 mutant in which the two glutamic acid residues in the EIE motif were replaced with alanine, designated LS/395–407(E400A/E402A). The LS/395–407 mutant was incapable of potentiating the integrin binding activity of LS/378–393, even at the highest concentrations used (Fig. 7C). No such synergism was observed in trans-complementation assays with αVβ3 integrin (supplemental Fig. S3), consistent with the specific role of the EIE motif in selective recognition of nephronectin by α8β1 integrin.

Involvement of the EIE Motif in α8β1 Integrin-dependent Cell Adhesion—The role of the EIE motif in nephronectin recognition by α8β1 integrin was further assessed by cell adhesion assays. To this end, we transfected HT1080 human fibrosarcoma cells with an α8 integrin cDNA and isolated stable transfecants expressing α8 integrin. Expression of the exogenous α8 integrin in the transfecants (designated HT1080-A8 cells) was

TABLE 3
Inhibition of α8β1-nephronectin interaction by synthetic peptides

| Peptides         | IC₅₀ (nM) |
|-----------------|---------|
| 23 AA           | 0.63 ± 0.14 |
| 23 AA-(AIA)     | 7.0 ± 0.9  |
| 23 AA-(RGE)     | 650 ± 50   |
| NN-RGD          | 1200 ± 200 |
| NN-RGE          | ND        |
| NN-EIE          | ND        |
| NN-RGD + NN-EIE | 1300 ± 500 |

* Determined based on the data in Fig. 5B. The values represent the mean ± S.D. of three independent determinations.

* ND, not determined.

FIGURE 7. Trans-complementation assays of recombinant nephronectin fragments. A, 96-well microtiter plates were coated with LS/378–407 (closed squares), LS/378–393 (open squares), and GST (open triangles), washed with PBS, and then coated a second time with increasing concentrations of LS/395–407 lacking the RGD motif. The plates were subjected to integrin binding assays using 1 nM α8β1 integrin as described under "Experimental Procedures." B, titration curves of α8β1 integrin bound to substrates coated with LS/378–393, followed by a second coating with LS/395–407 at 0 (diamonds), 1 (squares), 10 (triangles), and 100 nM (circles). Note that the affinities of α8β1 integrin for LS/378–393 are synergistically enhanced by the presence of LS/395–407. C, microtiter plates were coated with 10 nM LS/378–393, washed with PBS, and then coated a second time with increasing concentrations of LS/395–407 (closed diamonds), LS/395–407(E400A/E402A) (open squares), and GST (open triangles). The plates were subjected to integrin binding assays using 1 nM α8β1 integrin as described under "Experimental Procedures." The results represent the means of duplicate determinations.
controls incubated with mouse IgG instead of an anti-
HT1080-A8 cells to nephronectin was dependent on
whereas LS/378–403, which harbors both the RGD and EIE
motif to RGE resulted in an almost complete loss of the inhib-
ment in stimulating actin cytoskeleton reorganization through
binding to α8β1 integrin, and that the EIE motif within the
fragment is indispensable for stimulation of integrin-mediated
signaling events. Similar results were obtained in cell adhesion
assays using K562 erythroleukemic cells stably transfected with
chick α8 integrin (data not shown).

The importance of the EIE motif for α8β1 integrin-depend-
ent cell adhesion to nephronectin was further addressed by cell
adhesion inhibition assays using synthetic peptides. Adhesion
of HT1080-A8 cells to nephronectin was strongly inhibited by the
23-mer peptide with an apparent IC50 of 3.8 μM (Fig. 9).
Substitution of the two Glu residues in the EIE motif of LS/378–403 resulted in a markedly reduction in the
cell-adhesive activity, leading to comparable activity to that of
LS/378–393.

Integrin-mediated adhesion has been shown to transduce signals
that induce reorganization of the actin cytoskeleton, thereby leading
to cell spreading on substrates. Cells adhering to LS/378–403 exhibited
well spread polygonal shapes at cell coating concentrations of >40 μM,
whereas cells were poorly spread on LS/378–393 and LS/378–
403(E400/402A) even after coating at 320 μM (Fig. 8E). These results
dicate that LS/378–403 is competitive

verified by flow cytometry (Fig. 8A) and immunoblotting (Fig.
8B). HT1080-A8 cells were readily adherent to nephronectin
upon coating on substrates at >1 nM, whereas untransfected
HT1080 cells did not adhere to nephronectin even after coating
on substrates at 40 nM (Fig. 8C), confirming that the adhesion of
HT1080-A8 cells to nephronectin was dependent on α8β1
integrin.

To assess the α8β1 integrin binding activities of the RGD-
linker segment and its mutant proteins on the basis of cell
adhesion assays, HT1080-A8 cells were allowed to adhere to
substrates coated with increasing concentrations of the linker
segment and its mutants with deletions and/or amino acid sub-
stitutions. The RGD-linker was equally active to intact
nephronectin in promoting the adhesion of HT1080-A8 cells,
whereas LS/378–403, which harbors both the RGD and EIE
motifs and retains comparable α8β1 integrin binding activity to
that of intact nephronectin, was significantly less active than
intact nephronectin and the control RGD-linker in promoting
the adhesion of HT1080-A8 cells (Fig. 8D). The difference
between the cell-adhesive activities of the full-length RGD-linker and
LS/378–403 could be due to the involvement of non-integrin-type
adhesion receptors that possibly recognize the N-terminal region of
the linker segment absent from LS/378–403. LS/378–393, which
contains the RGD motif but not the EIE motif, was significantly less
active than LS/378–403. A similar reduction in cell-adhesive activity
was also observed with LS/378–393 in cell adhesion assays performed in
the presence of Mn2+ (data not shown), together corroborating the
importance of the EIE motif in the potent cell-adhesive activity of
nephronectin. In support of the critical

Bipartite Recognition of Nephronectin by Integrin α8β1

**DISCUSSION**

In the present study, we investigated the interactions of α8β1 integrin with a panel of RGD-containing proteins and found that α8β1 integrin binds strongly and preferentially to nephronectin with an ~100-fold higher affinity than those to other RGD-containing proteins including fibronection and vitronectin. In addition to the RGD-containing proteins examined, α8β1 integrin has been shown to bind to osteopontin, the latency-associated peptide of transforming growth factor-β, tenascin-W, MAEG, and QBRICK/Frem1 in RGD-dependent manners (9, 12, 31–33). Although the binding affinities for these ligand proteins remain to be determined, the following observations, together with our data, indicate that nephronectin is the most preferred ligand for α8β1 integrin with the highest binding affinity. Recombinant α8β1 integrin bound to nephronectin, but not to vitronectin or osteopontin, in far-Western blotting assays with tissue extracts (10). Mice deficient in nephronectin expression displayed kidney agenesis or hypoplasia and were associated with a marked reduction in glial cell line–derived neurotrophic factor expression in the metanephric mesenchyme, resembling the phenotypes observed in mice deficient in α8 integrin expression (5, 14). Consistent with the similarities in phenotypes between the nephronectin-deficient and α8 integrin-deficient mice, the distribution patterns of nephronectin in embryonic lungs and kidneys overlapped with those of α8β1 integrin (5, 10, 34, 35). Recently, we examined the distributions of α8β1 ligands by integrin overlay assays using frozen mouse tissues and found that the signals for α8β1 ligands significantly overlapped with those for nephronectin visualized by immunohistochemistry, further supporting the possibility that nephronectin is the physiological ligand for α8β1 integrin with the highest binding affinity among the RGD-containing proteins identified to date.

Our results showed that the RGD-linker segment connecting the N-terminal five epidermal growth factor-like repeats and the C-terminal MAM domain harbors full activity to bind to α8β1 integrin. Although the RGD motif in the linker segment is a prerequisite for the integrin binding activity, several lines of evidence indicate that the LFEIFEIER sequence at ~10 amino acid residues downstream of the RGD motif is required for the high-affinity binding of the linker segment to α8β1 integrin. First, deletion of the LFEIFEIER sequence from the linker segment resulted in a dramatic loss of the integrin binding activity, even though the RGD motif remained unperturbed. Second, trans-complementation assays demonstrated that almost full binding activity toward α8β1 integrin was restored to LS/378–393, a deletion mutant containing only the RGD motif, upon a second coating with LS/395–407, another deletion mutant containing only the LFEIFEIER sequence. Third, a synthetic 23-mer peptide covering the region from the RGD motif to the LFEIFEIER sequence strongly inhibited the binding of α8β1 integrin to nephronectin, whereas shorter peptides containing either the RGD motif or the LFEIFEIER sequence were only poorly inhibitory. Taken together, these results point to the conclusion that the LFEIFEIER sequence synergizes with the RGD motif to ensure the high-affinity binding of nephronectin to α8β1 integrin.

Alanine scanning mutagenesis of the LFEIFEIER sequence revealed that two Glu residues within the sequence, i.e., Glu-400 and Glu-402, play critical roles in the synergy of this sequence with the RGD motif. Alanine substitution of these individual Glu residues resulted in moderate decreases in the integrin binding activity of the linker segment, whereas double substitution of both Glu residues severely impaired the activity. In support of the critical roles of these two Glu residues, E400A/E402A double mutation abrogated the activity of LS/359–407, a mutant fragment containing the LFEIFEIER sequence but not the RGD motif, to potentiate the integrin binding activity of LS/378–393, a fragment containing only the RGD motif, in trans-complementation assays. In addition to these two Glu residues, several hydrophobic residues in the LFEIFEIER sequence may also be involved in nephronectin recognition by α8β1 integrin, because alanine substitution of Phe-396, Phe-399, and Ile-401 caused moderate reductions in the integrin binding activity of the RGD-linker segment. Recently, DiCaro et al. (19) reported that hydrophobic residues downstream of the RGD motif are involved in potentiation of the binding affinity of αVβ6 integrin toward RGD-containing oligopeptides modeled after its natural ligands, i.e., the coat protein of foot-and-mouth disease virus and the latency-associated peptides of transforming growth factor-β proteins. They demonstrated that oligopeptides capable of binding to αVβ6 integrin with high affin-

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3 M. Takeichi and D. Kiyozumi, unpublished observations.
ities had an RGDLXXL or RGDLXXI motif, of which LXX(L/I) forms a stable helix with two hydrophobic residues (i.e. Leu and Leu/Ile) exposed almost in apposition on one face of the helix, suggesting that these hydrophobic residues, together with the RGD motif, directly interact with the ligand binding pocket of \( \alpha V \beta 6 \) integrin (19). Given the similarity between nephronectin and the natural ligands for \( \alpha V \beta 6 \) integrin in the requirement for a post-RGD sequence for high-affinity integrin binding, the hydrophobic residues within the LFEIFEIEER sequence may also be directly involved in ligand recognition by \( \alpha 8 \beta 1 \) integrin. Consistent with this possibility, the LFEIFEIEER sequence was predicted to form a helix by PAPIA, an algorithm for protein secondary structure prediction (36).

The requirement for an auxiliary site for selective high-affinity recognition of RGD-containing ligands by integrins has been documented for other integrin-ligand pairs. Fibronectin has been the prototype for such ligands harboring a so-called synergy site, requiring not only the 10th type III module containing the RGD motif but also the preceding type III module (FNIII9) for its high-affinity binding to \( \alpha 5 \beta 1 \) integrin (18, 37). The PHSRN sequence in the FNIII9 module was shown to be critical for the synergistic activity (18). The natural ligands for \( \alpha V \beta 6 \) integrin also possess such an auxiliary site immediately on the C-terminal side of the RGD motif as described above (19). Despite the available evidence, including the data in the present study, for the requirement of synergy sites for high-affinity binding of RGD-containing ligands to integrins, it remains to be elucidated how the synergy sites potentiate the binding affinities between integrins and their RGD-containing ligands. This is in striking contrast to the interactions of the RGD motif with integrins, because determination of the crystal structures of integrins complexed with cyclic RGD-like peptides revealed that the arginine side chain of the RGD motif fits into a cleft in the \( \beta \)-propeller domain of integrin \( \alpha \) subunits, whereas the aspartate side chain coordinates the divalent cation at the metal ion-dependent adhesion site (designated MIDAS) of \( \beta \) subunits (38, 39). It should be noted, however, that in addition to the residues defined by the crystal structures to directly interact with the RGD motif, a group of residues located on the upper or side faces of the \( \beta \)-propeller domain of the \( \alpha \) subunits have also been implicated in ligand binding by integrins. Specifically, epitope mapping of function-blocking anti-integrin antibodies as well as mutational analyses of the integrin \( \alpha \) subunits indicated that the ligand-binding specificities of \( \alpha 5 \beta 1 \) and \( \alpha V \beta 1 \) integrins were dependent on residues in blades 2 and 3 of the \( \beta \)-propeller domain of their \( \alpha \) subunits (40 – 42), of which Tyr-208 and Ile-210 in blade 3 of the \( \alpha 5 \) subunit were shown to recognize the synergy site of fibronectin (43). Furthermore, mutations that disrupted ligand binding by \( \alpha 1 \beta 3 \) integrin were found to be clustered on the top or side of the \( \beta \)-propeller domain of the \( \alpha 1 \) subunit, and mostly located within blades 2 and 3 (44). Similarly, the acidic clusters located on blade 3 of the \( \beta \)-propeller domain of the \( \alpha 7 \) subunit were shown to determine the ligand-binding specificity of \( \alpha 7 \beta 1 \) integrin (45). Given the involvement of the upper or side faces of the \( \beta \)-propeller domain, particularly those involving blades 2 and 3, high-affinity ligand recognition by integrins, the same regions of the \( \beta \)-propeller domain of the \( \alpha 8 \) subunit may be involved in recognition of the LFEIFEIEER sequence, thereby ensuring the high-affinity binding of nephronectin to \( \alpha 8 \beta 1 \) integrin. It is interesting to note that the residues comprising the upper or side loops of the \( \beta \)-propeller domain differ significantly among RGD-recognizing integrins. The \( \alpha 8 \) integrin subunit is unique in that it possesses evolutionarily conserved basic amino acid clusters at the loops connecting blades 1 and 2 and within blade 3 of its \( \beta \)-propeller domain (supplemental Fig. S4), the latter being equivalent to the loops involved in the ligand-binding specificities of \( \alpha 5 \beta 1 \) and \( \alpha 7 \beta 1 \) integrins (43, 45). Because two Glu residues within the LFEIFEIEER sequence are required for high-affinity binding of nephronectin to \( \alpha 8 \beta 1 \) integrin and evolutionarily conserved among vertebrates (supplemental Fig. S1), it is tempting to speculate that the cluster of basic amino acid residues in the loop either between blades 1 and 2 or within blade 3 of the \( \alpha 8 \) \( \beta \)-propeller domain form salt bridges with the acidic residues of the EIE motif, thereby sustaining the high-affinity recognition of nephronectin by \( \alpha 8 \beta 1 \) integrin.

Synthetic RGD-containing peptides have been widely used as probes that specifically block the interactions of RGD-binding integrins with their ligands in a variety of biochemical and cell biological assays (46 – 48). However, the RGD peptides are equally inhibitory toward all RGD-binding integrins, and therefore cannot be used as specific probes for the biological consequences of interactions between defined RGD-binding integrins and their physiological ligands. Furthermore, the absence of the synergy sequences that potentiate the integrin binding affinities of RGD-containing ligands leads to a requirement for high concentrations of RGD peptides to block the integrin-ligand interactions. For instance, RGD peptide concentrations of \( > 1 \) mm are required to effectively block \( \alpha 5 \beta 1 \)-mediated cell adhesion onto fibronectin-coated substrates (46 – 48). In this regard, the 23-mer peptide containing both the RGD motif and the LFEIFEIEER sequence fully mimics the interaction of nephronectin with \( \alpha 8 \beta 1 \) integrin and completely blocks the interaction at a concentration of 10 nm, but has no inhibitory effects on the interaction between fibronectin and \( \alpha 5 \beta 1 \) integrin.\(^4\) Given the remarkable potency of the 23-mer peptide in specifically blocking the nephronectin-\( \alpha 8 \beta 1 \) integrin interaction at nanomolar concentrations, the 23-mer peptide represents a promising probe for elucidating the biological functions of this integrin-ligand interaction in both physiological and pathological processes.

In summary, we have shown that nephronectin is the most preferred high-affinity ligand for \( \alpha 8 \beta 1 \) integrin and that the specificity and high-affinity binding toward the integrin are fully recapitulated by a 23-amino acid residue fragment harboring both the RGD motif and the LFEIFEIEER sequence, the latter serving as an auxiliary site that ensures the specific high-affinity binding of nephronectin to \( \alpha 8 \beta 1 \) integrin in concert with the RGD motif. Although the mechanism by which the synergy site potentiates the integrin binding affinity remains to be validated by determination of the three-dimensional structure of the fully active RGD-linker segment at the atomic level, these results provide, for the first time, the molecular basis of the specific

\(^4\) Y. Sato, unpublished observations.
interaction between nephronectin and α8β1 integrin, and highlight the bipartite nature of the integrin recognition site of nephronectin.

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