Identification of the Ligand Binding Site for the Integrin α9β1 in the Third Fibronectin Type III Repeat of Tenascin-C*

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The integrin α9 subunit forms a single heterodimer, α9β1, that mediates cell adhesion to a site within the third fibronectin type III repeat of tenascin-C (TNfn3). In contrast to at least 3 other integrins that bind to this region of tenascin-C, α9β1 does not recognize the common integrin recognition motif, Arg-Gly-Asp (RGD). In this report, we have used substitution mutagenesis to identify a unique ligand recognition sequence in TNfn3. We introduced mutations substituting alanine for each of the acidic residues in or adjacent to each of the exposed loops predicted from the solved crystal structure. Most of these mutations had little or no effect on adhesion of α9-transfected SW480 colon carcinoma cells, but mutations of either of two acidic residues in the B-C loop region markedly reduced attachment of these cells. In contrast, cells expressing the integrin α9β4, previously reported to bind to the RGD sequence in the adjacent F-G loop, attached to all mutant fragments except one in which the RGD site was mutated to RAA. The peptide, AEIDGIEL, based on the sequence of human tenascin-C in this region blocked the binding of α9-transfected cells, but not β4-transfected cells to wild type TNfn3. This sequence contains a tripeptide, IDG, homologous to the sequences LVV, IDA, and LDA in fibronectin and IDS in VCAM-1 recognized by the closely related integrin α9β1. These findings support the idea that this tripeptide motif serves as a ligand binding site for the α9/α9 subfamily of integrins.

Integrins are cell surface heterodimers that play roles in essential biological processes including development and tissue remodeling (1–4). Ligand binding specificity depends in large part on the specific α and β subunit present in each heterodimer. To date, extracellular matrix proteins, cell surface immunoglobulin superfamily molecules, and cadherins have been identified as ligands for integrins. In most cases where the crystal structure of integrin ligands has been solved, the ligand binding site includes at least one acidic residue, generally displayed in an exposed peptide loop (5–7). The first short peptide sequence identified as an integrin recognition sequence was the tripeptide, Arg-Gly-Asp (RGD) (8), initially identified as a cell-recognition sequence in the large extracellular matrix protein, fibronectin (9, 10). Subsequently, this RGD sequence was found to be present in several integrin ligands and to serve as a recognition sequence for several different integrins (11). As the sequences of multiple integrin α subunits were solved, it became clear that these sequences could be divided into 3 subfamilies based on sequence homology (1). One family includes α subunits with a characteristic disulfide-linked cleavage site. A subset of these form heterodimers that recognize RGD-containing ligands. Another includes α subunits that contain an inserted domain close to the N terminus but no cleavage site. These integrins generally do not recognize RGD-containing ligands. Finally, a third family, including only two α subunits, α9 and α4, contains neither an inserted domain nor a disulfide-linked cleavage site (12).

We have previously identified the third fibronectin type III repeat in the extracellular matrix protein tenasin-C (TNfn3) as a ligand for the only known α9-containing integrin, α9β1 (13). TNfn3 is also recognized as a ligand by at least three other integrins (14, 15), α9β1 (16), α9β3 (17), and α9β4 (18). The crystal structure of TNfn3 has been solved and consists of a series of seven β strands separated by six exposed loops (5). α9β1, α9β3, and α9β4 all bind to the RGD tripeptide contained within the F-G loop. However, unlike each of these integrins, α9β1 could mediate adhesion to a fragment in which this site was mutated to RAA, and adhesion was unaffected by RGD peptides, suggesting that the ligand binding site was distinct from this RGD site (13). In the present study, we have used substitution mutagenesis and synthetic peptides to identify this site in an adjacent exposed loop on the same face of tenasin-C as the RGD loop.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Reagents—Human colon cancer cells (SW480) were stably transfected with either the expression plasmids pcDNAneo9, pcDNAneoβ3, or the empty vector pcDNAneo as described previously (19). Cells were maintained in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD) supplemented with 1 mg/ml neomycin analog, G418 (Life Technologies, Inc.). Anti-α9 monoclonal antibody Y9A2 was generated and characterized in our laboratory as described previously (20). Monoclonal antibody 15/7 that recognizes a ligand binding-dependent epitope on the integrin β1 subunit (21) was obtained from Ted Yednock (Athena Neurosciences, South San Francisco). The pGEX plasmids to express glutathione S-transferase-
Integrin α9β1 Recognizes AEIDGIEL

ase fusion proteins including the wild type third fibronectin type III repeat of chicken tenascin-C (TNfn3) or a mutant version in which the RGD site within the F-G loop was mutated to RAA (18) were obtained from Kathryn Crossin (The Scripps Research Institute, La Jolla, CA). Concentrations of recombinant proteins were determined by the Bradford assay (Pierce) using bovine serum albumin as a standard. Peptides were synthesized using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry on a peptide synthesizer (Model 432A, Perkin-Elmer) at Center Laboratory for Research and Education, Osaka University, followed by purification with C18-reversed phase column chromatography.

Cell Adhesion Assay—As described previously (22), wells of non-tissue culture-treated polystyrene 96-well flat-bottomed microtiter plates (Nunc Inc., Naperville, IL) were coated with incubation by 100 μl of recombinant wild type or mutant tenascin fragment in phosphate-buffered saline at 37 °C for 1 h. For blocking experiments, cells were incubated in the presence or absence of soluble peptide or Y9A2 on ice for 15 min before plating on tenascin fragments. Wells were washed with phosphate-buffered saline, then blocked with 1% bovine serum albumin in Dulbecco’s modified Eagle’s medium. 50,000 cells were added to each well in 200 μl of serum-free Dulbecco’s modified Eagle’s medium containing 0.5% bovine serum albumin. Plates were centrifuged at 10 × g for 1 min, then incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO2. Non-adherent cells were removed by centrifugation at 100 × g for 20 min at 4 °C. Cultures were then incubated with antibody 15/7 at 15 μg/ml for 20 min at 4 °C. The plates were then washed with PBS. The cells were solubilized in 50 μl of 2% Triton X-100 and quantified by measuring the absorbance at 595 nm in a Microplate Reader (Bio-Rad).

FIG. 1. The crystal structure of the third fibronectin type III repeat of tenascin-C (adapted from Ref. 5) consisting of six extended loops separating seven β strands. F-G loop contains the RGD sequence. The recognition sequence AEIDGIEL for α9β1 identified in this study includes portions of the B-C loop and the adjacent C strand.

FIG. 2. Alignment of tenascin amino acid sequences of human, chicken, pig, and mouse tenascin-C. The large arrow (►) indicates initially mutated series of acidic residues in each loop. Aspartic acid 816, 821, 825 were simultaneously mutated. The remaining 3 mutants generated for further experiments are indicated by small arrows (►). Asterisks (•••) indicate the RGD sequence. Two-headed arrows under the sequence alignment indicate predicted positions of β strands.

RESULTS

Attachment of α9 Transfectants to Mutant TNfn3—According to the published crystal structure of TNfn3 (5), there are six extended loops separating seven β strands (Fig. 1). The previously characterized RGD sequence is in the F-G loop. Since most integrin binding sites include at least one acidic residue in an extended loop structure, we made a series of alanine substitution mutations encompassing each acidic residue present in or near a predicted loop, including the potential loop at the N terminus of this repeat (Asp-775, Fig. 2). Fig. 2 shows the locations of each of the substitution mutations described in this study. We could identify acidic residues in or adjacent to five loops (all except the loop between the E and F strands). For efficiency, we sometimes made simultaneous mutations in more than one acidic residue.

We have previously reported that both α9- and β1-transfected SW480 cells adhere to wild type TNfn3, whereas mock-
fected cells do not adhere (19). To determine the effects of each mutation on \( \alpha_9 \beta_1 \)-mediated adhesion, we performed cell adhesion assays on plates coated with wild type TNfn3 or with TNfn3 expressing mutations in one or more loop. To determine the specificity of any decreases in adhesion of \( \alpha_9 \)-transfected cells, we also performed adhesion assays on the same fragments with \( \beta_3 \)-transfected SW480 cells. As previously reported, both \( \alpha_9 \)- and \( \beta_3 \)-transfected cells adhered to wild type TNfn3, but only \( \alpha_9 \)-transfected cells adhered to TNfn3 in which the RGD sequence in the F-G loop was mutated to RAA (Fig. 3).

Attachment of \( \alpha_9 \)-transfected cells to wild type TNfn3 was completely blocked by anti-\( \alpha_9 \) monoclonal antibody Y9A2. Mutations in acidic residues in the N terminus (D775A), the C-C' loop (D812A), or the C-C' and C'-E loops (D816A/D821A/D825A) had no effect on adhesion of either transfectant. However a mutation in the B-C loop (E802A) specifically and markedly reduced attachment of \( \alpha_9 \)-transfected SW480 cells (Fig. 3, panel A). The only other mutation that decreased adhesion of \( \alpha_9 \)-transfected cells was a mutation in the A-B loop (D787A), which reduced adhesion by approximately 40%. Mock-transfected SW480 cells did not adhere to any of the TNfn3 fragments used in this study (data not shown).
To examine the role of specific acidic residues in or adjacent to either the B-C or A-B loops, we generated fragments expressing each of 3 additional mutations. Mutations of the aspartic acid residue just proximal to the A-B loop (D784A) or of the most proximal glutamic acid residue in the B-C loop (E800A) had no effect on adhesion. However, mutation of the next glutamic acid residue in the adjacent C β strand (E805A) reduced adhesion of α9-transfected cells to the same degree as

Fig. 6. Effects of synthetic peptides on expression of a ligand binding-dependent epitope on the integrin β1 subunit. Panel A, α9- or mock-transfected SW480 cells were incubated with the synthetic peptides shown at 1000 μM, and 15E7 expression was analyzed by flow cytometry. The expression level of the ligand binding-dependent epitope is compared in the absence of peptides (open histogram) or in the presence of 1000 μM peptide (filled histogram). Panel B, expression level of the ligand binding-dependent epitope on α9-transfected SW480 cells (indicated by the mean fluorescent intensity) is compared for peptide concentrations of 0, 100, 300, or 1000 μM. Panel C, expression level of the ligand binding-dependent epitope on mock-transfected SW480 cells.
Effects of Synthetic Peptides on Cell Adhesion to TNfN3—In an attempt to further determine whether sites in the A-B loop or the B-C loop were critical for \( \alpha_9 \beta_1 \)-mediated adhesion to TNfN3, we synthesized linear peptides corresponding to the amino acid sequence of each of these loops of human TNfN3 and evaluated the ability of these peptides to inhibit adhesion of either \( \alpha_9 \)-transfected or \( \beta_1 \)-transfected SW480 cells to 3 \( \mu \)g/ml wild type TNfN3 (Fig. 5). We designed these peptides based on the human sequence to increase the likelihood that any effective peptide could serve as a starting point for drug design. The peptide Ala-Glu-Ile-Asp-Gly-Ile-Glu-Leu (AEIDGIEL) based on the B-C loop caused concentration dependent inhibition of adhesion of \( \alpha_9 \)-transfected cells with no effect on adhesion of the \( \beta_1 \)-transfected control cells (Fig. 5). In contrast, the peptide Val-Thr-Asp-Thr-Thr-Ala-Leu (VTDTTAL) corresponding to the A-B loop was without effect. In addition, the scrambled peptide GDLAEIEI, based on AEIDGIEL, had no effect on cell adhesion. These data further suggest that the B-C loop is critical for ligand binding.

**Discussion**

Previous work identified tenasin-C as a ligand for the integrin \( \alpha_9 \beta_1 \) and localized the binding site to TNfN3 (13). In the present study, we have used substitution mutagenesis and synthetic peptides to map the ligand binding site within this repeat in more detail. The dramatic reduction of adhesion of \( \alpha_9 \)-transfected, but not \( \beta_1 \)-transfected cells to wild type TNfN3, is most similar to the ligand binding dependent epitope on the \( \beta_1 \) subunit. It is thus likely that the D787A mutation inhibited adhesion by altering the conformation of a critical region of the B-C loop on the opposite face of TNfN3.

Several ligand binding sites for integrins have now been mapped within fibronectin type III repeats and immunoglobulin domains (23–27). Most of these sites have been mapped to short linear sequences involving exposed peptide loops. In every case these sequences have included a critical acidic residue. Many of these sites include the classic integrin recognition motif, RGD, but binding to RGD sites is largely restricted to integrins that contain the closely related \( \alpha \) subunits (\( \alpha_5 \), \( \alpha_4 \), \( \alpha_{

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J. Biol. Chem. 1998, 273:11423-11428.
doi: 10.1074/jbc.273.19.11423

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