The integrin \(\alpha_9\beta_1\) binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin*

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The integrin \(\alpha_9\beta_1\) mediates cell adhesion to tenasin-C and VCAM-1 by binding to sequences distinct from the common integrin-recognition sequence, arginine-glycine-aspartic acid (RGD). A thrombin-cleaved NH2-terminal fragment of osteopontin containing the RGD sequence has recently been shown to also be a ligand for \(\alpha_9\beta_1\). In this report, we used site-directed mutagenesis and synthetic peptides to identify the \(\alpha_9\beta_1\) recognition sequence in osteopontin. \(\alpha_6\)-transfected SW480, Chinese hamster ovary, and L-cells adhered to a recombinant NH2-terminal osteopontin fragment in which the RGD site was mutated to RAA (nOPN-RAA). Adhesion was completely inhibited by anti-\(\alpha_6\) monoclonal antibody Y9A2, indicating the presence of a non-RGD \(\alpha_6\beta_1\) recognition sequence within this fragment. Alanine substitution mutagenesis of 13 additional conserved negatively charged amino acid residues in this fragment had no effect on \(\alpha_6\beta_1\)-mediated adhesion, but adhesion was dramatically inhibited by either alanine substitution or deletion of tyrosine 165. A synthetic peptide, SVVYGLR, corresponding to the sequence surrounding Tyr165, blocked \(\alpha_6\beta_1\)-mediated adhesion to nOPN-RAA and exposed a ligand-binding-dependent epitope on the integrin \(\beta_1\) subunit on \(\alpha_6\)-transfected, but not on mock-transfected cells. These results demonstrate that the linear sequence SVVYGLR directly binds to \(\alpha_6\beta_1\) and is responsible for \(\alpha_6\beta_1\)-mediated cell adhesion to the NH2-terminal fragment of osteopontin.

Integrins are cell surface heterodimeric receptors that mediate cell-cell and cell-extracellular matrix adhesion (1, 2). Upon ligation by a wide variety of ligands, integrins can initiate signaling cascades that regulate cell growth, cell death, migration, polarization, and tissue remodeling (3). Integrins recognize a surprisingly large number of functionally diverse proteins as ligands, and the list of known integrin ligands continues to grow. New integrin ligands have been identified, and drugs targeting integrins have been developed as a consequence of the description of short linear amino acid sequences that directly bind to integrins. For example, the integrins \(\alpha_5\beta_1\), \(\alpha_6\beta_1\), \(\alpha_7\beta_1\), \(\alpha_8\beta_1\), \(\alpha_{10}\beta_3\), \(\alpha_{10}\beta_6\), \(\alpha_{10}\beta_8\), and \(\alpha_{10}\beta_{19}\) bind to sequences containing the tri-peptide sequence Arg-Gly-Asp (RGD). Several new and biologically important integrin ligands have been identified based on the presence of this sequence (4, 5). Drugs modeled on the structure of the RGD sequence are being used or tested to inhibit integrin function for treatment of thrombosis, inflammation, atherosclerosis, osteoporosis, and cancer (5). The RGD sequence has also been exploited to target cell surface integrins to enhance gene delivery (6). We have previously identified the recognition sequence for the integrin \(\alpha_6\beta_1\) in tenasin-C and found that this sequence did not include RGD, but was homologous to the \(\alpha_6\beta_1\) recognition sequence in the inducible endothelial adhesion molecule VCAM-1 (7). This finding led to our identification of \(\alpha_6\beta_1\) as a receptor for VCAM-1 (8).

Osteopontin is a phosphorylated acidic glycoprotein with diverse functions (9) including cell adhesion, chemotraction (10), and immunomodulation (11). Osteopontin is present at high concentrations in diseases associated with tissue remodeling, including granuloma formation (12) and coronary restenosis (13, 14), suggesting that this molecule might contribute to the process of remodeling. Osteopontin contains a predicted thrombin cleavage site (15) and appears to be cleaved at this site in vivo (16, 17). Several integrins have been identified as osteopontin receptors. Of these, \(\alpha_5\beta_1\) (18), \(\alpha_6\beta_1\) (19), \(\alpha_6\beta_2\) (20, 21), \(\alpha_7\beta_1\) (22), and \(\alpha_{10}\beta_5\) (20, 21) recognize an RGD sequence that is present within the NH2-terminal fragment of cleaved osteopontin. However, two integrins that generally bind to non-RGD sequences, \(\alpha_6\beta_1\) (23) and \(\alpha_6\beta_2\) (24), have also been reported to be osteopontin receptors. In contrast to the other integrin osteopontin receptors, \(\alpha_6\beta_1\) recognizes only the NH2-terminal fragment produced by thrombin cleavage, but does not appear to bind to full-length osteopontin, at least in vitro (24). In the present study we have used substitution and deletion mutagenesis and synthetic peptides to identify the sequence within this fragment that serves as the binding site for \(\alpha_6\beta_1\). The sequence identified (SVVYGLR) is a novel integrin-binding site that could serve as a basis for identifying...
additional α<sub>9</sub>β<sub>1</sub> ligands or for developing specific inhibitors of α<sub>9</sub>β<sub>1</sub> function.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Antibodies, and Reagents—**Human colon cancer cells (SW480), Chinese hamster ovary cells (CHO),<sup>7</sup> and mouse fibroblast L<sub>C</sub>-cells were obtained from ATCC and stably transfected with either the expression plasmid pCDNAIneo9 or the empty vector pCDNAIneo as described previously (25). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) supplemented with 1 mg/ml neomycin analog, G418 (Life Technologies, Inc.). Vitronectin was prepared from human plasma as described by Yatohgo and colleagues (26). Anti-α<sub>9</sub> monoclonal antibody Y9A2 was generated and characterized in our laboratory as described previously (27). Monoclonal antibody 15/7, which recognizes a ligand-binding-dependent epitope on the integrin β<sub>1</sub> subunit (28), was obtained from Ted Yednock (Elan Pharmaceuticals, South San Francisco, CA). cDNAs encoding splice variants of osteopontin, OPNa, OPNb, and OPNc, were obtained from human glioma cell lines and cloned in pCRII plasmid (Promega, Madison, WI) as described previously (29). Concentrations of recombinant proteins were determined by the Bradford assay (Pierce) using bovine serum albumin as a standard. Peptides were synthesized using Fmoc (9-fluorenyl) methoxycarbonyl)-chemistry on a peptide synthesizer (model 432A, Perkin Elmer, Foster City, CA) at Center Laboratory for Research and Education, Osaka University, followed by purification with C18-reversed phase column chromatography.

**Cell Adhesion Assays—**Wells of non-tissue culture-treated polystyrene 96-well flat-bottom microtiter plates (Nunc Inc., Naperville, IL) were coated by incubation with 100 μl of recombinant wild type or mutant osteopontin NH<sub>2</sub>-terminal 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 the NH<sub>2</sub>-terminal osteopontin fragments. Wells were washed with phosphate-buffered saline, then blocked with 1% bovine serum albumin in DMEM. 50,000 cells were added to each well in 200 μl of serum-free DMEM 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% CO<sub>2</sub>. Non-adherent cells were removed by centrifugation at 300 × g for 5 min. The attached cells were fixed with 1% formaldehyde, stained with 0.5% crystal violet, and excess dye was washed off with phosphate-buffered saline. 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).

**Mutagenesis—Site-directed mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene, San Diego, CA) as described previously (7).** Both strands of the expression plasmid were replicated by PCR using Pfu DNA polymerase with two complementary primers designed to introduce the desired mutation. The amplified product was treated with methylated DNA, to digest the parental DNA template. Then DH5α competent cells were transformed with the PCR-generated nicked plasmid. Plasmids from several isolated colonies were prepared with the QIAprep Spin miniprep kit (Qiagen, Hilden, Germany), and inserts were sequenced using an ALFred autosequencer (Amersham Pharmacia Biotech) with fluorescent-tagged primers flanking the polylinker of pCDNAIneo. QIAprep Spin miniprep kit (Qiagen, Hilden, Germany), and inserts were sequenced using an ALFred autosequencer (Amersham Pharmacia Biotech) with fluorescent-tagged primers flanking the polylinker of pCDNAIneo. QIAprep Spin miniprep kit (Qiagen, Hilden, Germany), and inserts were sequenced using an ALFred autosequencer (Amersham Pharmacia Biotech) with fluorescent-tagged primers flanking the polylinker of pCDNAIneo. The plasmid vector that had not been amplified by PCR.

**Expression of Recombinant Osteopontin Fragments—**cDNAs encoding the thrombin-cleaved NH<sub>2</sub>-terminal osteopontin fragment were amplified from the full-length cDNAs for OPNa, OPNb, and OPNc in pCRII by polymerase chain reaction with restriction site-tagged primers that amplify the same region as described previously (24), and then cloned between the BamHI and EcoRI sites within the multiple cloning site of the pGEX6P2 plasmid (Amersham Pharmacia Biotech). Wild type or variant recombinant NH<sub>2</sub>-terminal osteopontin proteins were prepared by bacterial expression with the pGEX6P2 plasmids as recommended by the manufacturer. Briefly, competent DH5α cells were transformed by heat shock and grown on ampicillin-containing plates. Individual colonies were picked and propagated overnight in 2 ml of 2×YT medium with 100 μg/ml ampicillin at 37 °C. 100 ml of 2×YT medium was inoculated with 1 ml of the bacteria, and incubated at 30 °C until OD<sub>600</sub> reached 0.5–2, at which time isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.1 mM. Cultures were grown for several more hours, cells were collected and sonicated, and glutathione S-transferase fusion proteins were affinity-purified with glutathione-Sepharose 4B beads and then cleaved off from glutathione S-transferase with PreScission protease (Amersham Pharmacia Biotech). Purity of the product was confirmed by 12.5% SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining.

**Flow Cytometric Assessment of Peptide Ligation of α<sub>9</sub>β<sub>1</sub>—**Binding of authentic or scrambled peptides to the ligand binding site of α<sub>9</sub>β<sub>1</sub> was assessed as described previously (7) using monoclonal antibody 15/7, which recognizes a ligand-binding-dependent epitope on the integrin β<sub>1</sub> subunit (28). Mock- or α<sub>9</sub>β<sub>1</sub>-transfected SW480 cells were incubated with the peptides in DMEM for 10 min at 4 °C. Cells were then incubated with antibody 15/7 at 15 μg/ml for 20 min at 4 °C, followed by incubation with secondary phycoerythrin-conjugated goat anti-mouse IgG. 15/7 expression was then quantified on 5,000 cells with a Becton Dickinson FACSort.

**RESULTS**

α<sub>9</sub>β<sub>1</sub>-mediated Attachment to the NH<sub>2</sub>-terminal Fragment of Osteopontin Does Not Require the Presence of an RGD Site—The only previous attempt to map the ligand binding site for α<sub>9</sub>β<sub>1</sub> in osteopontin suggested that α<sub>9</sub>β<sub>1</sub> might interact directly with the tri-peptide sequence RGD (30). However, we have previously shown that α<sub>9</sub>β<sub>1</sub>-mediated adhesion to another RGD-containing ligand, tenascin-C, does not require an intact RGD site (7, 25) and that α<sub>9</sub>β<sub>1</sub> mediates adhesion to a third

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<sup>7</sup>The abbreviations used are: CHO, Chinese hamster ovary; nOPN, NH<sub>2</sub>-terminal osteopontin; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction.
ligand, VCAM-1 (8), that does not contain an RGD site. We therefore re-examined the role of this site in the NH2-terminal fragment of osteopontin (nOPNa) by directly comparing adhesion of mock- or α9-transfected SW480 cells to wild type recombinant nOPNa or a mutant version in which the RGD site had been changed to RAA (nOPNa-RAA). Both cell lines attached to nOPNa (Fig. 1A), an expected result since SW480 cells express two other integrins, αvβ3 and αvβ5, that bind to the RGD site in this fragment. However, whereas mutation of the RGD site to RAA completely abolished adhesion of mock-transfected cells, attachment of α9-transfected cells was only slightly diminished. Furthermore, the adhesion of α9-transfected cells to nOPNa-RAA was completely abolished by anti-α9 blocking antibody Y9A2, suggesting that adhesion to this fragment was mediated entirely by α9β1 (Fig. 1A). To further confirm that adhesion of α9-transfected SW480 cells to the NH2-terminal osteopontin fragment was independent of the RGD site, we examined the effects of the synthetic peptide GRGDSP on the adhesion of mock- and α9-transfected SW480 cells to wild type nOPNa. GRGDSP completely inhibited adhesion of mock-transfected cells, but resulted in minimal inhibition of adhesion of α9-transfected cells (Fig. 1B). Adhesion of α9-transfected SW480 cells to the RGD-independent site in nOPNa was concentration-dependent (Fig. 1C). Over a range of coating concentrations from 0.3 to 10 μg/ml, adhesion of these cells to nOPNa-RAA or to wild type nOPNa in the presence of GRGDSP was essentially identical. At all concentrations examined, adhesion to nOPNa-RAA was completely inhibited by the α9β1 blocking antibody, Y9A2.

The experiments described above were performed with the colonic epithelial cell line SW480, whereas the previously reported experiments suggesting that α9β1 might bind directly to the RGD site were performed with the melanoma cell line, Mo. We therefore examined adhesion of two other mock- or α9-transfected cell lines, CHO and L-cells, to determine whether binding of α9β1 to a non-RGD site in nOPNa was specific for SW480 cells. As with SW480 cells, both mock- and α9-transfected CHO cells and L-cells adhered to nOPNa, but only α9-transfected cells adhered to nOPNa-RAA. Adhesion of both α9-transfected to nOPNa-RAA was completely blocked by Y9A2 (Fig. 2). Furthermore, adhesion of mock transfecants in both lines to wild type nOPNa was completely inhibited by GRGDSP, whereas adhesion of α9-transfected was only inhibited to the level of adhesion seen on nOPNa-RAA. As for SW480 cells, adhesion to both osteopontin fragments was concentration-dependent, and adhesion of α9-transfected to all concentrations of nOPNa-RAA and to wild type nOPNa in the presence of GRGDSP were essentially identical (data not shown).

α9β1 Mediates Adhesion to an RGD-Independent Site Present in Each of the Known Splice Variants of Osteopontin—In addition to full-length osteopontin (OPNa), there are two naturally occurring splice variants, OPNb and OPNc, derived from splice variants OPNa, OPNb, and OPNc (29), respectively. OPNb lacks the amino acid sequence encoded by exon 5 (3) and OPNc lacks exon 4 (3). Panel B shows adhesion of α9-transfected SW480 cells to the three variant NH2-terminal fragments (3). The RGD site in each of the fragments was changed to RAA to eliminate background adhesion due to RGD receptors on SW480 cells. Adhesion was tested in the presence (3) or absence of the anti-α9 antibody, Y9A2. Adhesion is expressed as absorbance at 595 nm. Each bar represents the mean (± S.D.) of triplicate wells.

α9β1-mediated Adhesion to Osteopontin Does Not Depend on the Presence of Conserved Glutamic or Aspartic Acid Residues—To perform fine mapping of the α9β1 binding site, we initially sought to identify sequences that were homologous to the EIDGLIEL motif that we have previously identified as the binding site for α9β1 in the third fibronectin type III repeat in
tenasin-C (7), but no homologous sites were apparent. Because most integrin binding sites contain a critical negatively charged amino acid, we next performed alanine substitution mutagenesis, replacing each of 13 aspartic acid and glutamic acid residues that are largely conserved within the required 110 amino acid region in human, bovine, rabbit, rat, and mouse osteopontin. For these experiments, we generated a series of five mutant fragments, each containing between two and four alanine substitutions, as diagrammed in Fig. 4A. Again, to eliminate background adhesion mediated by other RGD-binding integrins, each of these mutant fragments also included the sequence RAA at the RGD site. Each of these mutant fragments was made in the nOPNb splice variant. Surprisingly, each of these mutant fragments supported adhesion equally well, and the level of adhesion was equivalent to that supported by nOPNb-RAA (Fig. 4B). These data suggest that none of these negatively charged amino acids are required for $\alpha_\beta_1$-mediated adhesion.

Integrin $\alpha_\beta_1$-mediated Adhesion to NH2-terminal Osteopontin Fragments Requires the Presence of Conserved Residues Adjacent to the Thrombin Cleavage Site—Thrombin cleavage of osteopontin is critical for $\alpha_\beta_1$-mediated adhesion (24), suggesting that cleavage reveals a cryptic $\alpha_\beta_1$ recognition site in the NH2-terminal fragment. Because amino acid residues close to the cleavage site are likely to be especially affected, we next examined the possible role of the COOH-terminal region that is adjacent to the thrombin cleavage site in the NH2-terminal osteopontin fragment. Interestingly, the RGD sequence is in this region. In human, rabbit, bovine, rat, and mouse osteopontin, the GRGDS sequence is completely conserved as are three additional amino acids: Asp157, Tyr165, and Leu167 (Fig. 5A). Since we had already found no effect of replacing Asp157 with alanine in mutant 5, we next examined a fragment containing the RAA mutation combined with an alanine replacement of Tyr165. Adhesion of $\alpha_\beta_1$-transfected SW480 cells to this Y165A mutant fragment was dramatically reduced (Fig. 5). To determine the significance of other amino acids within the C-terminal region of this fragment, we performed additional mutagenesis on this region. In one mutant, we replaced Gly166 with alanine, and in two additional mutants we deleted either the last two amino acids (LR) or the last four amino acids (YGLR). The G166A mutation had no effect on adhesion, but deletion of either the last four or the last two amino acids completely abolished adhesion (Fig. 5). These results provide further evidence that this region is critical for $\alpha_\beta_1$-mediated adhesion.

Synthetic Peptide SVVYGLR Inhibits Cell Adhesion to the NH2-terminal Osteopontin Fragment and Reveals a Ligand Binding-dependent Epitope on the Integrin $\beta_1$ Subunit in $\alpha_\beta_1$-transfected SW480 Cells—Mutagenesis studies alone cannot definitively identify a ligand binding site, since the substitution and deletion mutants we made could have affected $\alpha_\beta_1$-mediated adhesion by changing the conformation of other regions in nOPN. In an attempt to further determine whether $\alpha_\beta_1$ interacts directly with the region containing the YGLR sequence, we synthesized a linear peptide Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR) corresponding to the C-terminal region of the osteopontin fragment and evaluated the ability of this peptide to...
Inhibit adhesion of α9-transfected SW480 cells to nOPNb-RAA. The SVVYGLR peptide caused concentration-dependent inhibition of adhesion to nOPNb-RAA, while the scrambled peptide VRVGLYS had no effect on adhesion (Fig. 6A). In contrast, adhesion to vitronectin (3 µg/ml) was unaffected by any concentration of the SVVYGLR peptide (Fig. 6B).

We have previously described that binding of a synthetic peptide corresponding to the ligand binding site for α9β1 in tenasin-C to α9-transfected SW480 cells induces recognition by monoclonal antibody 15/7 (7), an antibody that has previously been shown to be made accessible following direct interaction of β1-integrins with ligand (28). To determine whether the C-terminal region directly interacts with α9β1, we examined the effects of each peptide on expression of the epitope recognized by monoclonal antibody 15/7. To determine whether any peptide-induced expression of the 15/7 epitope was specifically due to interaction of peptides with α9β1, and not to interaction with other β1-integrins expressed on SW480 cells, we compared the effects of each peptide on α9-transfected and mock-transfected cells. The SVVYGLR peptide, but not the scrambled peptides VRVGLYS, significantly induced 15/7 expression on α9-transfected cells (Fig. 7). Neither of the peptides examined affected 15/7 expression on mock-transfected cells, demonstrating that induction of expression was due to specific interaction of the SVVYGLR peptide with α9β1.

**DISCUSSION**

Previous reports identified osteopontin as a ligand for the integrin α9β1 and localized the binding site to a thrombin-cleaved NH2-terminal osteopontin fragment (24). In the present study, we have mapped the ligand-binding region within this osteopontin fragment in more detail by site-directed mutagenesis and synthetic peptides. The dramatic reduction of adhesion of α9-transfected cells to the mutant fragment in which tyrosine 165 in the C-terminal region of the fragment was replaced with alanine identified this tyrosine residue as critical for ligand binding. Two different deletion mutations in the C-terminal region also abolished α9β1-mediated adhesion. The effects of the peptide corresponding to the region surrounding Tyr165, SVVYGLR, inhibition of adhesion of α9-transfected cells to the NH2-terminal osteopontin fragment, and induction of a ligand binding-dependent epitope on the integrin β1 subunit, provide further evidence that this region includes the ligand binding site.

One previous report suggested that the RGD sequence itself played an important role in α9β1-mediated adhesion to the NH2-terminal fragment of osteopontin (30). In that report, a melanoma cell line that did not express any α9 integrins (Mo) and had previously been shown to attach to the NH2-terminal fragment, demonstrated markedly less adhesion to a fragment in which the RGD site was changed to RAA. In that study, deletion of the carboxyl-terminal region of the fragment, including both the RGD sequence and the adjacent SVVYGLR completely abolished adhesion of these cells, whereas the RAA mutation partially inhibited adhesion. These results are consistent with a role for the SVVYGLR peptide in α9β1-mediated adhesion. In the current study, we also found some inhibition of adhesion of α9-expressing cells by the RAA mutation, but this effect could have been entirely due to the effects of this mutation on other RGD-binding integrins expressed on the cells we used. Our results do not allow us to completely exclude any contribution of the RGD site to α9β1-mediated adhesion, but any such contribution, if present, appears to be minimal. We cannot fully explain the difference in the magnitude of inhibition by the RAA mutation in our study and the previous report. One possible explanation would be differential effects of the RAA mutation on the conformation of the SVVYGLR binding site under the conditions used to purify the recombinant fragments in the two studies. Another possibility is that the cell line used in the previous report expresses higher levels of other RGD-binding integrins or lower levels of α9β1 than the three transfected cell lines used in the current study.

We have previously reported that α9β1 binds to two other ligands, tenasin-C (7, 25) and VCAM-1 (8), at sites that do not include an RGD sequence. In those studies it was possible to definitively exclude any contribution of RGD sites. Since VCAM-1 does not contain an RGD, the attachment of α9-transfected CHO cells and SW480 cells to recombinant VCAM-1 (8) is definitely RGD-independent. α9β1 mediates adhesion to the third fibronectin type III repeat in tenasin-C, a repeat that does contain an RGD site that serves as a ligand binding site for the integrins α9β1 (31), α9β3 (31, 32), and α9β4 (33). However, the α9-transfected SW480 cells we used to study interaction with tenasin-C do not express any of these integrins. These cells adhered equally well to a wild type tenasin-C fragment as to a fragment in which the RGD sequence had been changed to RAA, and an RGD-containing peptide had no effect on adhesion.

Most previously described integrin recognition motifs, including the EIDGIEL-recognition sequence we described in tenasin-C, include a central negatively charged amino acid residue. We were therefore surprised that mutation of each of

| A | 153 | hum | VDTYDGRDSVYYGLR |
|---|-----|-----|------------------|
|   |     | bov | ESANDGRDSVAYGLK |
|   |     | rab | VETYDGRDSVAYRLKR |
|   |     | rat | VDVPDGRDSLAYGLR |
|   |     | mou | VDVPNGRDSLAYGLR |

**FIG. 5.** Alignment of sequences around the RGD site in the osteopontin of human, bovine, rabbit, rat, and mouse (panel A), and adhesion of α9-transfected SW480 cells to the mutants in this region (panel B). In Panel A, numbers above the alignment indicate number of residues in human osteopontin. Conserved residues are in bold type. The arrow indicates Tyr165, which was replaced with alanine. RGD sequences are boxed. Panel B, adhesion of α9-transfected SW480 cells to four fragments with mutations in this region was compared with adhesion to nOPNb-RAA. RGD sites in these mutants have been mutated to RAA to eliminate the background adhesion due to RGD receptors on SW480 cells. Adhesion to nOPNb-RAA (25), and to nOPNb-RAA with additional mutations (26), Y165A, G166A substitution, LR or YGLR deletion at the C-terminal end, are shown. Adhesion is expressed as absorbance at 595 nm. Each bar represents the mean (± S.D.) of triplicate wells.
we identified did not contain any negatively charged amino acids. Rather, the critical sequence includes a central tyrosine residue. Thus far, the only integrin fragments for which there are solved crystal structures are the inserted (or I) domains present in a subset of integrin α subunits. In the case of these integrins, the negatively charged aspartic acid residue present in many integrin ligands has been suggested as one of the coordination sites for a metal ion predicted to be required at the ligand binding site (34). However, the α9 subunit does not contain an I domain (35), and the structural basis of interactions of non-I domain-containing integrins with their ligands remains to be determined. The identification of an integrin ligand binding site without any acidic residues suggests that alternative mechanisms must exist for integrin-ligand interactions. However, it is possible that the free carboxyl terminus could be an alternative source of a negative charge. In any case, the recognition sequence we have described could also allow identification of additional α9β1 ligands and improved design of specific inhibitors.

Based on the results of in vitro cell adhesion assays, the adhesive sequence in the NH2-terminal osteopontin fragment appears to be cryptic, and is not recognized by α9β1 in the full-length form of osteopontin (24). Osteopontin cleavage by thrombin thus appears to be critical for induction of accessibility to this site. Since the crystal structure of the osteopontin protein is not available at this time, we cannot predict the nature of any conformational change that results from thrombin cleavage. However, the localization of the recognition site to a linear peptide sequence immediately adjacent to the cleavage site fits well with the idea that this sequence is made accessible by thrombin cleavage.

The biological significance of the thrombin-cleaved fragment that is present in vivo has not been previously determined (16, 17). The results of this study together with the previous report provide insights into the potential physiological role of thrombin cleavage. We have previously shown that α9β1 is expressed on neutrophils (8), smooth muscle cells, and epithelial cells (35), and that ligation of α9β1 can contribute to cell migration (8) and proliferation (32). Upon thrombin cleavage of osteopontin at sites of inflammation or remodeling, the generation of an α9β1 ligand could thus enhance the cell migration and proliferation that are required for tissue remodeling to occur. In addition, osteopontin has another thrombin cleavage site at Arg160-Gly161 (16). The sequence of the fragment produced by cleavage at both sites would be GDSVVYGLR, a fragment that would be expected to inhibit α9β1-mediated effects on cell behavior, and thereby potentially contribute to the termination of the events described above.
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