Title: Identification of Dual $\alpha_4\beta_1$ Integrin Binding Sites Within a 38 Amino Acid Domain in the N-Terminal Thrombin Fragment of Human Osteopontin

Kayla J. Bayless and George E. Davis*

Department of Pathology and Laboratory Medicine,
Texas A&M University Health Science Center, College Station, Texas

Running title: Dual $\alpha_4\beta_1$ binding sites in osteopontin

* To whom correspondence should be addressed:

George E. Davis M.D., Ph.D.
Department of Pathology and Laboratory Medicine
Texas A&M University Health Science Center
255 Reynolds Medical Building
College Station, TX 77843-1114
Phone: (979) 845-0823
FAX: (979) 862-1229
email: gedavis@tamu.edu
SUMMARY

Previous work from our laboratory demonstrated that the $\alpha_4\beta_1$ integrin is an adhesion receptor for OPN and that $\alpha_4\beta_1$ binding site(s) are present in the N-terminal thrombin fragment of OPN\(^\text{1}\) (Bayless et al., *J. Cell Science*, 1998; 111, 1165-1174). The work presented here identifies two $\alpha_4\beta_1$ binding sites within a recombinantly produced N-terminal thrombin fragment of human OPN. Initial experiments, using wild-type OPN containing an RGD sequence or an OPN RGE mutant, showed identical $\alpha_4\beta_1$-dependent cell adhesive activity. A strategy to localize $\alpha_4\beta_1$ binding sites within the thrombin fragment of osteopontin involved performing a series of truncation analyses. Removal of the last 39 amino acids (a.a. 130-168) completely eliminated adhesion, indicating all binding activity was present within that portion of the molecule. Combined mutation and deletion analyses of this region revealed the involvement of dual $\alpha_4\beta_1$ binding sites. Synthetic peptides for both regions in OPN, ELVTDFPTDLPAT (a.a. 131-143) and SVVYGLR (a.a. 162-168), were found to block $\alpha_4\beta_1$-dependent adhesion. The first peptide when coupled to sepharose, bound the $\alpha_4\beta_1$ integrin directly, while a mutated ELVTEFPTELPA PET peptide showed a dramatically reduced ability to bind. These data collectively demonstrate that dual $\alpha_4\beta_1$ integrin binding sites are present in a 38 amino acid domain within the N-terminal thrombin fragment of OPN.

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\(^\text{1}\)The abbreviations used are: OPN, osteopontin; RGD, Arg-Gly-Asp; RGE, Arg-Gly-Glu; ELVTDFPTDLPATK, Glu-Leu-Val-Thr-Asp-Phe-Pro-Thr-Asp-Leu-Pro-Ala-Thr-Lys; ELVTEFPTELPA, Glu-Leu-Val-Thr-Asp-Phe-Pro-Thr-Asp-Leu-Pro-Ala-Thr-Lys; LDVP, Leu-Asp-Val-Pro; IDAPS, Ile-Asp-Ala-Pro-Ser; REDV, Arg-Glu-Asp-Val; QIDSPL, Gin-Ile-Asp-Ser-Pro-Leu; SVVYGLR, Ser-Val-Asp-Tyr-Gly-Leu-Arg; TBS, Tris buffered saline; PBS, phosphate buffered saline; PSA, Puck’s Saline A, BSA, bovine serum albumin; Tween 20, polyoxyethylene sorbitan monolaurate; PVDF, polyvinylidene difluoride.
INTRODUCTION

Integrins are a family of transmembrane heterodimeric cell adhesion receptors (1). The \( \alpha_4 \beta_1 \) integrin (VLA-4) is predominantly expressed on leukocytes (2-4). It is capable of existing in multiple activation states (5) to mediate cell-cell and cell-extracellular matrix interactions (6-14). Known binding sites for \( \alpha_4 \beta_1 \) include LDVP (8-10), IDAPS (15), REDV (16), QIDSPL (17-20), and under certain activating conditions, RGD (21). The \( \alpha_4 \beta_1 \) integrin is intricately involved in trafficking of mononuclear leukocytes into tissues during the normal inflammatory response as well as in pathological situations (22), such as encephalomyelitis (23), diabetes (24), and graft rejection (25). Also, increased expression of \( \alpha_4 \beta_1 \) is observed on smooth muscle cells within atherosclerotic plaques (26), and \( \alpha_4 \beta_1 \) plays a role in tumor metastasis (12,27).

Collectively, the \( \alpha_4 \beta_1 \) integrin appears to play a pathogenic role in inflammation, wound repair and tumor progression.

Osteopontin is an extracellular matrix protein originally isolated from bone (28), and much evidence has accumulated as to its role in bone physiology (29). OPN is also secreted by many epithelial surfaces (30), and one early study supported a role for OPN in host-response to bacterial infection (31). Accumulating evidence also indicates OPN secretion is involved in inflammation and tumor progression. OPN has previously been found to be upregulated in a variety of inflammatory, cardiovascular and infectious diseases (32-37) and is a major secreted product of macrophages in inflammatory settings (32-36). It is also associated with tumors (38-41), particularly at the tumor-host interface (42). Recent data from knockout mice show that OPN facilitates wound healing (43), aids in host defense against viral (44) and bacterial infections (44,45) and is involved in granuloma formation (44). Other studies have suggested
OPN may facilitate tumor cell metastasis (46) and decrease complement-mediated tumor cell
destruction (47). Based on these data, the presence of OPN in the wound environment likely
plays an important role in regulating disease progression in inflammatory and other conditions.
The molecular domains in OPN that mediate its effects in these phenomena remain to be defined.

The parallels between expression of the $\alpha_4\beta_1$ leukocyte integrin and expression of OPN
in wounds prompted a previous study by our laboratory to define osteopontin as a ligand for
the $\alpha_4\beta_1$ integrin (14). Here, we define two binding sites for the $\alpha_4\beta_1$ integrin in the recombinant
N-terminal thrombin fragment of human OPN using deletional and mutational analyses.

EXPERIMENTAL PROCEDURES

Preparation of recombinant osteopontin: cloning strategy

A full-length cDNA clone of the human osteopontin gene was obtained from the
American Type Culture Collection (ATCC; Rockville, Maryland) (48), and the sequence is
shown in Figure 1A. Sequences encoding the wild-type N-terminal thrombin fragment of
osteopontin (rOPN17-168) were amplified by PCR using the primers 5’-
TAGGATCCATACCAGTTAAACAGGCTGATTCTGGAAG-3’ and 5’-GTAAGC-
TTTACCTCAGTCCATAAACCACACTATCACCTCGGCCA-3’ (Genosys, The Woodlands,
TX). Sequences encoding a mutated N-terminal fragment (rOPN17-168E161) in which the single
RGD sequence at residues 159-161 was changed to RGE were obtained by substituting 5’-
TAAAGCTTTTACCTCAGTCCATAAACCACACTATCACCTCGGCCA-3’ for the
downstream primer used to generate the wild-type fragment. Restriction digests of the PCR
products and the pQE$_{30}$ vector (Qiagen) were carried out overnight with BamHI and HindIII
(Gibco-BRL). Digested vector and insert were purified, quantitated and ligated at an insert to
vector ratio of 4.5:1 overnight at 14°C (Boehringer-Manheim). These constructs encode a modified version of rOPN17-168 and rOPN17-168E\(^{161}\) where the sequence RGS\(\text{H}^{165}\text{H}^{166}\text{H}^{167}\text{H}^{168}\) replaces MRIAIVCFCLLGITCA at the N-terminus of wild type osteopontin. All positive clones were confirmed by sequence analysis at Lone Star Labs (Houston, TX). All subsequent constructs studied contained the RGE mutation at amino acid 161.

Additional recombinant constructs of OPN (Figure 1B) were produced using the following primer sets: rOPN55-168: 5’-TAAAGCTTTTACCTCAGTCCATAAACCACACTTCACCTGCA-3’; and 5’-AGGGATCCCTAGCCCCACAGAATGCTGTGTC-3’. The remaining constructs were constructed with the common upstream primer 5’-TAGGATCCATACCAGTGTTAAACACAGGCTGATTCTGGAAG-3’ and each individual downstream primer:

rOPN17-164 5’-AGAAGCTTTTAAACCACACTTTCAACC –3’;

rOPN17-129 5’- AGAAGCTTTTAAAGATTCATCAGAATGCTGTGAC-3’;

rOPN17-135 5’- CGAAGCTTTTATACGATGACCAGTTCTCAG-3’;

rOPN17-138 5’- AGAAGCTTTTACGTGGGAAAATCAGTGACC-3’;

rOPN17-138E\(^{135}\) 5’- AGAAGCTTTTACGTGGGAAAATCAGTGACCAGTC-3’;

rOPN17-142 5’- AGAAGCTTTTATGCTGGCAGGGGAAAATCAGTGACC-3’;

rOPN17-142E\(^{139}\) 5’- AGAAGCTTTTATGCTGGCAGGGGAAAATCAGTGACC-3’;

rOPN17-150 5’- AGAAGCTTTTATGCTGGCAGGGGAAAATCAGTGACC-3’.

Cloning experiments were performed as described above, and positive clones were confirmed by sequence analysis at Lone Star Labs (Houston, TX).
Production and characterization of recombinant wild-type and mutated N-terminal thrombin fragments of osteopontin

E. Coli strain RY2840 [MC4100 lacFq lac+ slyD Km'] (49) was transformed with plasmids encoding the His6-tagged OPN derivatives. Two ml of overnight cultures were innoculated into 200 ml of LB media (Gibco-BRL) containing 50 µg/ml ampicillin (Sigma). Cultures were grown to an A600 of 1.0 (approximately 2.5 hours) before induction with 0.5 mM isopropylthio-β-D-galactoside (IPTG-Gibco-BRL). Cultures were allowed to incubate for 3.5-4 hours at 37°C in a shaking incubator before being placed on ice for 15 minutes. Bacteria were pelleted, supernatants removed and pellets frozen at −80°C. Pellets were thawed at 25°C for 10 minutes, resuspended on ice in 20 ml Hepes buffered saline (pH 8.1) containing 25 mM Hepes, 150 mM NaCl and 1 mM 4-[(2-Aminoethyl) benzene sulfonyl-fluoride, HCl] (Calbiochem). Bacteria were lysed and debris pelleted (20,000xg at 4°C for 20 minutes) before adding supernatants to 2 ml TALON metal ion affinity column (Clontech) equilibrated with Hepes buffer. Columns were incubated for a minimum of 20 minutes at 4°C before washing with 20 column volumes of Hepes buffer. His-tagged proteins eluted with 0.2 M imidazole (Sigma) in Hepes buffer and fractions were dialyzed (M, cutoff 7,500 Daltons) against 8 liters of PBS. Purity of recombinant proteins was assessed by SDS-PAGE and western blot analysis. Protein concentration were estimated according to the method of Pace et. al. (50). Yields were approximately 6 mg per 200ml culture.

Cell Adhesion Assays

Cell adhesion assays were performed to determine the ability of OPN to promote leukocyte adhesion. Polystyrene microwells (Corning-Costar, Cambridge, MA) were coated
with 50 µl of bovine OPN purified as previously described (51) or recombinant fragments of OPN at a concentration of 20 µg/ml in TBS overnight at 4°C. After blocking with 100 µl of 10 mg/ml BSA (Sigma, St. Louis, MO) in TBS, wells were rinsed with PSA (Gibco-BRL, Grand Island, NY). HL-60 promyelocytic leukemia cells and Ramos cells (ATCC, Rockville, MD) were grown in RPMI-1640 (Gibco-BRL) and 10% fetal calf serum. Human umbilical vein endothelial cells were grown in M199 (Gibco) supplemented with heparin (Sigma), bovine brain extract (52) and 20% fetal calf serum (Gibco). Leukocytes were rinsed and resuspended in PSA at a density of 100,000 cells/well and endothelial cells at 35,000 cells per well. Media for adhesion in all leukocyte experiments contained a final concentration of 100 µg/ml BSA with physiological doses of CaCl2 (2mM) and MgCl2 (1 mM). HL-60 cells were activated with the β1-activating antibody, 8A2 (53) at a concentration of 1 µg/ml and a phorbol ester, 12-0-tetradecanoyl phorbol 13-acetate at a concentration of 50 ng/ml. Endothelial cells were allowed to attach in the presence of 100 µg/ml BSA with 1.5 mM CaCl2 and 1.5 mM MgCl2. After plating, cells were allowed to adhere for 30-60 minutes at which time they were rinsed and fixed with formalin. Plates were stained with 0.1% Amido Black for 5 minutes, rinsed and solubilized with 2 N NaOH to obtain an absorbance reading at 595 nm which corresponds directly to the number of cells stained in each well (54).

**Peptide Synthesis and Adhesion Blockade**

To confirm the findings of the truncation studies, the SVVYGLR peptide (corresponding to C-terminal amino acids 162-168) was synthesized (Sigma-Genosys). Also generated were the wild-type peptide ELVTDFPTDLPATK and aspartate mutant ELVTEFPTELPATK, representing amino acids 131-143. Molecular weight of each peptide was confirmed by mass-
spectral analysis (Sigma-Genosys). The synthetic peptides SVVYGLR, ELVTDFPTDLPATK and ELVTEFPTELPA{T were pre-incubated with cells at 250, 500 and 500 µg/ml, respectively under activating conditions in the presence of divalent cations for 15 minutes. Following the incubation period, cells were seeded and the assay performed as described above.

Direct Integrin Binding using Affinity Chromatography

To illustrate the integrin-binding capacity of osteopontin, the synthetic peptides ELVTDFPTDLPATK and ELVTEFPTELPA{T were coupled to cyanogen-bromide 4B (Sigma) at 5 mg/ml according to the manufacturer’s instructions. Ramos cells (ATCC) were surface biotinylated as described (55) and a 50 µl pellet of cells was extracted with 1 ml TBS containing 3% octylglucoside (ICN, Irvine, CA) in 1.5 mM Mg^{2+}, 1.5 mM Mn^{2+} and 10^{-3} M phenylmethane sulfonic acid. The HL-60 cell extracts were agitated at 5-10 min intervals with Sepharose columns (0.5 ml) over a 2h period at 0°C. The columns were washed with 5 ml of TBS containing 3% octylglucoside, 1.5 mM Mg^{2+} and 1.5 mM Mn^{2+}. This was followed with a 15 ml wash in TBS containing 1% octylglucoside, 1.5 mM Mg^{2+} and 1.5 mM Mn^{2+}. Integrins were eluted with 2 ml TBS with 1% octylglucoside + 10 mM EDTA (0.25 ml fractions). Forty µl of each fraction were loaded and run under non-reducing conditions on a 7% acrylamide gel and transferred to PVDF membrane (Millipore). The membrane was blocked overnight at 4°C with 5% milk in 0.1% Tween-20 saline containing 2.5 mM Tris-HCl, pH 7.5. Blots were washed and Streptavidin-alkaline phosphatase (Sigma) was added (1:1000) to 1% BSA in Tween-20 saline and incubated for 30 minutes followed by a 30-minute wash in Tween-20 saline. The alkaline phosphatase activity was developed using alkaline phosphatase development kit (Bio-Rad) and stopped with water.
Integrin Immunoprecipitation

Integrins which bound to the OPN-Sepharose column were identified using immunoprecipitation. Sepharose beads conjugated with goat anti-mouse IgG (Sigma) were rinsed and suspended 1:1 with 0.5% Triton X-100 in TBS. In 1.5 ml microcentrifuge tubes, 200 µl of the bead mixture was added to 5 µg of monoclonal antibodies against several human integrin subunits including α4 (HP2/1, Immunotech) (56), β1 (mAb13, Becton-Dickinson) (57) and α5 (IIA1, Pharmingen) (58). These mixtures were then combined with 280 µl of pooled EDTA eluate from OPN-Sepharose and 700 µl of 0.5% Triton X-100 in TBS. This mixture was rotated continuously at 4˚C overnight after which time tubes were centrifuged and rinsed six times with 1 ml of 0.5% Triton X-100 in TBS. Seventy five µl of 2X sample buffer was added to the beads and this mixture was boiled for 5 minutes. Thirty µl samples were run on 7% SDS-PAGE under non-reducing conditions, and blots were developed as described above.

RESULTS

To rule out the involvement of the RGD site in α4β1-dependent adhesion to OPN, the wild-type, RGD containing N-terminal thrombin fragment (rOPN17-168) and an RGE mutant (rOPN17-168E161), where Asp161 was mutated to Glu161, were produced. Each clone is described as rOPN followed by the amino acids coded for in the construct (e.g. a.a. 17-168) and finally the mutation incorporated into the clone (e.g. E161 for Asp161 mutated to Glu161). Clones were sequenced to confirm successful mutation, and recombinant proteins (>95% purity) were analyzed using SDS-PAGE (not shown). Western blotting experiments using a monoclonal antibody direct to the N-terminal Histidine tag revealed a pattern exactly matching staining results (not shown). Proteins were tested for their ability to promote αvβ3-dependent attachment
Wild-type rOPN17-168 promoted endothelial cell attachment dose-dependently, while no attachment occurred to the RGE mutant. This confirmed successful functional mutation of the RGD site in OPN. The ability of both recombinant OPN constructs to promote $\alpha_4\beta_1$-dependent adhesion was compared using the HL-60 promyelocytic cell line in the presence of physiologic divalent cations (2 mM Ca$^{2+}$, 1 mM Mg$^{2+}$). As shown in figure 2B, no differences were observed in the ability of either construct to promote HL-60 cell attachment. Additionally, both rOPN17-168 and rOPN17-168E$^{161}$ were comparable to bovine OPN with respect to their ability to promote $\alpha_4\beta_1$-dependent cell attachment (Figure 2C). Adhesion to both native OPN and recombinant constructs was completely inhibited by the $\alpha_4\beta_1$-specific LDV peptide. The control peptide, LEV, had lesser effects compared to control (no peptide). Minimal adhesion was observed to BSA substrate. Collectively, these data indicate that the RGD site in the N-terminus of OPN is not involved in $\alpha_4\beta_1$-dependent adhesion to recombinant OPN, as rOPN17-168 and rOPN17-168E$^{161}$ promote attachment similarly. Consequently, subsequent constructs described contain the RGE mutation (Glu$^{161}$) to rule out any influence from the RGD site in OPN, although the presence of this mutation is not reflected to simplify nomenclature.

Various deletions of the OPN molecule were introduced in an attempt to localize $\alpha_4\beta_1$ binding sites. SDS-PAGE analysis of the recombinant proteins revealed ~95% purity as visualized by Coomassie blue staining and all proteins contained an N-terminal histidine tag by western blot analysis (data not shown). The rOPN55-168, rOPN17-129, rOPN17-138, rOPN17-142, rOPN17-164 and rOPN17-168 constructs were tested for the ability to promote $\alpha_4\beta_1$-dependent attachment of HL-60 cells (Figure 3). These experiments revealed that removal of the first 39 amino acids (rOPN55-168) did not reduce cell binding compared to the entire thrombin fragment, rOPN17-168. All binding activity was removed by truncation of amino acids 130-168.
(rOPN17-129), with partial activity returning in the presence of amino acid residues 130-142 (rOPN17-138 and rOPN17-142). In addition, decreased adhesion occurred by deletion of the last 4 amino acids (rOPN17-164), which was previously shown to eliminate the $\alpha_9\beta_1$ integrin binding site in OPN (59).

A more detailed analysis of binding activity was conducted with the constructs shown in Figure 1B. Dose-response curves illustrating the ability of constructs to promote HL-60 cell attachment are shown in Figure 4. No binding was observed with truncation of the last 38 amino acids (rOPN17-129), and identical results were observed with the rOPN17-135 construct. Partial activity was restored with the addition of amino acids 130-138 (rOPN17-138) indicating the presence of a potential binding site. Activity remained at similar levels with the addition of amino acids 139-164 (rOPN17-142, rOPN17-150, rOPN17-164). Only in the presence of the last 4 amino acids did full activity return (rOPN17-168). Thus, deletion of the last 4 amino acids on the C-terminus of the thrombin fragment of OPN partially eliminated the ability of $\alpha_4\beta_1$-dependent cell attachment to occur. The remainder of binding activity was completely removed with further truncation of the molecule by ending at amino acid residue 129. These data strongly support the concept that two binding sites exist within residues 130-168. To examine in more detail whether the Asp$^{135}$ and Asp$^{139}$ residues located within the upstream binding region identified were important in $\alpha_4\beta_1$-dependent cell attachment, additional constructs incorporating mutations were generated (Figure 4). The rOPN17-138E$^{135}$ exhibited reduced adhesion compared to rOPN17-138. The same was true for rOPN17-142E$^{139}$ versus rOPN17-142. Interestingly, neither of the mutations completely abolished adhesion, indicating that the conservative substitution of glutamate for aspartate did not remove all activity. Collectively, these data indicate that there are dual $\alpha_4\beta_1$ binding sites in the N-terminal thrombin fragment of OPN.
To confirm both sequences in osteopontin were capable of binding the α₄β₁ integrin, the synthetic ELVTDFPTDLPATK and SVVYGLR peptides, corresponding to amino acids 131-143 (with a C-terminal lysine residue added) and 162-168, respectively, were tested for their ability to block α₄β₁-dependent attachment (Figure 5). Also, the synthetic peptide ELVTEFPTELPAKT was created containing two conservative Asp to Glu mutations to further examine whether the aspartate residues were involved in binding to α₄β₁. In the presence of both the ELVTDFPTDLPATK and SVVYGLR peptides, α₄β₁-dependent adhesion was inhibited significantly compared to control (P<0.001). The ELVTEFPTELPAKT peptide consistently had lesser effects compared to control, similar to that observed with the LEV peptide (see Fig. 2C).

As further evidence for direct interaction of amino acids 131-143 in OPN with the α₄β₁ integrin, affinity chromatography experiments were performed using surface labeled Ramos cell extracts (Figure 6A). Wild-type ELVTDFPTDLPATK-Sepharose, aspartate mutant ELVTEFPTELPAKT-Sepharose and blank-Sepharose beads were incubated with labeled extracts, and EDTA elutions were collected and analyzed (E1-E4). Results show strong binding of the α₄β₁ integrin to the wild-type peptide, while minimal binding occurred to the aspartate mutant. No binding of the α₄β₁ integrin was observed using blank-Sepharose. Immunoprecipitation of fractions from both wild-type and mutated peptide columns revealed the presence of α₄ and β₁ integrin subunits (Figure 6B), although much greater binding occurred to the wild-type peptide. Mutation of aspartate residues resulted in a reduced ability of α₄β₁ to bind, but did not completely eliminate binding activity. In both experiments, control integrin antibodies failed to immunoprecipitate integrins. Similar results were observed using surface labeled HL-60 cell extracts (data not shown).
DISCUSSION

Using sequential truncation analysis of the N-terminal thrombin fragment of OPN, we observed that dual $\alpha_4\beta_1$ integrin binding sites exist in a 38 amino acid C-terminal domain. Synthetic peptides encompassing either of these regions interfered with the ability of $\alpha_4\beta_1$-dependent attachment to occur, while a control peptide had lesser effects. Also, using affinity chromatography we were able to demonstrate direct binding of the $\alpha_4\beta_1$ integrin to the wild-type synthetic peptide coupled to sepharose, while a mutated peptide bound considerably less well. These results show that dual binding sites exist for the $\alpha_4\beta_1$ integrin in the N-terminal thrombin fragment of OPN.

Integrin binding sites in osteopontin

Numerous members of the integrin family have been reported to interact with OPN including $\alpha_v\beta_3$ (51,60-62), $\alpha_v\beta_1$ and $\alpha_v\beta_5$ (63,64), $\alpha_4\beta_1$ (14), $\alpha_5\beta_1$ (65), $\alpha_9\beta_1$ (66) and $\alpha_9\beta_1$ (67). The RGD site (68) has been reported to interact with the $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$ and $\alpha_9\beta_1$ integrins (51,60-62). The $\alpha_4\beta_1$ integrin has been shown to bind the SVVYGLR amino acid sequence (59), which comprises the last 7 C-terminal amino acids in the thrombin fragment of OPN. These results are interesting based on sequence homology, in that the $\alpha_9$ integrin subunit is most closely related to the $\alpha_4$ integrin subunit (69). Both the $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins have the ability to interact with VCAM-1 (11,70) and OPN (14,59,67), as well as sharing other common ligands (71). Here, we found that deletion of the four C-terminal amino acids of the N-terminal thrombin fragment of OPN reduced but did not eliminate $\alpha_4\beta_1$-dependent adhesion. Our result contrasts with those observed previously for $\alpha_9\beta_1$ interaction with OPN where deletion of the
YGLR sequence completely eliminated \(\alpha_\beta_1\)-dependent cell attachment, and the synthetic SVVYGLR peptide completely blocked \(\alpha_\beta_1\)-dependent cell attachment (59). Previous work by Barry et al. demonstrated that the synthetic SVVYGLR peptide also interfered with \(\alpha_\beta_1\)-dependent cell adhesion, reporting that the SVVYGLR site in OPN is a binding site for the \(\alpha_\beta_1\) integrin (72). In the same study, using recombinantly produced peptide sequences of the OPN molecule coupled to GST, it was postulated that an additional binding site may exist for \(\alpha_\beta_1\), yet this was not demonstrated convincingly, as the FPTDLPA synthetic peptide used in the study failed to block adhesion (72). The work presented here confirms that the SVVYGLR site in the N-terminal thrombin fragment of OPN is a binding site for the \(\alpha_\beta_1\) integrin. Truncation of the last 4 amino acids, YGLR, resulted in \(~50\%\) decrease in adhesion and the synthetic SVVYGLR peptide significantly blocked \(\alpha_\beta_1\) cell attachment. We also define a second binding site from amino acids 131-143, ELVTDFPTDLPAT, as demonstrated by truncation analyses, peptide blockade and direct integrin binding. Interestingly, this peptide contains a tandem sequence consisting of two copies of a consensus D-hydrophobic residue-P that is similar to the \(\alpha_\beta_1\) binding site LDVP seen in CS-1 FN (10). In addition, the related \(\alpha_\beta_1\) binding sequence hydrophobic residue-D-X-P is seen in other \(\alpha_\beta_1\) ligands such as QIDSPL in VCAM-1 and IDAPS in FN (15-20). The one common feature of these sequences is the presence of a proline residue two amino acids downstream of an aspartate residue.

Additionally, we present evidence for the direct involvement of aspartate residues in the affinity of \(\alpha_\beta_1\) for ELVTDFPTDLPAT based on evidence that a mutant synthetic peptide, containing glutamate substituted for aspartate residues, was less effective at blocking cell adhesion. It also minimally bound the \(\alpha_\beta_1\) integrin in affinity chromatography experiments.
Conservative substitution of glutamate for aspartate residues did not remove 100% activity in either peptide blocking studies or direct integrin binding experiments. These results are consistent with previous data from our laboratory where the control LEV peptide also had slight effects (ref. 14 and Figure 2C). These results correlate with previous evidence that the $\alpha_4\beta_1$ integrin recognizes a wide variety of motifs in FN, VCAM-1, OPN and denatured proteins (10,15-21). As was previously suggested, this integrin shows a broader ligand binding specificity than most other members of the integrin family (13).

Evidence that the thrombin fragment of OPN is a matricryptin

During tissue injury, considerable alterations occur in the ECM due to enzymatic breakdown, multimerization, adsorption, mechanical forces or denaturation to expose matricryptic sites (73). Matricryptic sites are defined as biologically active sites that are not revealed in the mature, secreted form of the ECM molecules, but become exposed after structural or conformational alterations, and matricryptins represent biologically active fragments of ECM that contain exposed matricryptic sites (73). Ample evidence exists for the presence of both OPN and thrombin in injury sites (38,74), therefore increasing the likelihood that thrombin cleavage of OPN occurs in these settings (38,74). Several studies support the contention that exposure of matricryptic sites occurs within OPN following thrombin cleavage (38,62,65,67,75) (Figure 7). Senger and colleagues have found the thrombin fragment of OPN is more potent at promoting RGD-dependent attachment than the native protein (62). The immobilized thrombin fragment of OPN also stimulated greater haptotactic migration of tumor cells compared to the intact molecule (75). The $\alpha_9\beta_1$ (67) and $\alpha_5\beta_1$ (65) integrins were unable to bind OPN without thrombin cleavage. These data support the concept that matricryptic sites liberated in OPN
following thrombin cleavage may be important in regulating inflammatory cell interactions with OPN in the wound environment.

An interesting feature of the above findings is that all three known integrin binding sites are located within a very limited 38 amino acid region of the thrombin fragment of OPN. The ELVTDFPDPAT (shown here), RGD (51,60-62,67) and SVVYGLR sites (shown here and in refs. 59,72) are all localized to a 38 amino acid region just proximal to the thrombin cleavage site (Figure 7). These sequences are highly conserved, particularly in large species of mammals, while they are less conserved or absent in rodents, particularly concerning the upstream binding site. The thrombin fragment appears to have altered biological activity compared to intact OPN and contains matricryptic sites (62,65,67,73,75). Localization of these integrin binding sites directly adjacent to the thrombin cleavage site strongly implicate the physiological importance of this region of osteopontin in inflammatory and wound repair responses.

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FIGURE LEGENDS

**Figure 1.** Production of the N-terminal thrombin fragment of human OPN. A.) Amino acid sequence reported for the N-terminal thrombin fragment of OPN (48). Bracketed signal sequence [ ] representing amino acids 1-16 is not present on constructs generated here. This sequence is replaced by RGSHHHHHHS sequence. The underlined sequences represent regions of OPN with affinity for $\alpha_4\beta_1$. B.) Combined truncation and mutation analyses generated various constructs listed by amino acid number. (*) indicates mutation of Asp to Glu.

**Figure 2.** Characterization of recombinantly produced N-terminal thrombin fragment of OPN. A.) Dose-response curves comparing the ability of wild-type rOPN17-168 and rOPN17-168E$^{161}$ mutant to promote RGD-dependent attachment of human endothelial cells. Experiments were performed as described in the methods. B.) Dose-response curves comparing the ability of wild-
type rOPN17-168 and rOPN17-168E\textsuperscript{161} mutant to promote $\alpha_4\beta_1$-dependent attachment of HL-60 cells. Experiments were performed as described in the methods. C.) Peptide blocking data indicating HL-60 cell adhesion to native and recombinantly produced OPN occurs through the $\alpha_4\beta_1$ integrin. Experiments were conducted in the presence of 250 $\mu$g/ml of the LDV and LEV peptides as described in the methods. bOPN: bovine OPN, BSA: control substrate. The data shown are representative experiments (n=3) performed in triplicate wells and values shown are mean absorbance ($\pm$ st. dev).

**Figure 3.** Deletional analysis of the N-terminal thrombin fragment of OPN indicating the ability of constructs to promote $\alpha_4\beta_1$-dependent attachment of HL-60 cells. Proteins were coated at concentrations of 5$\mu$g/ml, and cell binding assays were conducted and quantitated as described in the methods. Data shown are from a representative experiment (n=4) performed in triplicate wells. Data shown are absorbance readings normalized to control binding, rOPN17-168 ($\pm$ st.dev.)

**Figure 4.** Truncational analysis of the N-terminal thrombin fragment of OPN. Ability of constructs to promote $\alpha_4\beta_1$-dependent attachment of HL-60 cells. Proteins were coated at 20$\mu$g/ml and serially diluted two-fold. Cell binding assays were conducted and quantitated as described in the methods. Data shown are from a representative experiment (n=4) performed in triplicate wells. Data shown are actual absorbance readings ($\pm$ st.dev.)

**Figure 5.** Peptide blocking data confirming the involvement of dual $\alpha_4\beta_1$ integrin binding sites in OPN. Activated HL-60 cells were pre-incubated for 15 minutes at 37°C with
ELVTDFPTDLPATH (500µg/ml), ELVTEFPTELPATH (500µg/ml) and SVVYGLR (250 µg/ml) peptides, added at equimolar concentrations. Control indicates the absence of peptide. Experiments were performed as in figure 3. Values represent averaged absorbance readings compared to control from four separate experiments performed in triplicate wells (±st.dev). *P<0.001 compared to control using Student’s t-test.

**Figure 6.** Affinity chromatography data illustrating binding of the α4β1 integrin to synthetic OPN peptides. A.) ELVTDFPTDLPATH-, blank- and ELVTEFPTELPATH-Sepharose columns were incubated with surface-biotylated Ramos cell extracts as described in the methods. Half ml EDTA elution fractions were collected, analyzed using western blots and developed for alkaline phosphatase activity (see methods for detailed description). Elution pattern for all columns is shown (E1-E4). Upper arrows denote the α subunit, while lower arrows denote β subunit. B.) Immunoprecipitations were performed with fractions from both ELVTDFPTDLPATH- and ELVTEFPTELPATH-Sepharose columns. Monoclonal antibodies directed to the integrin subunits tested for are indicated above each figure. These are Ø (no antibody), α4 subunit (HP2/1), β1 subunit (mAb13) and α5 (IIA1). Upper arrows denote the α4 subunit, while lower arrows denote β1 subunit.

**Figure 7.** Schematic diagram of the entire human OPN molecule illustrating the localization of known integrin binding sites. OPN contains a poly-Aspartate region, a thrombin proteolytic cut site and integrin binding sites. The ELVTDFPTDLPATH, RGD and SVVYGLR sites are located in a 38 amino acid region just upstream from the thrombin cut site. Sequences of human (48), bovine (75), porcine (76) and rat OPN (68) are provided. Shaded boxes indicate three known...
regions of OPN with affinity for integrins. The open boxes indicate areas of sequence homology in the intervening amino acids between integrin binding sites.
A

1  [M R I A V I C F C L L G I T C A] I 17 P V K
21  Q A D S G S S E E K Q L Y N K Y P D A V
41  A T W L N P D P S Q K Q N L L 55 A P Q N A
61  V S S E E T N D F K Q E T L P S K S N E
81  S H D H M D D M D D E D D D D H V D S Q
101 D S I D S N D S D D V D D T D D S H Q S
121 D E S H H S D E S 129 D E L V T D 135 F P T 138 D L
141 P A 142 T E V F T P V V 150 P T V D T Y D G R G
161 D S V V 164 Y G L R 168

B

17  129
17  135
17  * 138 E 135
17  138
17  * 142 E 139
17  142
17  150
17  164
17  168
17  55
17  168
Protein Coated (5 µg/ml)

Percent Control Adhesion

rOPN55-168  rOPN17-129  rOPN17-138  rOPN17-142  rOPN17-164  rOPN17-168
% Control Binding

Peptide Present

Control
ELVTDFPTDLPATK
ELVTEFPTELPATK
SVVYGLR
A

Sepharose: ELVTDFPPTDLPAK

220 kDa

97 kDa

E1 E2 E3 E4 E1 E2 E3 E4 E1 E2 E3 E4

α4
β2

B

Sepharose: ELVTDFPPTDLPAK BLANK ELVTDFPPTDLPAK

220 kDa

97 kDa

∅ α4 β1 α5 ∅ α4 β1 α5

α
β1
poly Aspartate

Thrombin cut site

SVVYGLR

Human:
ELVTDFPTDLPATEVTPTVPVTVTDYTGRGD

Bovine:
EV. .DFPTDIPTIATVFPTFIPTESTANDGRGDSVVYGLKSR

Porcine:
ELVTDFPTDTPATDV.TPAPVTGDPNDRGD

Rat:
ESF. .TASTQADVTPTIAPTVDVPGRGD

SLAYGLR
Identification of dual $\alpha_4\beta_1$ integrin binding sites within a 38 amino acid domain in the N-terminal thrombin fragment of human osteopontin
Kayla J. Bayless and George E. Davis

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