Osteopontin N-terminal Domain Contains a Cryptic Adhesive Sequence Recognized by $\alpha_9\beta_1$ Integrin*

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Osteopontin is an adhesive glycoprotein implicated in numerous diseases associated with inflammation and remodeling. There are several structural domains in osteopontin that are of particular interest. The RGD motif is a cell attachment sequence shown to be critical for cell adhesion through $\alpha_\beta$-containing integrins. In close proximity to the RGD domain is the thrombin cleavage site. Previous observations suggest that thrombin cleavage of osteopontin occurs in vivo and may be physiologically important. To study the functional significance of osteopontin cleavage by thrombin, we made glutathione S-transferase-osteopontin fusion proteins. These proteins contain either the N- or C-terminal domains expected to be formed following thrombin cleavage at the Arg$^{160}$–Ser$^{170}$ peptide bond. We compared these osteopontin fragments with native osteopontin in their ability to support adhesion of several different cell lines and identified the receptors mediating these interactions. Our data show that the N-terminal osteopontin fragment, which contains the RGD domain, supports adhesion of a melanoma cell line that is unable to bind native osteopontin. This suggests that osteopontin adhesive interactions may be regulated by thrombin cleavage. We also demonstrate that osteopontin contains a cryptic binding activity, which can be recognized by a novel osteopontin receptor. This receptor has been identified as the $\alpha_9\beta_1$ integrin.

Osteopontin is a multifunctional glycoprotein that promotes cell adhesion and migration. Previous studies have suggested that osteopontin plays a role in bone resorption, tumorigenesis, and metastasis (1). More recently, osteopontin has been implicated in a number of disease states associated with inflammation and tissue remodeling (2–6).

Many cellular interactions with osteopontin are mediated through integrin receptors. Integrins are capable of generating signals that control many aspects of cell behavior including differentiation, adhesion, migration, and apoptosis (7, 8). The $\alpha_9\beta_1$ integrin allows a variety of cell types to adhere and migrate to osteopontin (9–13). In addition to $\alpha_9\beta_1$, the $\alpha_9\beta_1$ and $\alpha_9\beta_3$ integrins were recently found to mediate adhesion of human aortic smooth muscle cells (12) and human embryonic kidney cells to osteopontin (14). A weak interaction was also demonstrated between $\alpha_9\beta_3$ and osteopontin in the macrophage line, P388D1 (15). Interestingly, occupancy of osteopontin with different receptors has distinct functional consequences. For example, in smooth muscle cells, $\alpha_9\beta_3$, $\alpha_9\beta_1$, and $\alpha_9\beta_2$ mediate adhesion, but only $\alpha_9\beta_3$ can support migration (12).

Osteopontin contains several interesting structural domains. The RGD domain is an adhesive motif found in many matrix molecules (7) and is critical for $\alpha_9$ integrin-dependent cell adhesion and migration to osteopontin (12, 16). Osteopontin is also susceptible to proteolytic fragmentation. There are two conserved thrombin cleavage sites in human osteopontin. The major thrombin cleavage site is at residues Arg$^{160}$–Ser$^{170}$, which is 6 amino acids C-terminal from the RGD domain. A second potential thrombin-cleavage site is within the RGD domain (Arg$^{160}$–Gly$^{161}$) (17). Previous studies have shown that osteopontin proteolytic fragments are found in vivo and may have physiological importance (17, 18). In addition, both osteopontin and thrombin are likely to be localized together at sites of injury, inflammation, angiogenesis, and in tumors. The functional activity of cleaved osteopontin, however, is unclear. One report demonstrated that thrombin cleavage destroyed RGD-mediated cell adhesion (19). In contrast, a second report showed that thrombin treatment enhanced osteopontin cell adhesive activity (20). One explanation for the discrepancy is that the interaction with osteopontin fragments may be mediated through distinct receptors in different cell types. A key to understanding the function of osteopontin, therefore, is identifying the receptors that interact with not only the full-length molecule, but with any functional proteolytic fragments.

To study the functional significance of osteopontin fragmentation, we performed adhesion experiments using glutathione S-transferase-osteopontin (GST-OPN)$^3$ fusion proteins. For these studies, we created osteopontin peptides that contain either the N- or C-terminal domains expected to be formed following thrombin cleavage at the Arg$^{160}$–Ser$^{170}$ site, which is 6 amino acids C-terminal from the RGD adhesive motif. We compared the osteopontin fragments in their ability to support adhesion of several different cell lines with that of native osteopontin and identified the receptors mediating these interactions. These studies show that the N-terminal fragment of osteopontin contains a functional RGD domain recognized by $\alpha_9\beta_1$ integrins.

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$^3$ The abbreviations used are: GST, glutathione S-transferase; OPN, osteopontin; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting.
αβ₃, as well as a cryptic adhesive sequence recognized by the αβ₃ integrin.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Bovine aortic endothelial cells were isolated from bovine aortas as described previously (21). Mo and Mø, melanoma cells were maintained in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal calf serum. Mo and Mø were both derived from M21 melanoma cells. One subclone, Mø, expresses high levels of αβ₃. Mo is a subclone expressing no detectable levels of the α₃ subunit. These cell lines were provided to us by Dr. Mark H. Ginsberg (The Scripps Research Institute) and have previously been described (22).

**Antibodies and Peptides**—Monoclonal antibodies (mAbs) against human β₁ (P4C10) (23), α₁ (P1E6) (23), α₄ (P1B5) (24), α₁ (P1D6) (25), and αβ₃ (P1F6) (26) as well as integrin β₃ (SBBQF) (28), β₃ (LM534) (29), and αβ₃ (LM609) (30) were purchased from Chemicon International, Inc. Monoclonal antibody against human α₃ (5E5D0) (51) was purchased from Upstate Biotechnology, Inc., Lake Placid, New York. The anti-β₃ antibody (SZ-21) was purchased from Immunotech (Westbrook, ME). Anti-α₃ (L230) has been shown to block the function of all α₃ integrins (26). The anti-α₁ (LM142) (30) was provided by Dr. Cheresh. Anti-α₁β₃ antibody (D57) (32) was provided by Dr. Mark Ginsburg. Anti-αβ₃ (JB55) is a mouse monoclonal antibody that has been previously characterized and was provided by Dr. Caroline Damsky (33). Anti-α₁ antibody (1057) (34) and anti-α₁ antibody (19946) (35) are both affinity-purified polyclonal antibodies against the cytoplasmic domain of the corresponding integrin. The neutralizing α₁ monoclonal antibody (Y9A2) (36) and the anti-β₃ antibody (ETF6) (26) have been previously characterized.

**RESULTS**

**Expression of Recombinant Osteopontin Fragments in E. coli Cells**—The osteopontin proteins that were used in this study include native osteopontin, recombinant human full-length osteopontin, and recombinant N- and C-terminal human osteopontin fragments that would be formed following thrombin cleavage (Fig. 1). Two N-terminal fragments were used, 10N and 30N, which refer to two different splice variants of osteopontin. The 30N splice variant contains an additional 14 amino acids (NASVSEETNDQKE), which correspond to exon 5.

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osteopontin (Fig. 4 both cell lines adhered to the 30N and 10N osteopontin fragments. Interestingly, full-length recombinant osteopontin (not shown). Surprisingly, the native, full-length OPN used in these studies was purified from rat pup smooth muscle cells. The full-length human recombinant protein was prepared as a His-tagged protein (His-OPN). All other osteopontin molecules were prepared as human recombinant GST-fusion proteins. The N- and C-terminal domains are fragments that are expected to be formed following thrombin cleavage. The C-terminal osteopontin domain (10C) contains amino acids 170–317. The N-terminal osteopontin fragments (30N and 10N) include amino acids 17–169. The two N-terminal domains are alternatively spliced. The 30N splice variant contains an additional 14 amino acids (NAVSEEETNDFKQE), which correspond to exon 5.

beads were treated with biotinylated thrombin. The biotinylated thrombin was then separated from the osteopontin fragments by affinity chromatography on streptavidin-agarose beads. The resulting proteins were analyzed on SDS-PAGE and shown in Fig. 2. The C-terminal osteopontin domain (10C) contains amino acids 170–317 and has an apparent molecular mass of 25 kDa. The two alternatively spliced N-terminal fragments (30N and 10N), contain amino acids 17–169. The 30N fragment includes exon 5, while the 10N fragment lacks this exon. The apparent molecular masses are 30 and 26 kDa, respectively. The C-terminal osteopontin domain (10C) contains a lower molecular weight protein, which is most likely the result of truncated GST-10C translational products.

Cell Adhesion to Native and Recombinant Fragments of Osteopontin—To compare the adhesive function of full-length osteopontin with fragments that would be formed following thrombin cleavage at the Arg169–Ser170 peptide bond, we performed cell attachment assays with native osteopontin, recombinant human full-length osteopontin, and recombinant N- and C-terminal human osteopontin fragments. Adhesion assays were carried out with both bovine aortic endothelial cells and two different subpopulations of human melanoma cells. Bovine endothelial cells adhered to full-length native OPN and both splice variants of the N-terminal osteopontin fragment (30N and 10N). There was no adhesion to the C-terminal osteopontin domain (10C) or the GST, which was used as a control (Fig. 3).

We next performed adhesion assays with two subpopulations of human melanoma cell lines. The melanoma cell lines M21 and Mo were derived from M21 (22). Moαv was previously shown to express high levels of the αvβ3 integrin. Mo lacks expression of the αv subunit; therefore, these cells fail to express many of the known osteopontin receptors (αvβ3, αvβ1, and αvβ5). As expected, Moαv, but not Mo cells adhered to native osteopontin (Fig. 4A). Similar results were seen with human full-length recombinant osteopontin (not shown). Surprisingly, both cell lines adhered to the 30N and 10N osteopontin fragments (Fig. 4B). There was no adhesion to the C-terminal domain or the GST control. Because Mo cells interacted with the N-terminal domain of osteopontin and not the native protein or full-length recombinant protein, these data suggest that osteopontin adhesive interactions may be regulated by proteolytic fragmentation. Furthermore, the N-terminal region apparently contains a cryptic adhesive activity that is not exposed in the full-length molecule.

Moαv, Adhesion to Native Osteopontin and the N-terminal Domain

![Fig. 1. Schematic diagram of native osteopontin and human recombinant osteopontin fragments used for adhesion assays.](image)

The interaction between Moαv and the native osteopontin N-terminal domain was completely blocked by both the anti-αvβ3 mAb (LM609) and an anti-αv, mAb (L230) (Fig. 5A). This indicated that the osteopontin adhesive function was dependent on the αvβ3 integrin.

Adhesion of Moαv to native osteopontin was also αvβ3-dependent. The interaction between Moαv and the native osteopontin was completely blocked by anti-αvβ3 mAb LM609 but not by anti-αvβ1, an irrelevant antibody control (Fig. 5B). The adherence of untreated cells and anti-αvβ1-treated cells were similar.

As expected, the interaction of Moαv with the osteopontin N-terminal domain was mediated through the RGD sequence. GRGDSP peptide, but not GRGESP peptide, inhibited Moαv...
**α9β1 Integrin Binds Osteopontin N-terminal Domain**

**Fig. 3. Adhesion of bovine aortic endothelial cells to native osteopontin and recombinant human osteopontin fragments.** Bovine aortic endothelial cells (30,000/well) were allowed to attach for 1 h to wells coated with the indicated concentration of native OPN, osteopontin recombinant fragments (30N, 10N, and 10C), or GST control. The attached cells were fixed and stained with toluidine blue as described under “Experimental Procedures,” and the absorbance was measured at 595 nm. Each data point represents the mean ± S.D. of triplicate samples. Nonspecific cell adhesion as measured on BSA-coated wells was subtracted.

**Fig. 4. Adhesion of human melanoma cell lines, Mo and Moα9, to native osteopontin and osteopontin recombinant fragments.** Melanoma cells (100,000/well) were allowed to attach for 1 h to wells coated with 40 nM native OPN (A) or 40 nM osteopontin recombinant fragments (30N, 10N and 10C) (B). The attached cells were quantitated as described in the legend to Fig. 3. Each data point represents the mean ± S.D. of triplicate samples.

adhesion to the osteopontin N-terminal domain in a dose-dependent manner with an IC50 of 12 μM (Fig. 5C).

**Mo Cell Adhesion to the N-terminal Osteopontin Fragment Is β1-dependent and Poorly Blocked by RGD Peptides—Mo melanoma cells lack all α-integrin receptors, and αβ1, the interaction of Mo with the N-terminal fragment might be through αβ1 or a novel osteopontin receptor. Binding of Mo to the N-terminal fragment was cation-dependent (Fig. 6A), suggesting that this receptor was an integrin. To identify the integrin, Mo adhesion to the osteopontin N-terminal domain was carried out in the presence of integrin-neutralizing antibodies. As shown in Fig. 6B, the interaction between Mo and the N-terminal osteopontin domain was entirely blocked by P4C10, a neutralizing β1 mAb. A nonblocking β1 antibody, LM534, failed to inhibit adhesion. The α subunit mediating this interaction was not α1, α2, α5, or α6, because blocking antibodies to these subunits had no effect on Mo adhesion (Fig. 6C).

**Fig. 5. Adhesion of α9β1-expressing melanoma cell line Moα9 to osteopontin and the osteopontin N-terminal fragment in the presence of anti-integrin antibodies and RGD peptides.** Moα9 cells were preincubated with and without neutralizing antibodies directed against the indicated integrins (A and B) or with peptides (C) for 15 min at 37 °C before plating on wells coated with 40 nM N-terminal osteopontin fragment (10N) (A and C) or 40 nM native osteopontin (B). The monoclonal antibodies used were L230 (α5), 5E8D9 (α5), P1E6 (αv), P1B5 (α3), P4G9 (αv), P1D6 (α5), P4C10 (β1), LM609 (αβ1), P1F6 (αβ3), and D57 (αβ3β3), which were used as an irrelevant antibody control. The attached cells were quantitated as described in the legend to Fig. 5. Each data point represents the mean ± S.D. of triplicate samples.

The β1 interaction with the N-terminal osteopontin fragment was only partially dependent on RGD (Fig. 6D). The GRGDSP peptide inhibited adhesion with an IC50 of 250 μM, which was about 20 times higher than that observed for GRGDSP inhibition of Moα9 binding to the N-terminal fragment of osteopontin. These data suggest that the N-terminal osteopontin peptide may contain an additional adhesive domain, distinct from RGD, which is recognized by the β1 integrin on Mo cells.

Identification of Surface-expressed β1 Integrins from Mo Melanoma Cells—The antibody blocking studies suggested that the α subunit responsible for the β1-mediated adhesion of Mo to the N-terminal fragment was not α1, α2, α3, α5, or α6. There are four additional α subunits known to form heterodimers with the β1 integrin subunits: α6, α7, αv, and α9. It is unlikely that α9 mediates adhesion, because Mo cells express extremely low levels of this integrin by FACS analysis (Table 1). To determine if α9β1 was involved, we measured the adhesion of α9-containing 293 cell transfectants to the N-terminal osteopontin fragment in the presence of neutralizing antibodies. The adhesion was completely blocked by an anti-α9 antibody, suggesting that α9β1 does not mediate adhesion by itself (not shown). To determine if the α9 subunit was associated with surface-expressed β1 integrin, we immunoprecipitated surface-biotinylated cells with P4C10, a β1 mAb. Immunoprecipitation revealed multiple α chains with apparent molecular mass between 100 and 180 kDa associated with the β1 integrin (Fig. 7A). Two bands with molecular masses of approximately 140 and 115 kDa were particularly abundant. These sizes correspond to the molecular weight of the α9 and β1 subunits, respectively. To further identify these chains, an α9 antibody was used to immunoprecipitate surface-biotinylated proteins. Fig. 7B shows that the α9 antibody immunoprecipitated abundant levels of two proteins, corresponding to α9 and β1 subunits, respectively (34).

α9β1 Mediates Adhesion of α9-transfected SW480 and Mo Cells to the N-terminal Domain of Osteopontin—Because a significant level of α9β1 was expressed on Mo cells, we next determined if α9β1 can mediate cell adhesion to the N-terminal fragment by using a cell line that was stably transfected with α9. SW480 cells, human colon carcinoma cells that normally do not express α9β1 integrin, were stably transfected with a plasmid encoding for the α9 integrin subunit, pCDNA1neoα9. These cells have been shown to adhere to a tenascin fragment containing the third fibronectin type III repeat, while mock transfectants do not adhere to this fragment (41). The α9-transfectants also adhered to the N-terminal osteopontin fragment.
**αβ1 Integrin Binds Osteopontin N-terminal Domain**

Mo and Moα cells were stained with mAbs specific to αα (mAb 1880), α1 (5E8D9), α2 (P1E6), α3 (P1B5), α4 (P4G9), α5 (P1D6), α6 (J1B5), α9 (Y9A2), β1 (P4C10), β3 (SZ.21), β5 (ETP6), αβ2, αβ3, LM609, αβ5 (P1F6), or mouse IgG followed by phycoerythrin-labeled anti-mouse IgG and analyzed by FACS scan. Peak fluorescence intensity = linear fluorescence intensity (test antibody)/linear fluorescence intensity (control antibody).

| Antibody | Peak fluorescence intensity |
|----------|----------------------------|
| Mo       |                           |
| Moα      |                           |
| αα       | 1.90                      |
| αα       | 4.14                      |
| αα       | 3.10                      |
| αα       | 1.62                      |
| αα       | 1.26                      |
| αα       | 1.83                      |
| αα       | 7.02                      |
| αβ1      | 12.14                     |
| αβ2      | 1.02                      |
| αβ3      | 1.21                      |
| αβ3      | 1.17                      |
| αβ3      | 1.07                      |
| αβ3      | 3.88                      |

**DISCUSSION**

This study examined the potential role of proteolytic fragmentation on osteopontin function. We compared the N- and C-terminal GST-recombinant osteopontin fragments expected to be formed following thrombin cleavage at the Arg169-Ser170 site with native osteopontin in their ability to mediate adhesion of several cell lines. The results demonstrated that: 1) osteopontin adhesive interactions may be regulated by proteolytic fragmentation such as seen with thrombin, 2) two different splice variants of the N-terminal osteopontin fragments have identical adhesive properties, and 3) N-terminal osteopontin fragment contains two distinct integrin-binding activities. One is the RGD-dependent αβ3-binding activity. The second is a cryptic binding activity for the ααβ1 integrin. Thus, proteolytic fragmentation may be a way of controlling or altering osteopontin’s receptor specificity and thus its function.

Using N- and C-terminal recombinant fragments, we have shown that two splice variants of the N-terminal, but not the C-terminal, domain can support adhesion of bovine aortic endothelial cells and two subpopulations of human melanoma cell lines. The two subpopulations of melanoma cells differ in αα integrin expression. Moα contains high levels of the ααβ1 in-
osteopontin, the αβ₁ binding domain in tenasin appears to be distinct from the RGD adhesion motif (41). However, Mo adhesion to the osteopontin fragment is at least partially inhibited by RGD, suggesting that either αβ₁ recognizes osteopontin and tenasin by somewhat different mechanisms or that other RGD-dependent receptors are involved.

In normal adult tissue, osteopontin and α₃ are expressed on most epithelia and could potentially colocalize (34, 43–45). In diseased tissue, osteopontin is highly up-regulated. Abundant osteopontin is found at the interface between malignant and normal tissue and at sites of inflammation and tissue remodeling (5, 46). These are also sites where thrombin and thrombin-cleaved fragments of osteopontin are likely to be found. It would be interesting if α₃ is coordinately up-regulated at these sites. If so, the ability of osteopontin to promote adhesion, migration, or other cellular functions may be regulated in the presence of thrombin by exposing the cryptic domain.

Cryptic integrin-mediated binding activities have also been identified in other adhesive proteins. For example, laminin contains a cryptic peptide site that becomes functional after proteolysis and supports αβ₁-mediated adhesion of rat osteoclasts (47). Collagen also contains a cryptic site that is exposed following denaturation. Native type I collagen in its helical conformation supports α₁β₁-, α₁β₃-, and α₁β₅-mediated adhesion. These interactions are disrupted by heating or proteolysis of collagen, revealing a cryptic α₁β₅ binding activity (48). The exposure of novel binding activities following proteolytic fragmentation is particularly relevant in remodeling tissues where proteases are active.

Both Mo and Moα₉ express equal amounts of surface α₉ integrin, but only the Mo cells use this receptor for N-terminal osteopontin interactions. There are several possible explanations for this phenomenon. First, the activation state of α₃β₁ on the two cell lines may differ. It is known that β₁ integrins can exist in different activation states, which can affect the affinity of ligand binding (49–52). The second possibility is that α₃β₁ function could be regulated by the ligation of another integrin. For example, the interaction of α₁β₅ with ligand could transmit a signal that inhibits the affinity modulation and/or function of α₁β₅. This type of “cross-talk” between integrins has previously been reported in several cell types (53–55).

Two previous reports have examined the functional consequences of osteopontin cleavage with thrombin. One report demonstrated that thrombin cleavage destroyed RGD-mediated adhesion (19). In contrast, a second report showed that thrombin treatment enhanced osteopontin-mediated adhesion (20). In agreement with the second report, our studies demonstrate that the N-terminal fragment that is expected to be formed following thrombin cleavage can support adhesion. It is clear from these studies as well as others that the interaction of cells with osteopontin and osteopontin fragments is mediated through distinct receptors in different cell types. This could explain the discrepancy between different studies. Another possible explanation is that the conditions used to cleave osteopontin with thrombin may result in partial or complete proteolysis at an additional thrombin cleavage site. A second potential cleavage site is at residues Arg₁⁰⁰-Gly₁⁰¹, which is within the RGD domain. If the RGD were destroyed by proteolytic cleavage, it is likely that α₁β₅-mediated interactions would not take place.

There are several different splice variants of osteopontin. Two of the variants are due to variable usage of exon 5, which contains 14 amino acids. The functional significance of alternative splicing in this gene is not known. In this study we compared the adhesive function of recombinant osteopontin N-terminal fragments of each splice variant. Our results dem-

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2 L. L. Smith, H.-K. Cheung, L. E. Ling, J. Chen, D. Sheppard, R. Pytel, and C. M. Giachelli, unpublished observations.
onstrate that both splice variants had identical adhesive functions. Exon 5 contains the sequence NAVSSEETNDFKQE. The two serine residues are potential sites for phosphorylation or O-linked glycosylation; therefore, our data do not rule out the possibility that there is a functional difference following post-translational modification.

In conclusion, we have demonstrated that the N-terminal fragment of osteopontin contains two distinct activities. We predict from these results that osteopontin fragmentation by proteases, such as thrombin, are important in the regulation of receptor specificity and, thus, the function of osteopontin.

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