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Non-RGD Domains of Osteopontin Promote Cell Adhesion Without Involving αv Integrins

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Abstract Osteopontin (OPN) is an integrin-binding secreted protein that contains an Arg-Gly-Asp (RGD) amino acid sequence and binds to various cell types via RGD-mediated interaction with the αvβ3 integrin. We have identified a cell line whose binding to OPN does not require RGD or αv interactions. We compared the ability of two murine cell lines, L929 fibroblastic cells and B16-BL6 melanoma cells, to interact with OPN (from human milk, and recombinant human and mouse OPN) as well as recombinant OPN prepared to include either the N-terminal or C-terminal halves but lacking the RGD sequence. Both cell lines adhered to GRGDS peptides coupled to BSA, and these interactions were inhibited by addition of GRGDS (but not GRGES) peptides or a monoclonal antibody specific to the αv integrin subunit. Adhesion of L929 cells to OPN was also dependent on the RGD sequence and the αv integrin subunit. However, the binding of B16-BL6 cells was not inhibited by either GRGDS peptides or the anti-αv antibody. B16-BL6 (but not L929) cells were also able to adhere to and spread on both N-terminal and C-terminal OPN proteins that lack the RGD sequence, and these interactions were not inhibited by either GRGDS peptides or anti-αv antibody. Together these results indicate that B16-BL6 cells can adhere to OPN by interactions that are independent of either the RGD sequence or the αv integrin subunit, and suggest that some cells can interact with additional, non-RGD binding sites in OPN.

Key words: OPN, binding site, integrin

INTRODUCTION

The tripeptide sequence Arg-Gly-Asp (RGD) within osteopontin (OPN) provides a crucial structural site for cell binding and is recognized by the cell surface receptor, αvβ3 integrin [Oldberg et al., 1980; Bulter, 1989; Miyauchi et al., 1991]. Various extracellular matrix (ECM) molecules such as fibronectin (FN) and collagen or soluble proteins found in plasma such as vitronectin (VN), thrombospondin, fibrinogen, and von Willebrand factor have also been shown to contain an RGD sequence [Yamada, 1991]. Cell surface receptors for these RGD containing molecules are members of the integrin family, including αvβ3. However, additional adhesive recognition sequences have been identified in various molecules such as FN, laminin, fibrinogen, von Willebrand factor, collagen, and thrombospondin [Yamada, 1991; Bowditch et al., 1991; de Boer et al., 1992; Kosfeld et al., 1991; Gehlsen et al., 1992]. Those studies indicated that the αvβ3 integrin can recognize RGD as well as non-RGD sequences within molecules and a single protein molecule can interact with cells through several distinct cell binding sites. More recently, it was shown that rat OPN may contain a second cell binding site [Van Dijk et al., 1993]. This second site consists of a non-RGD sequence or undefined structure in the carboxy terminal half of OPN that can bind to the αvβ3 integrin. In this paper, we report that the binding of murine B16-BL6 melanoma cells to OPN occurs through an RGD- and αv-independent process and provide evidence for the existence of additional cell binding sites within the C-terminal and N-terminal halves of OPN molecule.

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MATERIALS AND METHODS

Reagents

A rat monoclonal antibody (mAb) (RMV7, rat IgG2c) reacting to mouse αv integrin was kindly supplied by Drs. Yagita and Okumura, Juntendo University, School of Medicine, Tokyo, Japan [Takahashi et al., 1990]. A rat mAb (7G12) reacting to murine Thy-1 established in our laboratory was also used as an isotype matched control. The synthetic peptides, GRGDS and GRGES and the peptide-protein conjugate, GRGDS coupled to bovine serum albumin (BSA) were purchased from Iwaki Glass Inc. (Tokyo, Japan). Human plasma fibronectin and murine laminin were purchased from Koken, Inc. (Tokyo, Japan). Human vitronectin was purchased from Iwaki Glass Inc. Fetuin (type III) was purchased from Sigma Chemical Co. (St. Louis, MI). Antiserum specifically reacting to OPN was prepared as follows. A rabbit was immunized with bovine serum albumin (BSA) coupled to the synthetic peptide (EQYPDATDEDLTSHM), which corresponds to an internal amino acid sequence of mouse OPN. Immunoglobulin (Ig)G fraction was obtained from the serum of the immunized rabbit and was extensively absorbed with BSA-coupled Sepharose. The resulting IgG was further applied to a synthetic peptide (EQYPDATDEDLTSHM)-coupled Sepharose column. After extensive washing, the bound IgG fraction was eluted and used as anti-OPN antibody. This antibody reacts with milk OPN, recombinant hOPN, and recombinant mOPN. However, this antibody does not react with BSA, FN, or VN.

Cell Lines

Highly metastatic B16-BL6 and B16-F10 melanoma cells originally provided by Dr. I.J. Fidler, M.D. Anderson Cancer Center, Houston, Texas, were maintained in the Institute of Immunological Science, Hokkaido University, Sapporo, Japan [Matsumoto et al., 1991]. Murine fibroblastic cell line L929 was also used in this study [Minamoto et al., 1990]. These cells were maintained as monolayer cultures in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum.

Construction of the GST-OPN Fusion Plasmid

Oligonucleotides used in this study were synthesized based on published murine OPN cDNA sequence [Craig et al., 1989]; primer 1, (5′ AAAGGATCCCCCTCCCGGTGAAAGTGACT 3′) containing the *Bam* HI restriction site and cDNA sequences of the first six amino acids of mature OPN; primer 2, (5′ TTTCCCGGGTCAGCCGTTGGGGACG [original T was complemented by G to create a *Sal* I site; no amino acid change resulted from this substitution] TCGACTGTAGG3′] complementary to the cDNA sequence of the eight amino acids prior to $R_{128}G_{129}D_{130}$, plus a stop codon and a *Sma*I restriction site; primer 3, (5′ AAAGGATCCGCTTGGCTTATGGACTGAGG3′) containing a *Bam* HI restriction site and the cDNA sequence of the six amino acids starting from L_{132}; primer 4, (5′ TTTCCCGGGTTAGTTGACCTCAGAAGATGA 3′) complementary to the cDNA sequence of the last six amino acids of mature OPN, plus a stop codon and a *Sma*I restriction site. PCR reactions were conducted using full length murine OPN cDNA as a template directed by appropriate pairs of the above primers. PCR products were purified and cloned into pCRII vector according to previously described methods [Staar and Quaranta, 1992]. The OPN cDNA cloned by PCR was completely sequenced and inserted into pGEX-3X vector in the same reading frame as the carrier gene (glutathione S-transferase, EC 2.5.1.18) [Smith and Johnson, 1988] and transformed in *E. coli* DH5 α cells. Thus three murine GST-OPN were produced; GST fused to mature murine OPN (r-mOPN; L_1-N_{293}), an N-terminal half of mOPN which lacks $R_{128}G_{129}D_{130}$ (r-mOPNdeltaN; L_1-G_{127}), and a C-terminal half of mOPN which lacks $R_{128}G_{129}D_{130}$ (r-mOPNdeltaC; L_{132}-N_{293}). The human GST-OPN (r-hOPN) fusion plasmid was constructed as described previously [Xuan et al., 1994].

Protein Purification

The various recombinant GST-OPN fusion proteins were prepared in *E. coli* as described previously [Xuan et al., 1994]. The GST fusion proteins were purified on glutathione-Sepharose columns as described [Smith and Johnson, 1988]. OPN from human milk was purified as follows; fifty ml of human colostrum (milk-OPN) was applied to a DE52 column (2.5 × 15 cm) equilibrated with 50 mM Tris-HCl buffer pH 7.2 containing 6M urea and eluted with a linear gradient of NaCl from 0 to 0.7 M in the starting buffer. The eluted proteins were pooled as 3 ml individual fractions and then subjected to sodium dodecyl sulfate (SDS)-polyacrymalide gel electrophoresis (PAGE) using Laemmli's sys-

tem. The presence of OPN was estimated by staining the gel with Coomassie Brilliant Blue R-250 (CBB) and was confirmed by immunoblotting as described below. The fraction containing OPN was rechromatographed as described above. The protein of interest then was subjected to gel filtration chromatography on a 2.5 cm × 100 cm Sephadex G-150 column equilibrated with 50 mM Tris-HCl buffer pH 7.2 containing 4 M guanidine HCl and eluted with the same buffer. The OPN fraction was dialyzed against phosphate buffered saline (PBS) and diluted to 1:10 with 0.1% trifluoroacetic acid (TFA) in water. The OPN protein was applied to reverse phase column chromatography on a PRoRPC 5/2 column and eluted with a 15 ml linear gradient from 0 to 90% acetonitrile (each containing 0.1 ml TFA per 100 ml) using Fast Protein Liquid Chromatography system (Pharmacia Fine Chem., Tokyo, Japan). The purity of protein was analyzed by SDS-PAGE as described above. Polyacrylamide gels were then stained with CBB or the cationic carbocyanine dye "Stains-all" (Nakarai Chem., Kyoto, Japan), which stains sialoglycoproteins and phosphoproteins blue and all other proteins red [Campbell et al., 1983]. In some experiments, after SDS-PAGE, proteins were blotted to PVDF membranes and immunostained with the monospecific antibody raised against the synthetic peptide EQYPDATDEDLTSHM corresponding to an internal sequence of OPN. In some experiments, the purity of protein was evaluated by amino acid sequence analysis.

Amino Acid Sequence

Amino acid sequence analysis was performed on an ABI 477A automated amino acid sequencer (Applied Biosystems, Foster City, CA).

Cell Binding Assay

Ninety-six well plates were precoated with various concentrations of OPN, GST, GST-OPN, VN, or GRGDS coupled to BSA, overnight at 4°C followed by treatment with 0.5% BSA in RPMI-1640 medium for 10 min at 37°C to block nonspecific binding. B16-BL6 melanoma cells were suspended in DMEM containing 0.25% BSA and 200 μl cell suspension (at a cell density of 5×10^4 cells/well) were applied to 96 well plates precoated with OPN, VN, GST, GST-OPN, or GRGDS coupled to BSA, and incubated for 1 h at 37°C. The medium was removed from the plates, and all wells were washed twice with PBS. The adherent cells were fixed and stained with 0.5% crystal violet in 20% methanol for 30 min. All wells were rinsed three times with water and adherent cells were then lysed with 20% acetic acid. Absorbance at 595 nm were measured from the resulting supernatants from each well, using an immunoreader, to determine the relative number of cells attached per well. In most experiments, cell adhesion was expressed as a percentage of maximum binding to the precoated plates without added synthetic peptides or mAb as a inhibitor. All assays were performed in triplicate and at least three separate experiments were done to obtain data. Each value represents the mean of at least three separate experiments. In some experiments, assays were performed to determine whether binding and interaction with OPN resulted in cell spreading as described by Guan and Hynes [1990]. Spreading of attached cells to OPN was evaluated under the microscopic observation and was photographed.

Flow Cytometry

Cells were incubated with a saturating amount of mAb reacting to murine αv integrin for 30 min at 4°C. Cells were washed with PBS, then incubated with FITC conjugated murine anti-rat *k* chain (MARK-1, mouse IgG1) for 30 min at 4°C. After washing, cells were analyzed using a fluorescence-activated cell sorter (FACS; Becton Dickinson & Co., Mountain View, CA).

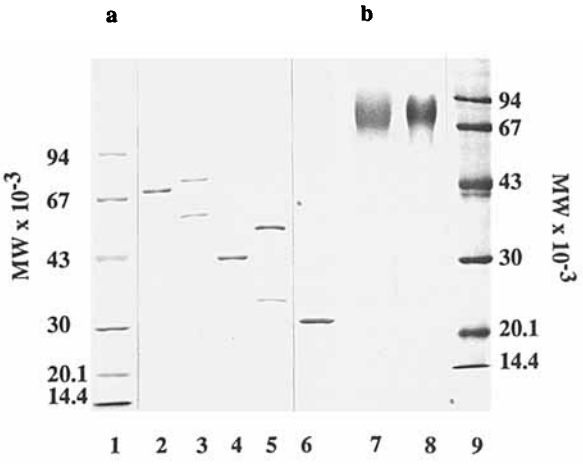

Fig. 1. SDS-PAGE analysis of purified OPN. Purified OPN was electrophoresed on SDS-polyacrylamide gels (**a:** 4–20%. **b:** 12.5%) under reducing conditions. The gels were stained with CBB (*lanes 1–7 and 9*) or stains all (*lane 8*). 1 and 9: molecular markers. 2: r-hOPN. 3: r-mOPN. 4: r-mOPNdeltaN. 5: r-mOPNdeltaC. 6: GST. 7 and 8: milk-OPN.

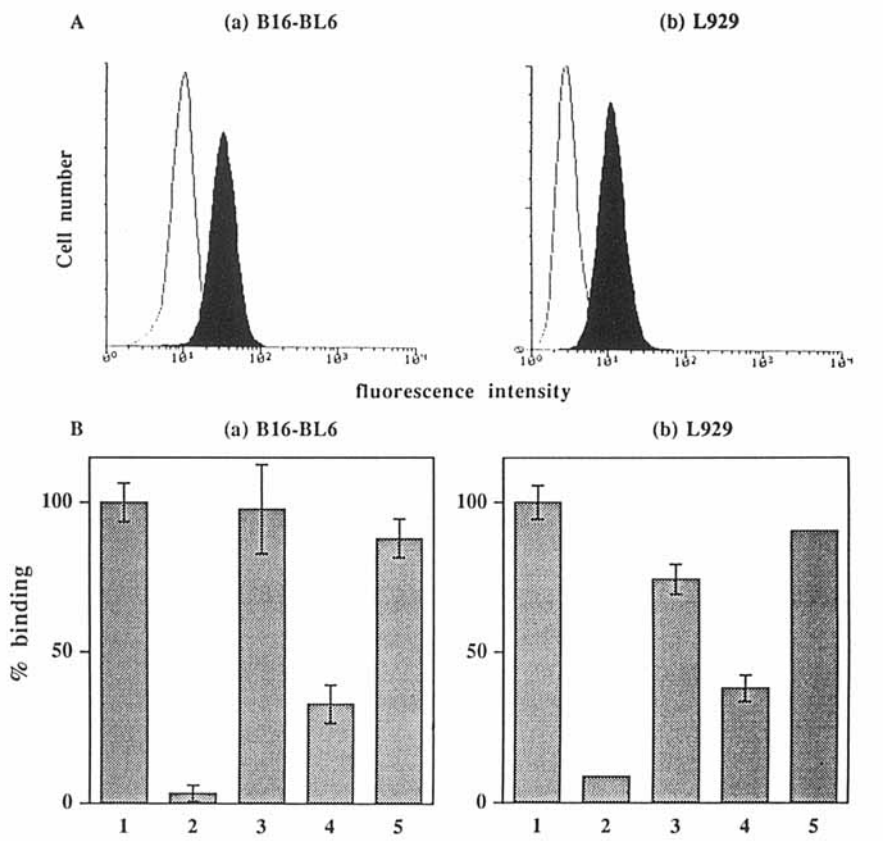

Fig. 2. The expression of functional αv integrin on B16-BL6 and L929 cells. **A:** B16-BL6 **(a)** and L929 **(b)** cells were stained with rat anti-mouse αv (RMV7) mAb followed by fluorescein isothiocyanate (FITC)-labeled mouse anti-rat k mAb (solid peaks) or by FITC-labeled mouse anti-rat k mAb alone as a negative control (open peaks) and analysed by flow cytometry analysis. **B:** The binding of B16-BL6 **(a)** or L929 **(b)** cells to GRGDS coupled to BSA was studied. Plates were precoated with 40 μg/ml of GRGDS coupled to BSA, overnight at 4°C. Cell binding assays were performed in the presence of medium (*1*), 100 μg/ml GRGDS peptide (*2*), 100 μg/ml GRGES peptide (*3*), 100 μg/ml anti-mouse αv (RMV7) mAb (*4*), or 100 μg/ml isotype matched anti-thy-1 (7G12) mAb (*5*) and the cell binding was quantitated as described in Materials and Methods. Maximum binding of B16-BL6 and L929 cells as measured by the absorbance (A595 nm) from cells bound to GRGDS coupled to BSA was 0.846 and 0.501, respectively.

RESULTS

Purification of Osteopontin

After the several purification steps described in Materials and Methods, the purity of the samples was evaluated by SDS-PAGE analysis. Gels were then stained by CBB or Stains-all. As shown in Figure 1, the various GST-OPN and milk-OPN proteins were highly purified. After blotting, these bands (except lanes 1, 4, and 6) were clearly stained by an antibody raised against an internal sequence of OPN (data not shown). The purity of milk-OPN was also verified by the detection of a single amino-terminal sequence that is IPVKQADS.

Expression of Functional αv Integrin Receptor on Murine Melanoma and Fibroblastic Cell Lines

B16-BL6 cells and L929 cells were first examined by flow cytometry to determine if these cells express cell surface αv integrin. As shown in Figure 2A, both cells expressed αv integrin. To test whether the αv integrins were functional, cell binding assays were performed (Fig. 2B). Both cells bound to GRGDS coupled to BSA (lanes 1). The binding of B16-BL6 and L929 cells to GRGDS coupled to BSA was strongly inhibited by GRGDS (lanes 2) or mAb reacting with murine αv (lanes 4), but not by either GRGES (lanes 3) or an irrelevant isotype matched mAb (lanes 5). Thus, both cells expressed functional αv integrin.

The Binding of B16-BL6 Cells to OPN

First, we examined whether various cells can bind to both glycosylated (milk OPN) and unglycosylated (recombinant human OPN; r-hOPN) forms of OPN. As shown in Figure 3, B16-BL6,

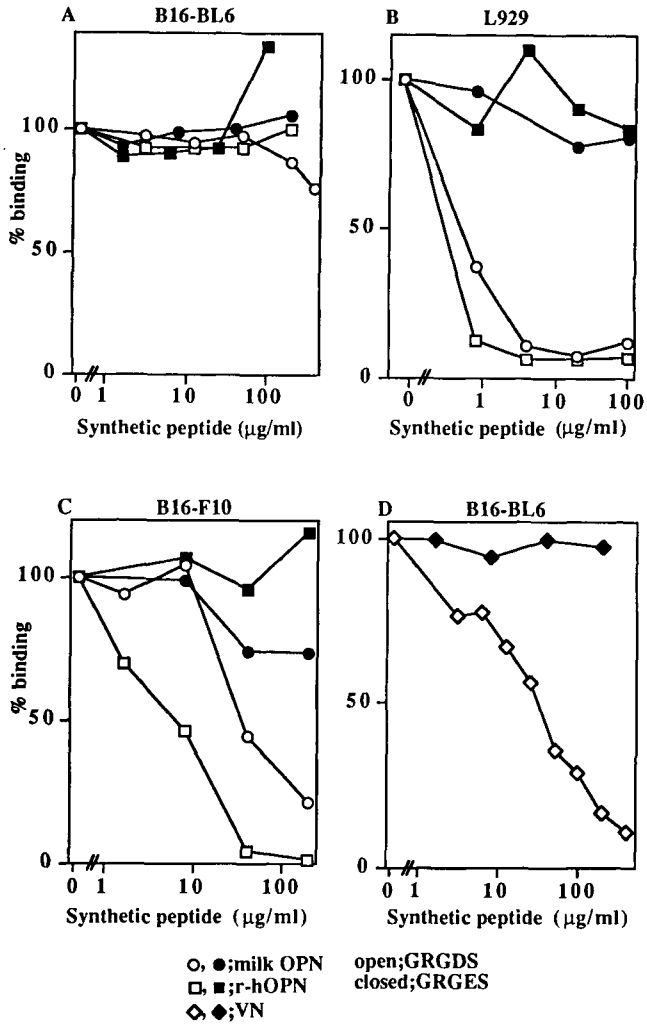

Fig. 3. The binding of B16-BL6 cells to OPN in an RGD-independent manner. The binding of B16-BL6 (**A and D**), L929 (**B**), or B16-F10 (**C**) cells to plates precoated with various concentrations of milk-OPN, r-hOPN, or VN was studied in the presence of various concentrations of GRGDS (open symbols) or GRGES (closed symbols) synthetic peptides. A: Plates were precoated with 1 μg/ml milk-OPN or 1 μg/ml r-hOPN in a volume of 50 μl. Maximum binding of B16-BL6 cells as measured by the absorbance (A595) to milk-OPN and r-hOPN was 0.980 and 1.056, respectively. B: Plates were precoated with 4 μg/ml milk OPN or 10 μg/ml r-hOPN in a volume of 50 μl. Maximum binding of L929 cells as measured by the absorvance to milk OPN and r-hOPN was 0.501 and 0.400, respectively. C: Plates were precoated with 10 μg/ml milk OPN or 1 μg/ml r-hOPN in a volume of 50 μl. Maximum binding of B16-F10 cells as measured by the absorbance to milk OPN and r-hOPN was 0.739 and 0.607, respectively. D: Plates were precoated with 1 μg/ml VN in a volume of 50 μl. Maximum binding of B16-BL6 cells as measured by the absorbance to VN was 0.748.

L929, and B16-F10 cells bound to both glycosylated and unglycosylated forms of OPN.

Second, we examined whether the binding of cells to milk OPN and recombinant human OPN was RGD-dependent. As shown in Figure 3A, the binding of B16-BL6 cells to milk and recombinant OPN was not inhibited by excess amount of synthetic GRGDS peptides, thus indicating that the B16-BL6 bound to OPN in RGD-independent manner. L929 (Fig. 3B) and B16-F10 (Fig. 3C) cells also bound to milk as well as recombinant OPN. However, the binding of L929 and B16-F10 cells to milk and recombinant OPN was abrogated by synthetic GRGDS peptides, but not by GRGES (Fig. 3B and C), indicating that L929 and B16-F10 cells bound to OPN through interaction of the RGD tripeptide sequence within the OPN molecule. In addition, we tested the binding of B16-BL6 cells to another RGD-containing molecule, VN. The binding to VN was specifically inhibited by the addition of synthetic GRGDS peptides, but not by synthetic GRGES peptides (Fig. 3D), demonstrating that the binding of B16-BL6 cells to VN

(in contrast to their binding to OPN; Fig. 3A) is RGD-dependent.

Third, we examined whether the binding of B16-BL6 cells to OPN was αv integrin-dependent. As shown in Figure 4A, the binding of B16-BL6 cells to milk OPN (1), r-hOPN (2), r-mOPN (3), r-mOPNdeltaN (4), and r-mOPNdeltaC (5) was not inhibited by excess amount of anti-αv mAb. The same concentration of anti-αv mAb strongly inhibited the binding of B16-BL6 cells to GRGDS-BSA (Fig. 2B [a]; lane 4). As a control, the binding of L929 cells to r-mOPN and milk OPN was tested in the presence of mAb. As shown in Figure 4B, the binding of L929 cells to either r-mOPN or milk OPN was clearly inhibited by anti-αv mAb, RMV7, but not by a control mAb, 7G12.

The Binding of B16-BL6 Cells to OPN Fragments

In order to determine whether the carboxy-terminal half or amino-terminal half of OPN provide an additional cell binding site for B16-BL6 cells, we prepared various recombinant mouse OPN proteins that included the amino-terminal or carboxy-terminal halves of the protein but lacked the RGD sequence. B16-BL6 cells bound equally well to full-length recombinant human and mouse OPN (Fig. 5A). When the binding of B16-BL6 cells to the amino-terminal half (r-mOPNdeltaN) of OPN was examined, the binding was comparable to that to the carboxy-terminal half of OPN (r-mOPNdeltaC). Both r-mOPNdeltaN and r-mOPNdeltaC lack an RGD sequence. It should be noted that B16-BL6 cells did not bind significantly to a negatively charged protein, fetuin. We also tested whether L929 cells can bind to the either the carboxy-terminal half or amino-terminal half of OPN. As shown in Figure 5B, L929 cells failed to bind to either r-mOPNdeltaC or r-mOPNdeltaN. In addition, the binding of B16-BL6 to wild type r-mOPN, r-mOPNdeltaN, or r-mOPNdeltaC was not inhibited by excess amount of synthetic peptide GRGDS, whereas the binding of B16-BL6 cells to VN was clearly inhibited by excess amount of GRGDS (Fig. 5C). Finally, we analyzed whether the incubation of B16-BL6 cells on various OPN and control proteins resulted in the cell spreading. As shown in Figure 6, the binding of B16-BL6 cells to milk OPN, r-mOPN, r-mOPNdeltaC, or r-mOPNdeltaN resulted in cell spreading.

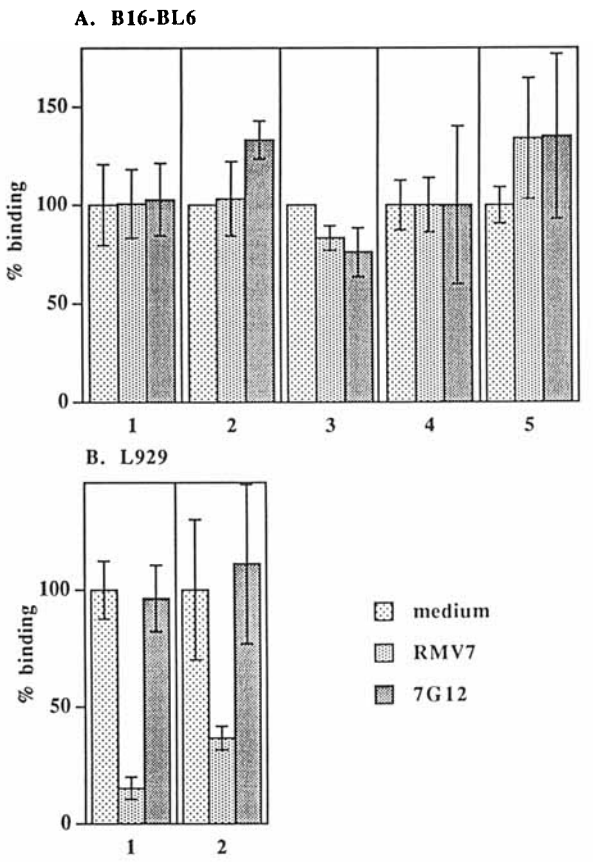

Fig. 4. The binding of B16-BL6 cells to OPN in an αv-independent manner. **A:** The binding of B16-BL6 cells to milk OPN (*1*), r-hOPN (*2*), r-mOPN (*3*), r-mOPNdeltaN (*4*), or r-mOPNdeltaC (*5*) was studied in the presence of medium, 100 μg/ml anti-αv mAb, RMV7, or 100 μg/ml isotype matched control mAb, 7G12. Plates were pre-coated with 4 μg/ml milk OPN, 1 μg/ml r-hOPN, 1 μg/ml r-mOPN, 20 μg/ml r-mOPNdeltaC or 20 μg/ml r-mOPNdeltaN overnight at 4°C. Maximum binding of B16-BL6 cells as measured by the absorbance to milk-OPN, r-hOPN, r-mOPN, r-mOPNdeltaN, or r-mOPNdeltaC was 0.980, 1.056, 0.487, 0.254, or 0.206 (A595), respectively. **B:** The binding of L929 cells to r-mOPN (*1*) or milk OPN (*2*) was studied in the presence of medium, 100 μg/ml anti-αv mAb, RMV7, or 100 μg/ml isotype matched control mAb, 7G12. Plates were pre-coated with 2 r-mOPN μg/ml or 5 μg/ml overnight at 4°C. Maximum binding of L929 cells to r-mOPN and milk OPN was 0.445 an 0.363 (A595), respectively.

DISCUSSION

It has previously been shown that the binding of various cells to OPN can be mediated through interaction of the RGD tripeptide sequence within the OPN molecule and αvβ3 integrin on cell surface [Oldberg et al., 1980; Bulter, 1989; Miyauchi et al., 1991; Liaw et al., 1994]. Therefore, the binding of various cell types to OPN is both RGD- and αv-dependent. Recently, it was shown that the carboxy-terminal half of the rat

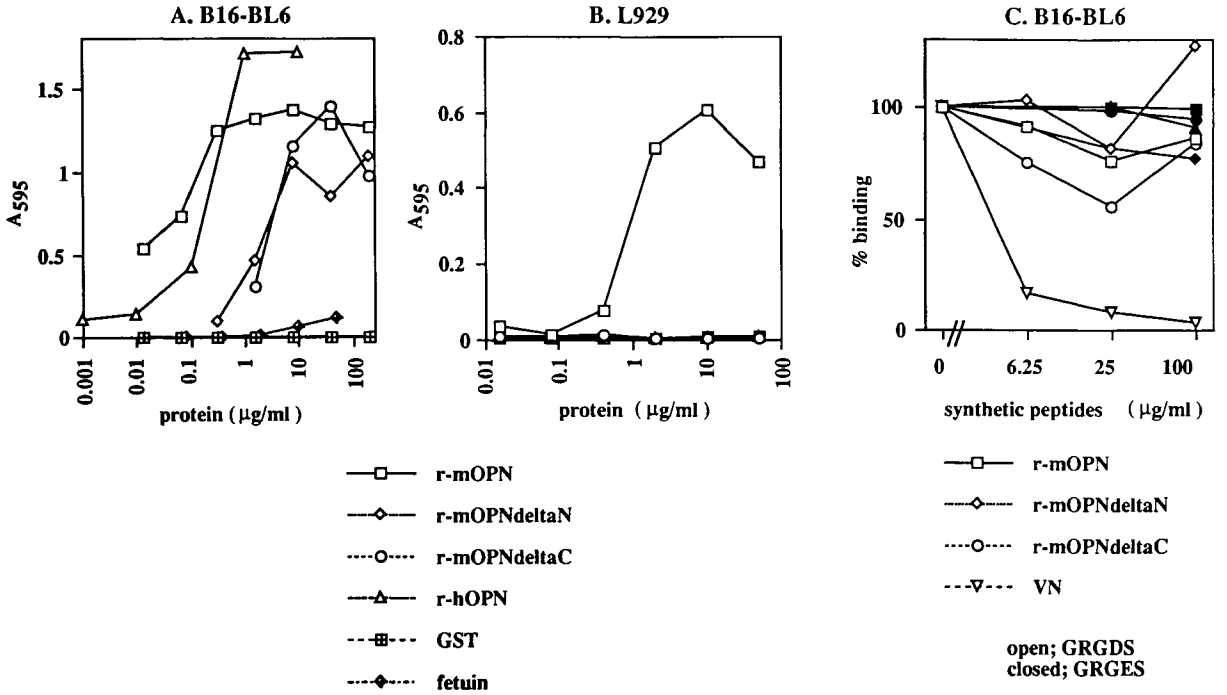

Fig. 5. The binding of B16-BL6 and L929 cells to N-terminal and C-terminal OPN fragments. **A:** The binding of B16-BL6 cells to various concentrations of recombinant OPN such as r-mOPN, r-mOPNdeltaN, r-mOPNdeltaC, r-hOPN, or fetuin was tested. **B:** The binding of L929 cells to various concentrations of r-mOPN, r-mOPNdeltaN, or r-mOPNdeltaC was tested. **C:** Plates were precoated with 2 μg/ml OPN, 1 μg/ml VN, or r-mOPNdeltaC and r-mOPNdeltaN (20 μg/ml protein solution) overnight at 4°C. The binding of B16-BL cells to OPN or VN was tested in the presence of various concentrations of GRGDS (open symbols) or GRGES (closed symbols) synthetic peptides.

OPN molecule may contain an additional cell binding site [Van Dijk et al., 1993]. In that study, OPN was digested with endoproteinase Arg-C, a cysteine proteinase specific for cleavage at the carboxy-terminal side of arginine residues. A resulting 28 kilodalton carboxy-terminal fragment of rat OPN that does not contain the RGD sequence was found to interact with the αvβ3 integrin on the cell surface, thus suggesting the presence of a second cell binding domain in OPN [Van Dijk et al., 1993].

We demonstrated in this study that human and mouse OPN possess additional cell binding sites that do not contain the RGD sequence. L929 cells bound to OPN through interactions of the RGD tripeptide sequence within OPN and αvβ3 integrins on the cell surface. However, the binding of B16-BL6 cells to OPN was found to be both RGD- and αv-independent. This RGD-independent binding of B16-BL6 cells to OPN is unlikely to be due to nonspecific binding since the binding of the same cells to the RGD-containing VN was clearly RGD-dependent. We also demonstrated that the additional cell binding sites are located in both the carboxy-terminal and amino-terminal half of the OPN molecule that do not contain an RGD sequence and interact with a cell surface molecule(s) that is(are) distinct from an αv-containing integrin. These results thus suggest an additional mechanism by which OPN may interact with some cells, and that the cell binding sites involved are distinct from the second cell binding site described in rat OPN [Van Dijk et al., 1993].

The contribution of the additional cell binding sites to the adhesive function of OPN may be significant in some cases, since the adhesion of B16-BL6 cells to milk and r-hOPN was not affected by the addition of excess amount of GRGDS peptide or an antibody against αv, which presumably abrogate RGD-dependent and αv-dependent binding. The incubation of B16-BL6 cells on plates coated with GRGDS-BSA or purified OPN in the presence of GRGDS peptide resulted in adhesion and subsequent cell spreading, indicating that the new cell binding sites, through interaction with cell surface receptors, provide appropriate signalling for cell spreading which requires reorganization of the cytoskeleton. It should be noted that cell surface mol-

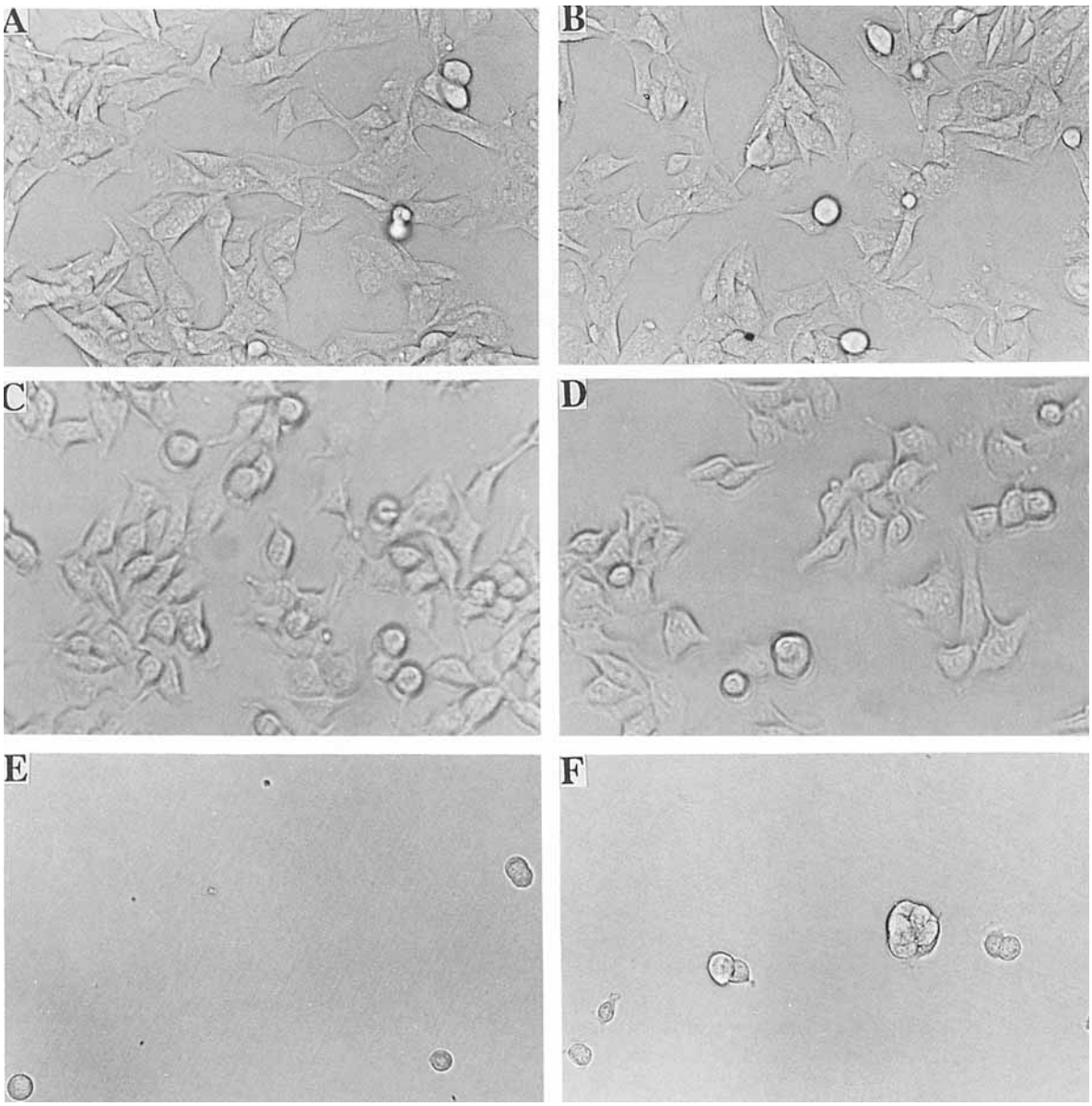

Fig. 6. Cell spreading assays. Plates were precoated with 2 μg/ml milk OPN (**A**), 2 μg/ml r-mOPN (**B**), 20 μg/ml r-mOPNdeltaC (**C**), 20 μg/ml r-mOPNdeltaN (**D**), 50 μg/ml GST (**E**), or 50 μg/ml fetuin (**F**), in a volume of 50 μl and 5×10^4 B16-BL6 cells were added to each well. After incubation for 1 h at 37°C, all wells were washed twice with PBS and photographs were taken. The photomicrographs are representatives of several independent experiments (magnification, ×240).

ecules which bind to the new cell binding sites may be present in only certain cell types, as we found evidence for this interaction in B16-BL6 but not L929 and B16-F10 cells. Similarly, a Leu-Arg-Glu (LRE) sequence within the laminin molecule shows adhesive activity for only ciliary ganglion cells [Yamada, 1989].

It was previously shown that the heparin binding domain of FN also contains a sequence that shows adhesive activity and OPN-contains two heparin binding domains in the C-terminal half [Denhardt and Guo, 1993]. VN also contains a functional heparin binding domain which can bind to cells through interaction with heparan sulfate proteoglycans [de Boer et al., 1992]. However, the binding of B16-BL6 cells to VN was strongly inhibited by GRGDS peptide, indicating that the participation of the heparin-binding domain in adhesion of B16-BL6 cells to VN is minimal if any. Therefore, it is unlikely that the

binding of B16-BL6 cells to OPN is mainly mediated by heparin binding domains of OPN. In a related murine melanoma cell line, B16F1, occupancy of both an integrin and a cell surface lectin is required for spreading on glycosylated laminin [Chandrasekaran et al., 1994a; Chandrasekaran et al., 1994b]. In this regard, it has been shown that OPN is highly glycosylated and phosphorylated [Senger et al., 1989]. We demonstrated that B16-BL6 cells can bind to nonglycosylated, nonphosphorylated forms of OPN (recombinant OPN prepared in *E. coli*) in an RGD- and αv-independent manner. Our results thus indicate that some cells can adhere to the carboxy-terminal as well as amino-terminal halves of OPN via an RGD- and αv-independent mechanism.

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