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Site-Directed Mutagenesis of the Arginine-Glycine-Aspartic Acid Sequence in Osteopontin Destroys Cell Adhesion and Migration Functions

Jian-Wu Xuan, Charulata Hota, Yoichiro Shigeyama, John A. D'Errico, Martha J. Somerman, and Ann F. Chambers

London Regional Cancer Centre (J.-W.X., C.H., A.F.C.), Department of Oncology, University of Western Ontario (A.F.C.), London, Ontario, Canada N6A 4L6; Department of Periodontics/Preventative/Geriatrics, Dental School (Y.S., J.A.D., M.J.S.) and Department of Pharmacology (M.J.S.), University of Michigan, Ann Arbor, Michigan 48109-1078

Abstract Osteopontin (OPN) is a secreted calcium-binding phosphoprotein produced in a variety of normal and pathological contexts, including tissue mineralization and cancer. OPN contains a conserved RGD (arg-gly-asp) amino acid sequence that has been implicated in binding of OPN to cell surface integrins. To determine whether the RGD sequence in OPN is required for adhesive and chemotactic functions, we have introduced two site-directed mutations in the RGD site of the mouse OPN cDNA, in which the RGD sequence was either deleted or mutated to RGE (arg-gly-glu). In order to test the effect of these mutations on OPN function, we expressed control and mutated mouse OPN in *E. coli* as recombinant glutathione-S-transferase (GST)-OPN fusion proteins. Control mouse GST-OPN was functional in cell adhesion assays, supporting attachment and spreading of mouse (malignant PAP2 *ras*-transformed NIH 3T3, and, to a lesser extent, normal NIH 3T3 fibroblasts) and human (MDA-MB-435 breast cancer, and normal gingival fibroblast) cells. In contrast, neither of the RGD-mutated OPN proteins ("delRGD" or "RGE") supported adhesion of any of the cell lines, even when used at high concentrations or for long assay times. GRGDS (gly-arg-gly-asp-ser) peptides inhibited cell adhesion to intact GST-OPN, as well as to fibronectin and vitronectin. In chemotaxis assays, GST-OPN promoted directed cell migration of both malignant (PAP2, MDA-MB-435) and normal (gingival fibroblast, and NIH 3T3) cells, while RGD-mutated OPN proteins did not. Together these results suggest that the conserved RGD sequence in OPN is required for the majority of the protein's cell attachment and migration-stimulating functions.

Key words: osteopontin, OPN, site-directed mutagenesis, RGD integrin binding sequence, cell adhesion, cell migration, chemotaxis, recombinant proteins

Osteopontin (OPN) is a secreted phosphoprotein produced in a variety of normal and pathological contexts [reviews, 1,2]. It was originally described as a transformation-associated protein [review, 3], and then as a developmentally regulated component of mineralized tissues [review, 1]. Increased levels of OPN are produced by NIH 3T3 cells transformed with the H-*ras* oncogene [4–6] and high levels are found in other tumor cell lines [review, 3]. In addition, OPN is produced by a number of normal tissues and cells, including smooth muscle cells, kidney cells, and activated T cells and macrophages [reviews, 1,2,7]. OPN also has been identified as early T-lymphocyte activation-1 (Eta-1), and the murine OPN gene maps to the same genetic locus as the *Rickettsia* resistance gene, implicating OPN in immune functions [review, 7]. OPN is secreted into various body fluids, including milk, serum, urine, and cochlear fluid. While many functions have been proposed, OPN's function in any of these contexts has remained elusive.

OPN proteins from all species studied have a GRGDS amino acid sequence, typical of proteins able to bind to cell surface integrins. An adjacent thrombin cleavage site (6–8 amino acids COOH-terminal from the RGD (arg-gly-asp) sequence, depending on the species) also is highly con-

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Address reprint requests to Dr. Ann F. Chambers, London Regional Cancer Centre, 790 Commissioners Road East, London, Ontario, Canada N6A 4L6.

served. Conservation of these sequences suggests that they may be important to OPN function. OPN has been shown to have cell adhesion properties and is capable of supporting attachment of a variety of cell types. The RGD sequence has been implicated in this process by studies in which RGD-containing peptides, or antibodies against subunits of the αvβ3 integrin (vitronectin receptor—to which OPN can bind), have been shown to block cell attachment to OPN [8–17]. In addition, alternative receptors for OPN, as well as other binding domains in OPN, may contribute to its cell binding ability [14,16,18].

Here we tested the hypothesis that the RGD sequence in OPN is required for cell adhesive and chemotactic function. We previously reported that recombinant human OPN, expressed as a glutathione-S-transferase (GST)-fusion protein, is functional and supports cell attachment of PAP2 cells (malignant, *ras*-transformed murine NIH 3T3) to a greater extent than control NIH 3T3 cells [19], as was found for adhesion of these lines to native bovine bone OPN [12]. We also found that a human breast cancer cell line (MDA-MB-435) is able to adhere to the recombinant GST-OPN protein, as well as to native bone OPN [19]. For the present study, we designed and constructed two mutant OPN proteins, in which the RGD sequence was deleted ("delRGD") or mutated to a sequence (RGE, arg-gly-glu) that binds poorly to cell surface integrins. This approach has been used successfully to study the role of RGD sequences in fibronectin, von Willebrand factor, and vitronectin [20–23]. Using site-directed mutagenesis, we generated mouse OPN cDNA constructs containing mutations in the RGD sequence, and then expressed these proteins in *Escherichia coli* as fusion proteins with GST, using procedures we described previously for normal human OPN [19].

We tested the ability of malignant (murine PAP2, *ras*-transformed NIH 3T3; MDA-MB-435 human breast cancer) and nontransformed (immortalized murine NIH 3T3; early passage human gingival fibroblast) cells to bind to surfaces coated with mutated vs. control GST-OPN fusion proteins. The abilities of these cells to bind to native OPN has been documented previously [8,12,19,24]. We found that neither of the mutant OPN proteins ("delRGD," "RGE") had adhesive function for any of the cell lines, even when tested at high concentrations or for extended assay periods. In contrast, all of the cell lines were able to bind to control OPN that contains an intact RGD sequence. Adhesion to control OPN was blocked by added GRGDS peptides, confirming our previous results with recombinant human OPN and native bone OPN [12,18,19]. These results establish that the conserved RGD sequence in OPN is required for cell attachment function, for both normal and transformed cells.

We also assessed the ability of control and RGD-mutated OPN proteins to stimulate directed migration of malignant (PAP2, MDA-MB-435), as well as nontransformed (NIH 3T3, gingival fibroblast) cells. OPN has been shown to induce migration of macrophages [9] and smooth muscle cells [16]. The ability of OPN to induce chemotaxis in cancer cells or normal fibroblasts has not been reported previously. We found that control recombinant OPN stimulated migration of these cells, in a dose-dependent fashion. In contrast, neither of the RGD-mutated OPN proteins stimulated migration of any of the cells.

MATERIALS AND METHODS

Cells and Cell Culture

PAP2 cells (malignant, T24-H-*ras*-transformed NIH 3T3 cells) [25,26] and NIH 3T3 mouse fibroblasts were maintained in DMEM (Gibco, Burlington, Ontario, Canada) supplemented with 10% calf serum (Gibco). MDA-MB-435 human mammary carcinoma cells [27], a kind gift of Dr. Janet Price, M.D., Anderson Cancer Center, Houston, TX, were grown in α+MEM with 10% fetal calf serum (Gibco). Human gingival fibroblasts were cultured as explants from tissues obtained from patients undergoing extraction of premolars for orthodontic reasons, as described in detail elsewhere [28]. Gingival fibroblasts were grown in DMEM, 10% fetal calf serum, and antibiotics, as described [28].

Construction of the Murine GST-OPN Fusion Plasmid

The strategy used is outlined in Figure 1A. The mouse osteopontin cDNA clone pGEM4-2ar was obtained from Dr. D.T. Denhardt [29]. The 1.4 kb *Bam*HI insert from pGEM4-2ar, containing a single *Hin*dIII site, was cloned into the *Bam*HI site of pBluescript (Stratagene, La Jolla, CA). A single *Hpa*II site is located one amino acid codon downstream of the excision site of the

mature mouse OPN protein. Two partially complementary oligonucleotide primers were synthesized, a 12mer (GATCCTCCCTCC) and a 10mer (GAGGGAGGGC). As shown in Figure 1A, after annealing of these two primers, cohesive ends for *Bam*HI and *Hpa*II restriction enzymes were created. The annealed double stranded linker was ligated with *Bam*HI to the linearized *E. coli* GST (glutathione S-transferase, EC 2.5.1.18) expression vector pGEX-2T (Pharmacia, Montreal, Quebec, Canada) and a 1.1kb *Hpa*II fragment of pBluescript-mouse OPN cDNA containing the murine 2ar/OPN coding region. The recombinant clone maintains the correct reading frame for both GST and the mature mouse OPN protein.

Site-Directed Mutagenesis of Mouse OPN cDNA

The RGD sequence of osteopontin was mutated in two ways: mutation of the RGD to RGE (arg-gly-glu) by a single base change, GAT (asp) to GAG (glu), designated as "RGE;" and deletion of the RGD sequence, designated as "delRGD." Oligonucleotide-directed mutagenesis was performed using the Altered Sites in vitro Mutagenesis System (Promega, Mississauga, Ontario, Canada), using procedures specified by the manufacturer. A ~500 bp *Hin*dIII-*Sph*I fragment that includes the RGD site of pGEM4-2ar (mouse OPN) cDNA (Fig. 1B) was cloned into the pALTER-1 vector and single stranded DNA was prepared from this clone for use as a template. Two oligonucleotide primers, a 20mer (GCCAAGCTCT CACCTCGGCC) and a 50mer (TTGACCTCAG TCCATAAGCC AAGCTGCCGT TGGGGACATC GACTGTAGGG), complementary to the desired sequences (Fig. 1B), were synthesized to introduce the RGE and delRGD mutations, respectively. Site-directed mutagenesis was performed according to the protocol provided by Promega. In addition to the ampicillin resistance screening, the delRGD mutant also was verified by *Hae*III digestion, due to deletion of the *Hae*III site at the RGD sequence. The mutations were confirmed by complete sequencing of the full ~500 bp fragment for each mutant. The *Sph*I-*Hin*dIII fragments carrying the mutated sequences were isolated and used to replace the *Sph*I-*Hin*dIII fragment of the GST-mOPN (mouse OPN) clone, above.

Expression and Purification of GST-OPN Proteins

Recombinant GST-OPN fusion proteins (control GST-mOPN, RGE and delRGD mutants, ~58.8 kDa by sequence) were prepared in *E. coli* as described previously for recombinant GST-human OPN [19]. The GST fusion proteins were purified on glutathione-Sepharose columns as described [19]. The recombinant pGEX-2T/OPN clones generate GST-fusion proteins that include the full sequence of mature OPN plus three additional amino acids added during cloning (gly-ser-ser). The OPN moiety can be cleaved from GST by thrombin (at the *Bam*HI site, Fig. 1A). (Thrombin also cleaves internally in OPN [19,30]). The recombinant proteins, generally as intact GST-fusion proteins or cleaved with thrombin as described [19], were analyzed by 15% polyacrylamide gel electrophoresis.

Cell Adhesion Assays

Adhesion of cells to 96- or 24-well dishes was assessed as described previously [8,12,19,24]. Wells were coated either for 1 h, or dried overnight, as described [8,12,19,24], using various substrates as indicated. Murine GST-OPN (control and mutated) were prepared as described above; 50 μg/ml is ~850 nM. Fibronectin was purchased from Collaborative Biomedical Products (Bedford, MA), and vitronectin from Gibco. Synthetic GRGDS and GRGESP peptides (Telios Pharmaceuticals, San Diego, CA) were added at 100 μM as described [12,19]. Cells were added to coated wells, in DMEM plus BSA (1 mg/ml) at 2×10^4 cells/96-well (NIH 3T3, PAP2), 4×10^4 cells/96-well (MDA-MB-435), or 2×10^4 cells/24-well (gingival fibroblasts). Adhesion was assessed either by staining with hematoxylin and counting by eye (for PAP2, NIH 3T3, and MDA-MB-435 cells) [12] or by trypsinization and counting by Coulter counter (for gingival fibroblasts) [8,24], after the times specified.

Cell Migration Assays

Cell migration assays were performed as modified from Melchiori et al. [31], using 24-well Transwell chambers with polycarbonate filters of 8 μm pore size (Costar, Cambridge, MA). Gelatin (Sigma) was applied at 5 μg/filter and air dried. The gelatin was rehydrated with 100 μl of serum-free DMEM at room temperature for 90 min. Lower wells contained 800 μl of DMEM plus 0.1% BSA, with or without test proteins; in some cases, as specified, proteins were added to the upper wells, in order to assess directed migration. Cells were added to the upper wells and incubated at 37°C; for each cell line, the incubation time was based on preliminary experiments in which optimal time was

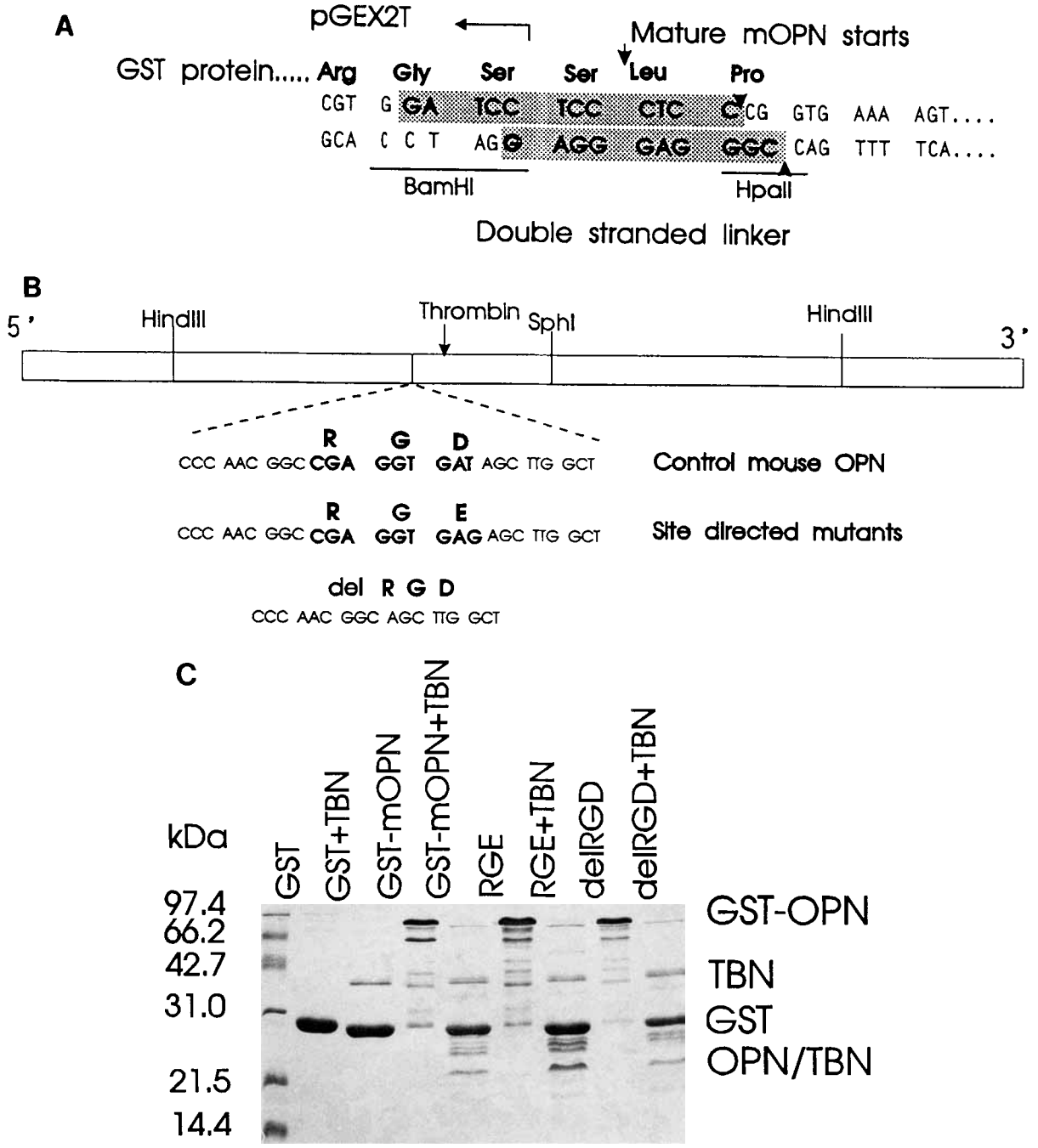

Fig. 1. Generation of recombinant GST-mouse OPN fusion protein with deleted or mutated RGD sequence. **A:** Strategy for cloning of the mature mouse OPN cDNA into the *E. coli* expression vector pGEX-2T. The two primers used, as described in Methods, are shaded. **B:** Strategy for site-directed mutagenesis of mouse OPN cDNA in pGEX-2T, to delete the RGD sequence or mutate it to RGE. **C:** 15% SDS-PAGE analysis of GST-mOPN fusion proteins. GST, control GST protein; GST-mOPN, nonmutated GST-mouse OPN fusion protein; RGE and delRGD, mutated OPN proteins, as GST-fusion proteins; +TBN indicates proteins that have been cleaved with thrombin. Migration of the GST-OPN proteins, thrombin (TBN), GST alone, and the two fragments of thrombin-cleaved OPN (OPN/TBN) are indicated at the right of the figure.

determined. PAP2 and NIH 3T3 cells were harvested and resuspended in DMEM plus 0.1% BSA at a concentration of 5×10^5 cells/ml and 100 μl of cell suspension was placed in each upper well. The cells were allowed to incubate 7 h. For MDA-MB-435 cells, the cells were resuspended in α + MEM plus 0.1% BSA, added at 5×10^4 cells/well, and the wells were incubated for 24 h. Gingival fibroblasts were resuspended in DMEM plus 0.1% BSA, added at 2–3 × 10^4

cells/well, and were incubated for 6 h. At the end of the specified incubation time the cells that had migrated to the lower surface of the filters were fixed in place with glutaraldehyde and stained with hamatoxylin. Cells that had not migrated and were attached to the upper surface of the filters were removed from the filters with wet Q-Tips. The lower surfaces of the filters were examined microscopically and six randomly chosen areas were counted per filter, to determine the number of cells that had migrated through the filters. Adhesion and migration assays were performed in duplicate or triplicate. Statistical differences between groups were assessed using the Mann-Whitney test, *t*-test, or ANOVA, using SigmaStat (Jandel Scientific, San Rafael, CA) statistical software.

RESULTS

Expression of Recombinant Mouse OPN and RGD-Mutant Proteins in *E. coli* Cells

As shown in Figure 1C, mouse osteopontin was expressed in *E. coli* as a fusion protein with the carrier protein GST. Control GST-mOPN and the two mutated proteins ("RGE" and "delRGD") all appeared as an ~85 kDa protein when analyzed by 15% SDS-PAGE, similar to results reported previously for GST-human OPN [19]. Several less abundant bands, slightly smaller than 85 kDa, were also described previously, and may represent truncated fusion proteins [19]. Thrombin cleavage of control GST-mOPN and the two mutated proteins (lanes labelled + TBN) resulted in two major OPN bands (~23 and ~25–26 kDa), as well as minor, similarly-sized additional bands, which likely represent thrombin cleavage products of the truncated GST-OPN proteins. Thrombin also released intact GST (~27 kDa); thrombin itself ran as a band of ~37 kDa. These results are consistent with the thrombin cleavage patterns described previously for GST-human OPN [19].

Cell Adhesion to Control and Mutated ("RGE" and "delRGD") GST-OPN Proteins

The ability of control and mutated OPN proteins to support cell adhesion of normal and malignant cells was assessed. As shown in Figure 2, PAP2 cells adhered well, and NIH 3T3 cells less well, to control GST-mOPN, while both lines adhered to the same extent to fibronectin, as we found earlier with these cells [12,19]. In contrast to these results, neither PAP2 nor NIH 3T3 cells were able to adhere to the RGD-mutated OPN proteins, "RGE" or "delRGD" (Fig. 2). These cells did not adhere to either mutated protein, even when the proteins were used at high concentrations (Fig. 3A) or the adhesion assays were conducted for extended periods of time (Fig. 3B).

Similar results were obtained for malignant human MDA-MB-435 cells (Fig. 4). These cells adhered well to control GST-mOPN, while they did not adhere to either the "RGE" and "delRGD" mutated proteins. We previously reported that MDA-MB-435 cells adhere to GST-human OPN and to bovine bone OPN [19].

Normal human gingival fibroblasts adhered well to nonmutated recombinant GST-mOPN protein (Fig. 5), consistent with previous results for these cells adhering to native bovine and rat OPN [8,24]. As observed for the other cell lines tested here, human gingival fibroblasts did not adhere to either of the mutated OPN proteins, "RGE" or "delRGD" (Fig. 5).

Adhesion to GST-mOPN protein was inhibited by addition of GRGDS peptides, but not by control GRGESP peptides (Fig. 6). No adhesion was observed to the mutated proteins, and no effect of added peptides was seen for the mutated proteins. Adhesion to fibronectin (FN) and vitronectin (VN) were similarly inhibited by GRGDS but not GRGESP peptides (Fig. 6).

Cell Migration in Response to Control and Mutated ("RGE" and "delRGD") GST-OPN Proteins

Control GST-mOPN stimulated chemotactic migration of all four cell lines tested, as did

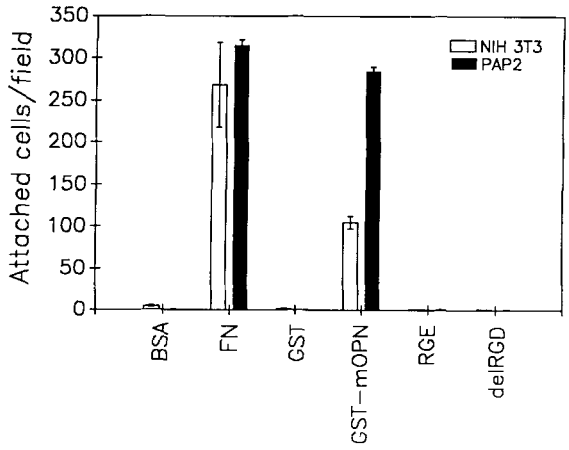

Fig. 2. Adhesion of PAP2 and NIH 3T3 cells to surfaces coated with nonmutated GST-mOPN and mutated GST-mOPN proteins (RGE, delRGD). OPN proteins and control GST protein were coated at 50 μg/ml, and fibronectin (FN) at 10 μg/ml. Adhesion was determined as number of attached cells/microscope field, after 90 min, as detailed in Methods. Bars are mean values ± S.D.

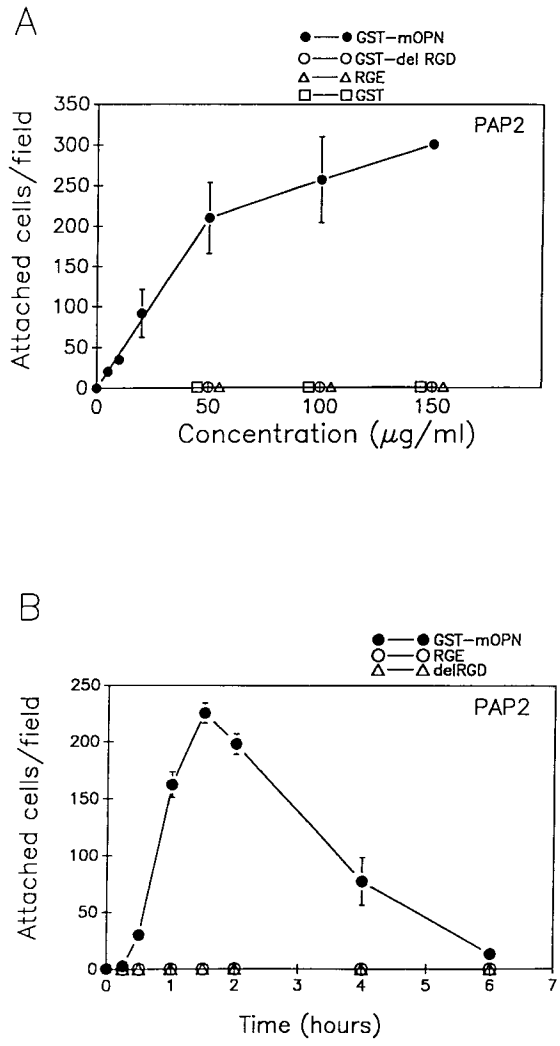

Fig. 3. Adhesion of PAP2 cells to GST-fusion proteins (GST-mOPN, mutant RGE, and del RGD) and control GST proteins. **A:** Concentration dependence of coating proteins, adhesion for 90 min. **B:** Time course, with proteins coated on wells at 50 μg/ml. Kinetics of adhesion to GST-mOPN are comparable to that reported previously for recombinant human and native bovine OPN [12,19]. No adhesion to the mutated OPN proteins was seen at either high coating concentrations or extended assay times. Points are mean values from 3 wells ± S.D.

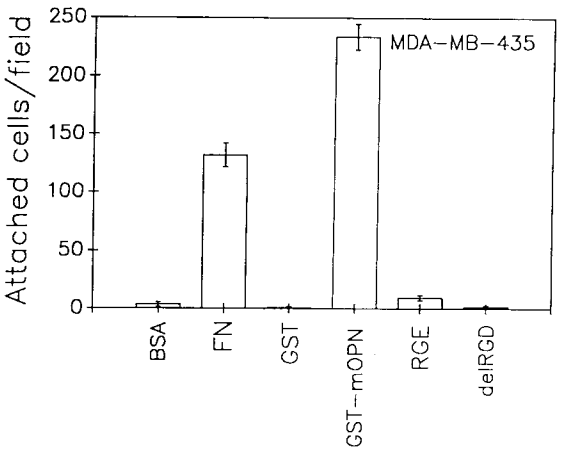

Fig. 4. Adhesion of MDA-MB-435 human mammary carcinoma cells to surfaces coated with proteins, as in Figure 2. Adhesion was determined after 18 h. Bars are mean values from 3 wells ± S.D. Adhesion to GST-mOPN differed significantly from adhesion to control (BSA, GST) and mutated OPN (RGE, delRGD) proteins ($P < 0.05$).

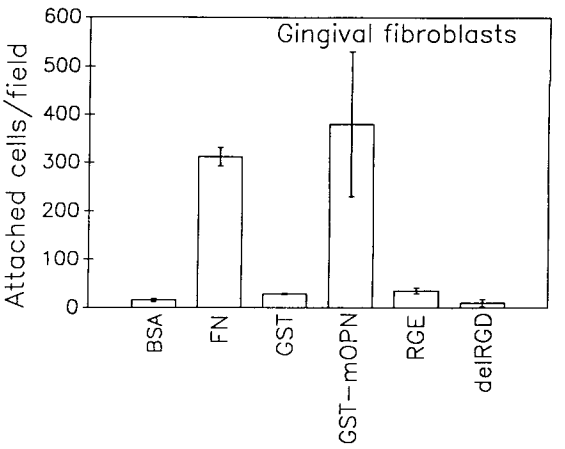

Fig. 5. Adhesion of human gingival fibroblasts to surfaces coated with proteins, as in Figure 2. Adhesion was determined after 2 h. Adhesion to GST-mOPN differed significantly from adhesion to control (BSA, GST) and mutated OPN (RGE, delRGD) proteins ($P < 0.05$).

fibronectin (Fig. 7). PAP2 cells were stimulated to migrate to GST-mOPN to a greater extent than were NIH 3T3 cells (Fig. 7A). In contrast to results with control GST-mOPN, the mutated OPN proteins "RGE" and "delRGD" failed to stimulate migration of any of the cells tested, suggesting that an intact RGD sequence is required for chemoattractant effects of OPN.

Stimulation of migration to control GST-mOPN was dependent on the concentration of OPN present in the lower wells of the chemoinvasion chambers (Fig. 8). We determined that the migration seen was indeed a directed chemoattraction, and not simply stimulation of undirected migration, by experiments in which GST-mOPN was added to the top well only, the bottom well only, both wells, or neither well (Fig. 9). The greatest degree of migration was observed when OPN was added to only the lower well.

DISCUSSION

The RGD amino acid sequence of OPN is absolutely conserved (as GRGDS) in all species examined to date [32; see Fig. 6], and this sequence is thought to be important for the protein's ability to promote cell adhesion. Exog-

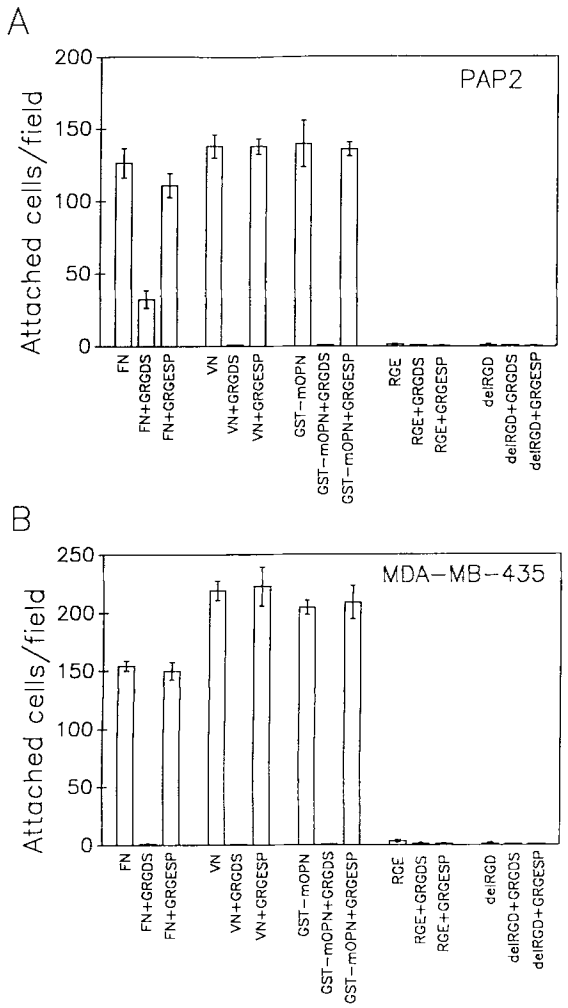

Fig. 6. Effect on adhesion of PAP2 (**A**) and MDA-MB-435 (**B**) cells to specified proteins of added peptides (GRGDS, control GRGESP). Peptides were used at 100 μM; coating proteins were applied at 50 μg/ml (GST-mOPN, RGE, delRGD), 10 μg/ml (VN [vitronectin] and FN). Adhesion to FN, VN, and GST-mOPN was significantly reduced by GRGDS but not GRGESP peptides ($P < 0.05$).

enously added RGD-containing peptides have been shown to inhibit adhesion to OPN, as have antibodies to the αvβ3 integrin [8–17,19]. In addition, we have found that thrombin cleavage of OPN reduces the ability of OPN to support cell adhesion [19]. The conserved thrombin cleavage site in OPN is 6–8 amino acids C-terminal from the RGD sequence, and we have postulated that altered adhesive function of thrombin-cleaved OPN may be due to conformational alteration of the nearby RGD sequence [19]. Consistent with the idea that thrombin cleavage may be an important regulator of RGD-mediated OPN function, we recently have developed a monoclonal antibody to OPN that recognizes OPN before, but not after, cleavage with throm-

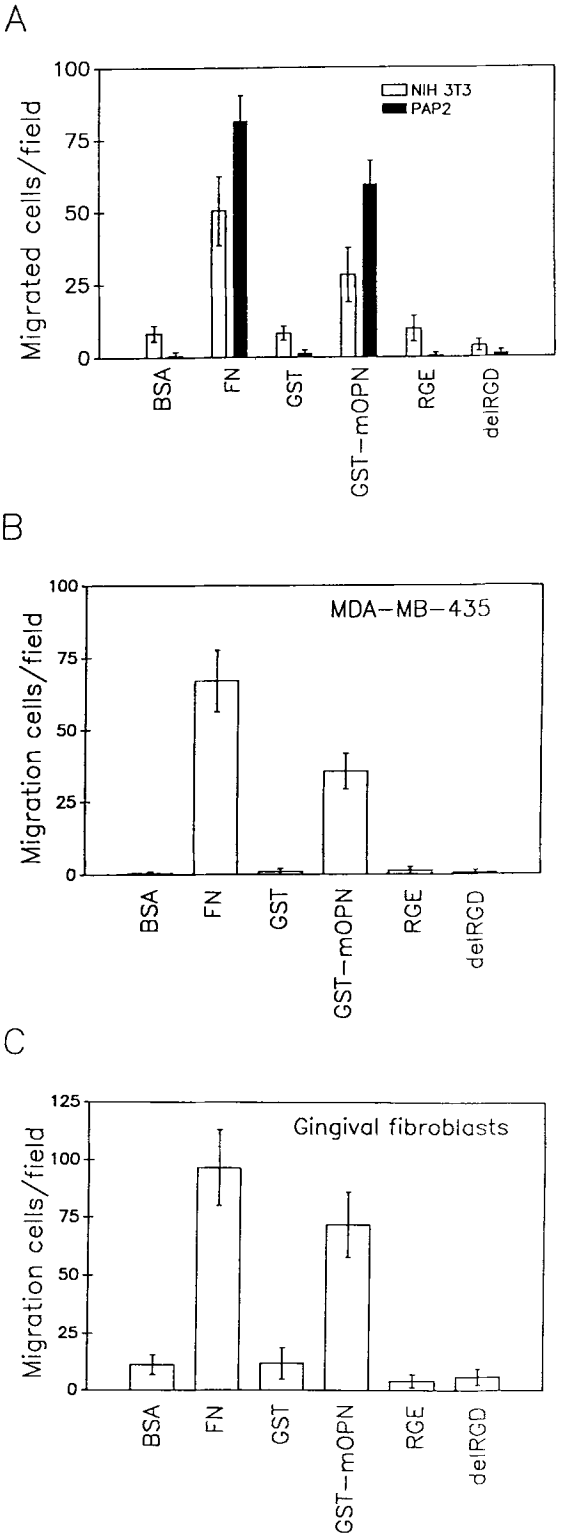

Fig. 7. Cell migration to proteins in lower chambers of Transwells. GST-mOPN, mutants RGE and delRGD, and GST were used at 50 μg/ml; FN at 10 μg/ml; and BSA at 0.1%. Incubation times were as specified in Methods. **A:** PAP2 and NIH 3T3 cells. **B:** MDA-MB-435 cells. **C:** Gingival fibroblasts. In all cases, adhesion to GST-mOPN differed significantly from adhesion to control (BSA, GST) and mutated OPN (RGE, delRGD) proteins ($P < 0.05$).

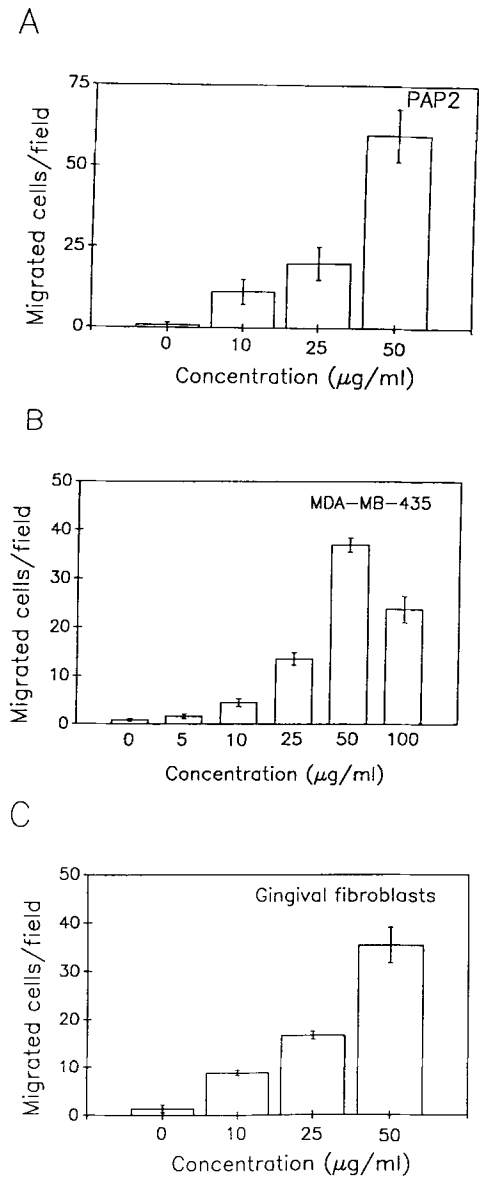

Fig. 8. Concentration dependence of cell migration to GST-mOPN protein in lower chambers of Transwells. Incubation times were as specified in Methods. **A:** PAP2 cells. **B:** MDA-MB-435 cells. **C:** Gingival fibroblasts. Multiple pairwise comparisons indicated that migration to GST-mOPN at concentrations ≥ 10 μg/ml (for PAP2), 25 μg/ml (for MDA-MB-435), and 25 μg/ml (for gingival fibroblasts), differed significantly ($P < 0.05$) from migration to other concentrations of protein.

bin, and which also inhibits RGD-mediated adhesion of cells to OPN [32]. Interestingly, Senger et al. have reported that thrombin cleaved OPN from human milk or rat cells in culture supports cell adhesion better than intact OPN [17]. Together these results suggest that regulation of RGD-mediated OPN activity may be complex. The RGD-containing region of OPN thus has been implicated by several lines of evidence as a key region in the regulation of OPN function.

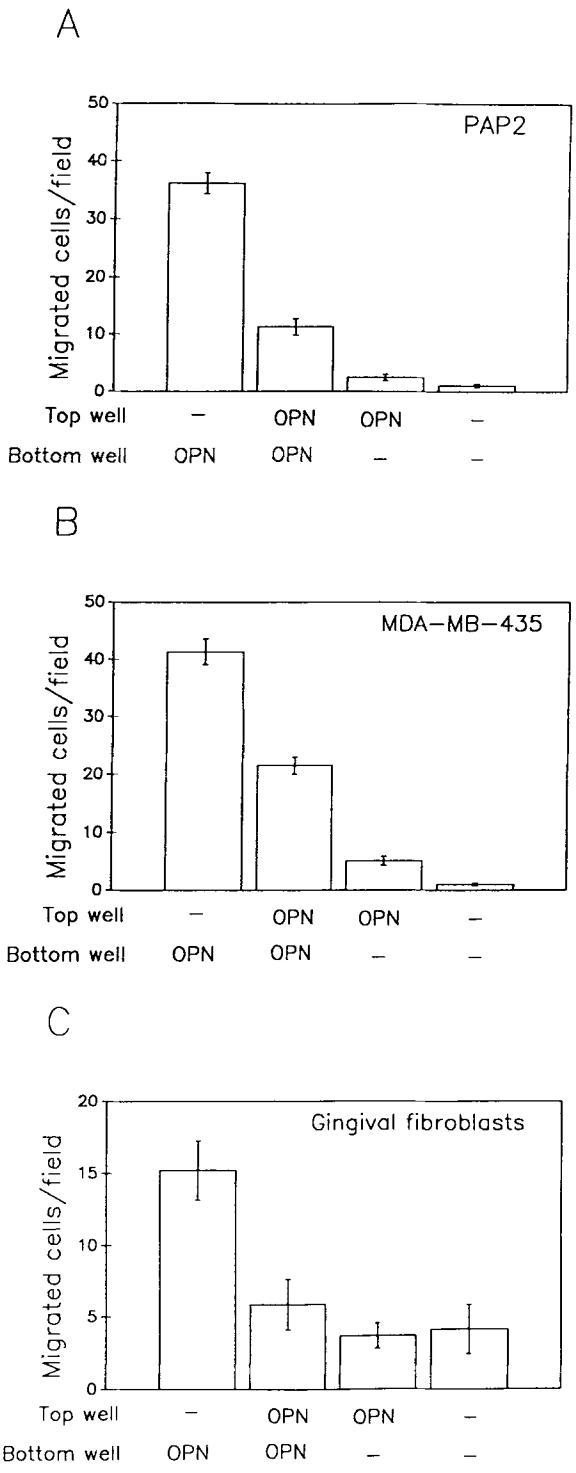

Fig. 9. "Checkerboard" analysis of cell migration to GST-mOPN protein (50 μg/ml) in lower chambers of Transwells, both chambers, upper chamber, or neither chamber. Incubation times were as specified in Methods. **A:** PAP2 cells. **B:** MDA-MB-435 cells. **C:** Gingival fibroblasts. Multiple pairwise comparisons indicated that, for PAP2 cells (A), all values were statistically different ($P < 0.05$) except OPN in the top chamber vs. no OPN; for MDA-MD-435 cells (B), all values differed from one another ($P < 0.05$); and for gingival fibroblasts (C), migration to OPN in the bottom well differed from other conditions ($P < 0.05$).

The present study tested directly the hypothesis that the RGD region is important to OPN function, by site-directed mutagenesis of this sequence. Our results demonstrate that the RGD sequence is crucial for the ability of OPN to support both cell adhesion and chemotaxis.

We generated site-directed mutant OPN proteins, as recombinant GST-fusion proteins, in which the RGD sequence was completely deleted or mutated to RGE, and tested the ability of these proteins to function in assays of cell adhesion and induction of directed cell migration. Four cell lines were used, malignant (MDA-MB-435 human mammary carcinoma cells, and PAP2 *ras*-transformed murine NIH 3T3 cells) and nontransformed (normal human gingival fibroblasts, and "normal," immortalized NIH 3T3 cells), in order to assess whether normal and transformed cells adhere to OPN via similar mechanisms. The ability of OPN to support cell adhesion of a variety of normal and malignant cell types has been well documented [review, 2]. The ability of OPN to induce chemotaxis has been reported for macrophages [9], and more recently for smooth muscle cells [16].

We found that adhesion to GST-mOPN, for all of the cell types tested, was dependent on having an intact RGD sequence in the protein. Mutation of the RGD sequence to RGE, or deletion of the RGD sequence, resulted in protein that did not support cell adhesion (Figs. 2–6), even when used at high concentration or for extended assay times (e.g., Fig. 3).

We also found that all of the cell lines tested were induced to undergo directed cell migration in response to GST-mOPN protein. This chemotactic response required an intact RGD sequence, as neither the "RGE" nor the "del-RGD" mutant OPN proteins induced chemotaxis in any of the cells tested (Figs. 7–9). This is the first report of OPN-induced chemotaxis for malignant cells or normal fibroblasts, suggesting that many cell types, in addition to macrophages [9] and smooth muscle cells [16], can be induced to migrate toward OPN.

Cell activities that depend on adhesion to, or migration toward, OPN thus require an intact RGD sequence for maximal function. Loss of the RGD sequence, either experimentally by mutation (as we show here), or in some cases functionally by, for example, thrombin present in the circulation, can result in loss of the various functions of OPN in vitro and also perhaps in vivo. The role of OPN in its diverse contexts remains the subject of much ongoing research [2,7,33]. Our results do not negate the potential importance of other domains of OPN, or various posttranslational modifications (phosphorylation, glycosylation), in modulating OPN function [e.g., 14,34]. For example, site-directed mutagenesis of fibronectin has identified non-RGD sequences that can influence cell binding of that protein [35]. Sequence-based structural analysis of OPN suggests that the RGD sequence may be located at a turn in two regions of β-sheet structure [36; see also 2 for review]; presumably changes in primary structure or posttranslational modifications that alter the conformation of the RGD-containing region of OPN could result in altered RGD-mediated function.

Research over the past several years on integrin-mediated signal transduction suggests that many ligand-integrin interactions result in transmission of a signal to cells [37,38]. OPN, like other integrin-binding proteins, may function both as an attachment factor as well as an inducer of integrin-mediated signals. Binding or migration of cells to OPN may be only an initial step, followed by integrin-mediated signal transduction and induction of an appropriate cellular response. At present, the cell surface receptor(s) involved in OPN cell surface binding and signal transduction are the subject of much ongoing research, although receptor blocking experiments suggest that OPN can bind to the αvβ3 integrin on osteoclasts, smooth muscle cells, some transformed cell lines, and gingival fibroblasts [10,13,15–18]. Adhesion and migration to OPN, both of which we show to be dependent on the RGD sequence of the protein, may result in down-stream consequences of RGD-mediated interactions of OPN with cell surface integrins, including changes in gene expression and cell behavior [e.g., 10,33,39,40]. The results of our site-directed mutagenesis studies on OPN suggest that in the absence of these RGD interactions, those OPN functions that require integrin interactions may be severely compromised. In addition, it will be of interest to determine the effects of RGD-mutations in OPN on other reported functions of OPN, including its ability to complex with osteocalcin [41], to act as a substrate for transglutaminase [42], and to inhibit mineralization [43–45]. The results presented here indicate that OPN can function as an adhesive and chemotactic protein for both normal and malignant cells, and that these interactions (and any consequences of these interactions) depend on an intact RGD sequence.

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