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Recombinant GST-Human Osteopontin Fusion Protein Is Functional in RGD-Dependent Cell Adhesion

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Abstract Osteopontin (OPN) is a secreted phosphoprotein expressed by many tumor cells, as well as a limited set of normal cells. Native OPN has been shown to support cell adhesion in an RGD-peptide-inhibitable fashion. Here we expressed human OPN in *E. coli* as a recombinant fusion protein with glutathione-S-transferase (GST). We report that the GST-OPN fusion protein has functional activity. PAP2 (*ras*-transformed, metastatic murine NIH 3T3) and MDA-MB-435 human mammary carcinoma cells bound to GST-OPN in an in vitro cell adhesion assay nearly as well as to native bovine OPN. Adhesion to the recombinant fusion protein was blocked by addition of GRGDS peptide, suggesting that the cells adhere to the recombinant and native OPN proteins by similar, integrin-mediated mechanisms. Adhesion to both sources of OPN also was inhibited by thrombin treatment of the protein. Thrombin cleaves GST from OPN in the fusion protein, and also cleaves internally in OPN, adjacent to the RGD sequence of the protein. Our results suggest that (a) thrombin cleavage of native OPN may be a natural regulator of OPN function, and (b) the majority of OPN cell binding activity is mediated by the RGD sequence in the protein backbone, with little or no requirement for post-translational modifications that occur in native OPN for adhesive function as measured here. © 1994 Wiley-Liss, Inc.

Key words: OPN, secreted phosphoprotein, tumor cells, normal cells, *E. coli*

Osteopontin (OPN) is a secreted phosphoprotein produced by a limited number of normal cells and tissues (e.g., during bone development, in lactating mammary gland, kidney, activated T-cells and macrophages, and smooth muscle) [review 1,2–9]. OPN also has been detected in milk, serum and urine [10–12]. In addition, OPN has been identified as a tumor-associated protein [10,11,13–17]. The function of OPN in either normal or tumor cells is poorly understood, although some work suggests that it has adhesive function. OPN proteins from all species studied have a conserved GRGDS amino acid sequence [15,18–26] typical of integrin-binding adhesive proteins [27,28], and consistent with possible adhesive function. A number of cell types have been shown to bind OPN, in an RGD peptide inhibitable fashion [21,29–33]. OPN is believed to bind to cells via the $\alpha v\beta 3$ (vitronectin receptor) integrin [31,34], and perhaps to other less well-characterized receptors [35]. Binding of OPN to osteoclasts through the $\alpha v\beta 3$ integrin has been shown to result in signal transduction leading to cytosolic calcium fluxes [31]. Adhesion and spreading of gingival fibroblasts on OPN leads to increased expression of heat shock proteins [36].

We have shown previously that metastatic, *ras*-transformed NIH 3T3 fibroblasts express increased levels of OPN [16,17,37] and bind to bovine OPN-coated surfaces better than do control, non-transformed NIH 3T3 cells [33]. We found further that blocking of cell surface RGD binding sites inhibits the adhesive interactions of these cells with OPN [33].

Here we describe the cloning, production in *E. coli*, and characterization of a recombinant GST (glutathione-S-transferase)-human OPN fusion protein that has functional activity. The GST-OPN protein supports adhesion of PAP2 (murine *ras*-transformed NIH 3T3) and MDA-MB-435 human mammary carcinoma cells. This adhesion was inhibited by addition of RGD-containing peptides and by thrombin cleavage of the protein.

MATERIALS AND METHODS

Cells and Cell Culture

Murine fibroblast cells NIH 3T3 and PAP2 (T24-H-*ras*-transformed NIH 3T3) [38,39] were

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maintained in DMEM (Gibco, Burlington, Ontario, Canada) with 10% calf serum (Gibco). MDA-MB-435 human mammary carcinoma cells [40,41], 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).

Construction of the GST-OPN Fusion Plasmid

The strategy used is outlined in Figure 1A. Two primers located near the 5′- and 3′-ends of the mature human osteopontin cDNA sequence [19,42] were synthesized: *P1* (GGGTTGGATC CATACCAGTT AAACAGGC) containing the *Bam*HI restriction site and cDNA sequences of the first six amino acids of mature OPN; *P2* (TTAATTGACC TCAGAAG) complementary to cDNA sequences of the last five amino acids and stop codon of the OPN protein. The PCR reaction was conducted using plasmid OP-10 [19] (human OPN cDNA clone, a kind gift from Dr. Larry W. Fisher, Bone Research Branch, NIH) directed by the above two primers. The PCR product was purified by Prep-a-Gene (BioRad, Mississauga, Ontario, Canada), treated with *Bam*HI, Klenow fragment enzyme, phosphorylated, and cloned into pBluescript vector according to previously described methods [43]. The human OPN mature cDNA sequence cloned by PCR was completely sequenced and inserted into the pGEX-2T (Pharmacia, Montreal, Quebec) vector in the same reading frame as the carrier gene (glutathione S-transferase, EC 2.5.1.18) [44] and transformed in *E. coli* BL21 cells (F^-*omp*T $r_B^- m_B^-$, Novagen, Madison, WI). The recombinant OPN protein as expressed in *E. coli* has two additional amino acids (Gly-Ser) inserted in the N-terminal sequence when excised and separated from GST protein by thrombin digestion, due to introduction of a *Bam*HI restriction site at the 5′ end of the mature OPN cDNA.

Expression and Purification of GST-OPN Protein

Expression of the GST-OPN fusion protein was induced from the IPTG-inducible *tac* promoter by 0.5 mM IPTG [44], in transformants grown to a cell density of OD_{600} 1.5, by incubation for 90 min at 37°C. GST-OPN protein was purified as described [44]. Briefly, *E. coli* cells (frozen and thawed) were incubated with 1 mg/ml lysozyme, 1 mM PMSF in phosphate-buffered saline (PBS) for 20 min at 0°C, followed by treatment with 1% Triton X-100 and sonication (4 × 20 s). The cell lysate was cleared by centrifugation (10,000*g*) and 0.45 μm filtration. The pellet after centrifugation was dissolved in 8 M urea, 0.1 M glycine-NaOH (pH 9.0), and desalted by passage through a spin column of Sephadex G-25. Affinity chromatography with a glutathione sepharose 4B column (Pharmacia) was used according to the manufacturer's instructions for the final purification of the GST-OPN fusion protein. Protein content was measured using a Bio-Rad protein assay kit.

Metabolic Labelling of Mammalian OPN

Native OPN protein secreted by human MDA-MB-435 cells was labeled metabolically following procedures as previously described [17,33]. Briefly, monolayers of cells were washed with phosphate-free, serum-free DMEM (Gibco) and labelled with [^{32}P] orthophosphoric acid. ^{32}P-labeled OPN secreted into the medium was purified from unincorporated $^{32}P_i$ by passage through Centricon 30 columns (Amicon, Beverly, MA).

Thrombin Cleavage

For thrombin digestion, 50 μl of concentrated medium containing metabolically labelled OPN was incubated with thrombin enzyme (Boehringer Mannheim, Montreal, Quebec) (0.25U) in the presence of 0.1 M Tris (pH 8.0), 0.15 M NaCl, 0.0025 M $CaCl_2$ at room temperature for 2 h. GST-OPN fusion protein was digested by thrombin at 10 μg protein/0.1U enzyme, incubated overnight at room temperature. The digested samples were analyzed by 15% SDS-PAGE followed by autoradiography. ^{14}C-labeled protein markers (Gibco-BRL) were used as molecular weight standards.

Adhesion Assay

For NIH 3T3 and PAP2 cells, the attachment to 96-well polystyrene plates coated with specified substrates was measured as described previously, using the assay for nonlabeled cells (2×10^4/well) [33]. For MDA-MB-435 cells, cells were trypsinized and treated with soyabean trypsin inhibitor, then washed twice with PBS and resuspended in α+ MEM with 2 mg/ml BSA. Cells (4×10^4/well) were added to 96-well plates and adhesion to various substrates was assayed as for NIH 3T3 and PAP2 cells. Substrates used were the GST-OPN fusion protein, native bovine OPN [45,46] (a kind gift from Dr. Charles Prince, University of Alabama at Birmingham), and human fibronectin (Collaborative Biomedical Products, Bedford, MA). In some experi-

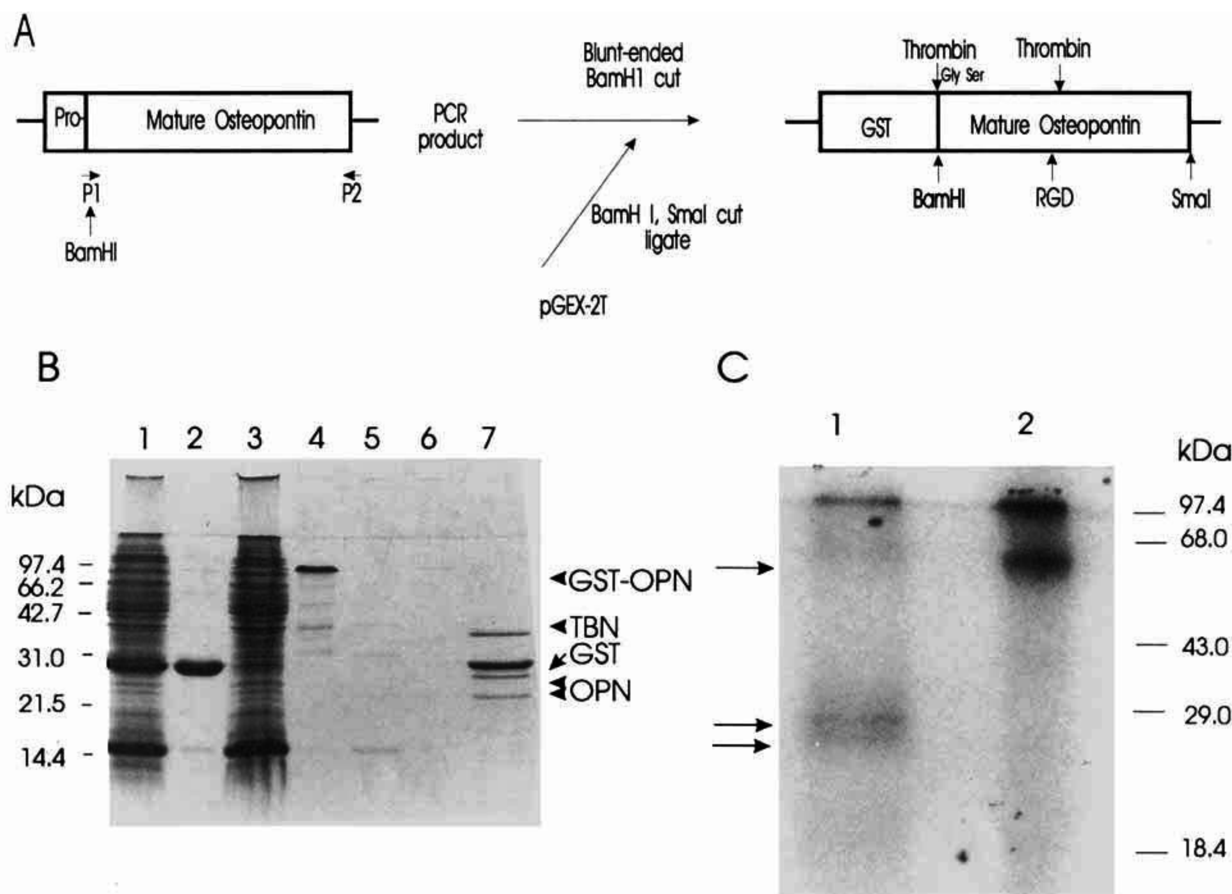

Fig. 1. **A:** Strategy use for PCR cloning of the cDNA encoding human OPN mature protein into the *E. coli* expression vector pGEX-2T. The PCR product, containing a *Bam*HI site at the 5′ end, was cloned into pGEX-2T. "Pro" indicates the signal sequence of OPN. Two amino acids ("Gly Ser") were added, which are part of the thrombin digestion site (Pro Arg ↓ Gly Ser) used to cleave GST from OPN and encoded by the *Bam*HI linker. **B:** 15% SDS PAGE analysis of recombinant GST-OPN protein produced by *E. coli*. **Lane 1:** Total protein from *E. coli* cell lysate of control pGEX-2T vector transformants before affinity column purification. **Lane 2:** After column purification. **Lane 3:** Total protein of *E. coli* cells harboring the recombinant GST-OPN plasmid before purification. **Lane 4:** After column purification; precipitates of *E. coli* cell cytosol, vector (**lane 5**) and recombinant GST-OPN (**lane 6**) transformants. **Lane 7:** GST-OPN cut by thrombin. **C:** 15% SDS-PAGE analysis of metabolically labelled [^{32}P]-OPN secreted by MDA-MB-435 human mammary carcinoma cells, cut by thrombin (**lane 1**), and without thrombin cleavage (**lane 2**).

ments, OPN proteins were cleaved with thrombin, as described above, prior to application to wells. Substrates were applied to the wells as described previously [33]. When used, synthetic GRGDS peptides (Telios Pharmaceuticals, San Diego, CA) were added at 100 μM as described [33].

RESULTS

Expression of GST-Human Osteopontin cDNA in *E. coli* Cells

As shown in Figure 1A, human osteopontin cDNA excluding the signal peptide (amino acids 1–16) was amplified by PCR and inserted in the same reading frame at the 3′ end of the GST gene in vector pGEX-2T. Expression of the GST-OPN fusion protein is regulated by the IPTG-inducible *tac* promotor [44]. Induction of the GST-OPN fusion plasmid for 90 min resulted in the abundant synthesis of a ~85 kDa protein as analyzed by 15% SDS-PAGE (Fig. 1B, lane 4). Approximately 30 mg of column-purified GST-OPN could be obtained per liter of bacterial stock. The 85 kDa GST-OPN protein band was seen only in *E. coli* cell proteins from transformants with the GST-OPN plasmid (Fig. 1B; lane 3, total protein; lane 4, protein purified by glutathione sepharose 4B affinity chromatography). A 27 kDa GST band was seen only in proteins from control transformants, with the unmodified pGEX-2T vector (Fig. 1B; lane 1, total protein; lane 2, purified protein). Minor

bands that co-purified with GST-OPN protein were obtained (see lane 4). These bands were not removed by a second round of affinity column purification (data not shown), suggesting that these small proteins might be truncated GST-OPN recombinant translational products. The induced proteins were not found in the insoluble protein fraction of cell lysates of transformants, for either the vector pGEX-2T (lane 5) or the recombinant clones (lane 6).

The vector pGEX-2T was designed to have a thrombin recognition site between the carrier protein GST and insertion proteins. The purified GST-OPN protein thus was digested by thrombin in order to separate OPN from GST. As shown in lane 7, Figure 1B, thrombin digestion resulted in a strong 27 kDa band (the size of the GST protein, cf. lane 2), one thrombin protein band (37 kDa) and two OPN bands (~23 kDa and 25 kDa). These results are consistent with the reported ability of thrombin to cleave OPN [10,14] discussed below.

In order to demonstrate that these two bands are thrombin fragments of human OPN, OPN protein secreted by human mammary carcinoma cells (MDA-MB-435) was metabolically labelled, partially purified and digested by thrombin. Figure 1C (lane 1) shows two thrombin digestion bands of native human OPN, of ~24 and ~26 kDa, sizes similar to those of the thrombin-cut GST-OPN (cf. Fig. 1B, lane 7).

Adhesion of Mouse Fibroblast PAP2 and NIH 3T3 Cells to Recombinant Human GST-OPN Protein

We tested the biological function of recombinant human OPN protein (GST-OPN) expressed in *E. coli* cells, by assaying the ability of GST-OPN protein to support cell adhesion (Fig. 2). We assayed the ability of PAP2 and NIH 3T3 cells to adhere to and spread on plastic surfaces coated with recombinant GST-OPN, compared with adhesion to native bovine OPN (bOPN), with controls of BSA (negative control), fibronectin (FN, positive control), and GST alone (control for GST-OPN). As shown in Figure 2, GST-OPN was functional in this assay and was capable of supporting adhesion of PAP2 cells at a level similar to that of bovine OPN, while NIH 3T3 cells adhered poorly to either source of OPN, consistent with our previous results with these cells and bovine OPN [33]. Both cell lines adhered well to FN and poorly to the negative BSA control, as reported previously [33], and poorly to GST protein alone. In this experiment, we used a slightly higher concentration of GST-OPN (50 μg/ml) than bOPN (40 μg/ml) to coat the wells, to account for possible truncated GST-OPN protein, as described above.

Adhesion of PAP2 and NIH 3T3 cells to GST-OPN was blocked by addition of GRGDS peptides (Fig. 2), as we observed previously for adhesion to bovine OPN [33], suggesting that adhesion of these cells to both native and recombinant OPN is dependent on RGD-mediated interactions. Adhesion of these cells to both GST-OPN and bovine OPN also were completely inhibited by thrombin cleavage of the coating proteins (Fig. 2). As discussed below, this finding is consistent with the expected cleavage by thrombin of OPN near the RGD site in the protein.

The concentration dependence (Fig. 3A) and time course of adhesion (Fig. 3B) of PAP2 and NIH 3T3 cells to recombinant GST-OPN were examined. The ability of both PAP2 and NIH 3T3 cells to adhere to GST-OPN coated surfaces was dependent on the GST-OPN concentration (Fig. 3A). As also seen in Figure 2, PAP2 cells adhered better to GST-OPN than did NIH 3T3

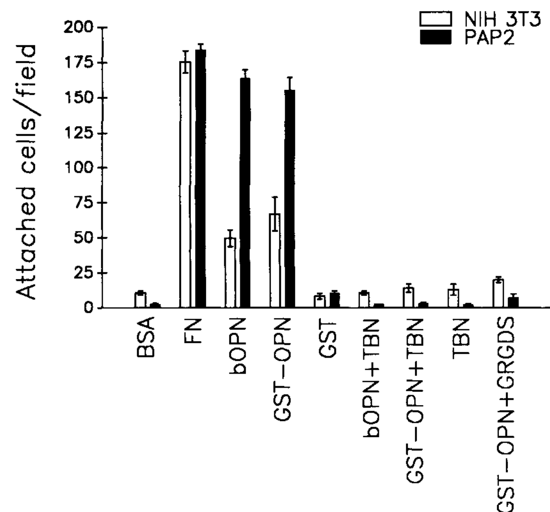

Fig. 2. Adhesion of NIH 3T3 and PAP2 cells to coated surfaces. Cells were added to 96-well dishes that had been coated with BSA (2 mg/ml), fibronectin (FN; 10 μg/ml), bovine OPN (bOPN; 40 μg/ml), recombinant GST-OPN (50 μg/ml), control GST protein (50 μg/ml), bovine OPN cleaved with thrombin (bOPN + TBN), GST-OPN cleaved with thrombin (GST-OPN + TBN), thrombin control (TBN), and GST-OPN plus GRGDS peptide (100 μM) (GST-OPN + GRGDS). Cells were allowed to adhere to the coated plastic surfaces for 1 h and the plates were washed, fixed, stained, and counted using the non-labelled method as previously [33]. Numbers of attached and spread cells per microscope field (0.49 mm^2) were counted; bars show mean values for 3 wells per condition ± SD.

cells. These results are similar to the concentration dependence of adhesion of these cells to bovine OPN [33], with maximal adhesion of PAP2 cells to GST-OPN at ~50 μg/ml and to bovine OPN at ~20 μg/ml.

Adhesion of PAP2 and NIH 3T3 cells to GST-OPN (Fig. 3B) was transient. Maximal adhesion and spreading was found at ~60 min, with most cells rounding up and detaching from the substrate after ~120–180 min. These results parallel the adhesion kinetics for these cells to bovine OPN, reported earlier [33].

Adhesion of MDA-MB-435 Human Mammary Carcinoma Cells to Recombinant Human GST-OPN Protein

We compared the ability of MDA-MB-435 cells to adhere to GST-OPN and bovine OPN, as well as to fibronectin. A concentration dependence similar to that seen for PAP2 and NIH 3T3 cells was observed for MDA-MB-435 cell adhesion to both sources of OPN (Fig. 4A), with maximal adhesion at a concentration of ~50 μg/ml. Adhesion to FN was also concentration dependent, but the maximal concentration had not been reached by 75 μg/ml (Fig. 4B). However, in contrast to the rapid and transient adhesion of PAP2 and NIH 3T3 cells to these substrates, MDA-MB-435 cells attached and spread much more slowly and their adhesion to GST-OPN and bovine OPN (Fig. 5A), as well as to fibronectin (Fig. 5B), was persistent rather than transient. As with PAP2 and NIH 3T3 cells, a given concentration of bovine OPN supported adhesion of MDA-MB-435 cells somewhat better than did the same concentration of GST-OPN. In addition, adhesion of MDA-MB-435 cells to GST-OPN and bovine OPN (Fig. 5A), as well as to fibronectin (Fig. 5B), was completely inhibited by GRGDS peptides.

DISCUSSION

Results presented here indicate that the majority of cell adhesive function of OPN, as measured by the ability of cells to adhere to OPN in vitro, is mediated by the RGD sequence in the protein backbone. Native OPN undergoes a variety of post-translation modifications, which can vary under different physiological conditions [47,48]. The functions of these modifications are not well understood. Our results suggest that these post-translational modifications do not contribute significantly to adhesion to OPN as measured by in vitro assay. Clearly there are subtleties in adhesive interactions that could be mediated by phosphorylation, glycosylation, etc., and the resulting changes in conformation and charge. These sorts of interactions will be important to document. However, the results presented here indicate that the majority of attachment function of OPN can be attributed to the RGD sequence in the protein backbone. Phosphorylation may negatively regulate the adhesive function of OPN, as suggested by a study showing that a synthetic GRGDSL peptide with a phosphorylated serine was less adhesive to cells than the non-phosphorylated peptide [49].

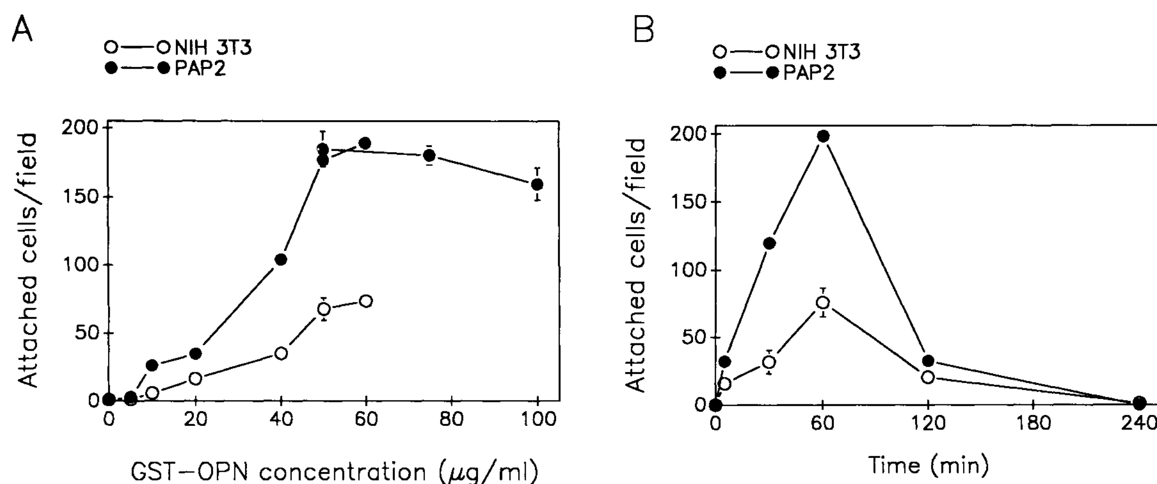

Fig. 3. Concentration dependence and time course of adhesion of GST-OPN to murine NIH 3T3 and PAP2 cells. **A:** Adhesion to wells coated with the specified concentrations of OPN were assessed after 1 h, as in Figure 2. **B:** Adhesion to GST-OPN (50 μg/ml) after stated times. Each point represents the mean of 3 values ± SD.

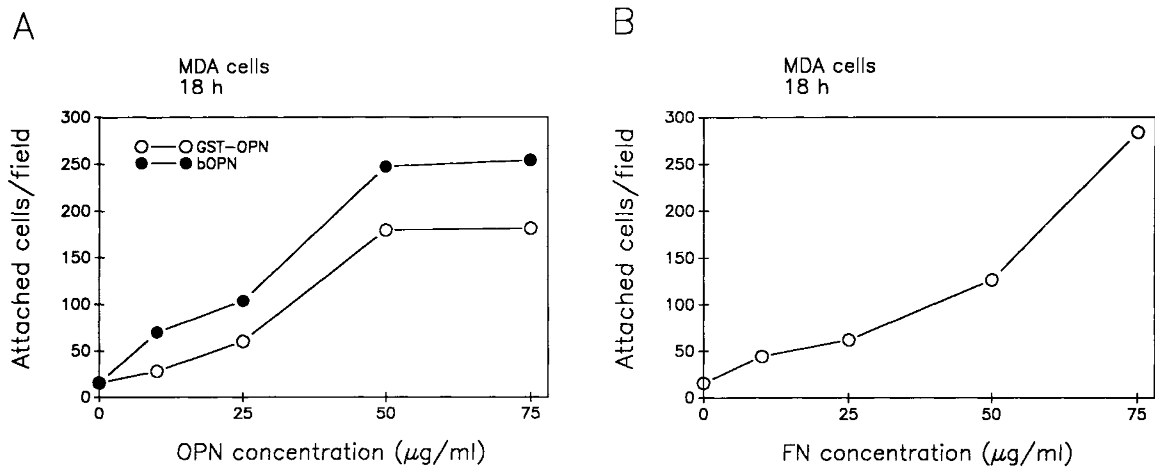

Fig. 4. Concentration dependence of adhesion of MDA-MB-435 human breast carcinoma cells, to wells coated with bOPN or GST-OPN (**A**) and fibronectin (**B**). Adhesion was assessed after 18 h, as described in Materials and Methods. Each point represents the mean of 3 values ± SD.

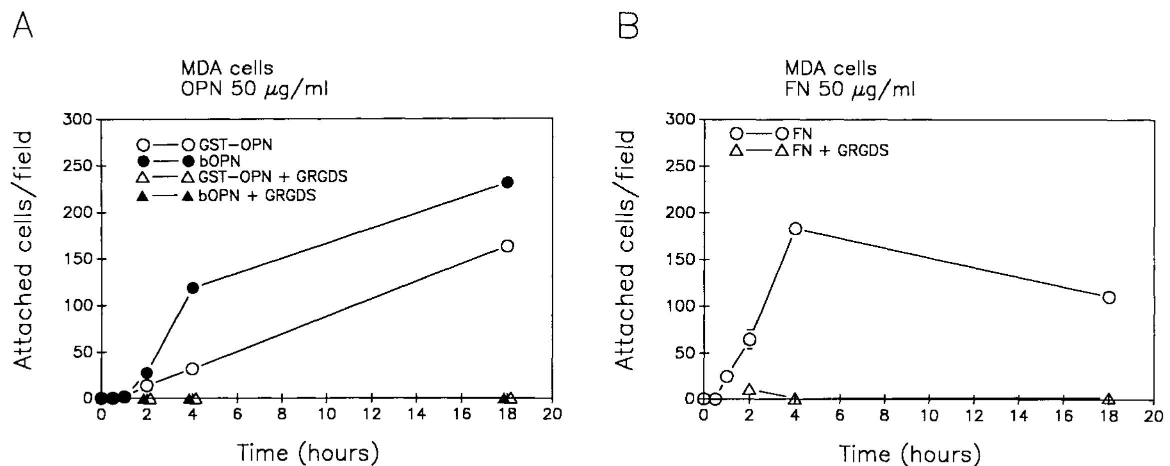

Fig. 5. Time course of adhesion of MDA-MB-435 human breast carcinoma cells to wells coated with 50 μg/ml GST-OPN or bOPN (**A**) and fibronectin (**B**), assessed as described in Materials and Methods. Each point represents the mean of 3 values ± SD.

Adhesive function of OPN was preserved in the fusion protein, containing the GST sequences. Availability of GST-OPN protein with functional activity offers several advantages. The fusion protein can be readily prepared in ample quantities (~30 mg/liter of *E. coli* stock), compared with more tedious purification procedures and considerably lower yields of native protein from various sources. For example, it has been reported that ~30 mg OPN can be prepared from 800 rat leg bones [35]. Calculation from reported yields indicate that ~3–10 liters of human milk [10], or ~120 liters of tissue culture medium [10], would be required for comparable yields from these sources. The availability of functional recombinant OPN protein also will permit clarification of the roles of various post-translational modifications to the protein.

Recently, Ashkar et al. [50] reported generation a murine GST-OPN fusion protein in another GST vector, cleavable from GST by factor Xa. They used this recombinant protein to document in vitro phosphorylation of the protein by casein kinase II. They confirmed that thrombin cleavage of murine OPN generated a peptide with an amino-terminal sequence homologous to that for thrombin cleavage fragments identified previously by Senger [10,14] for rat (with

cleavage after arginine 153) and human OPN (after arginine 154), and consistent with our results. OPN from all species analyzed to date (human [18,19], mouse [15,20], rat [21], chicken [22,23], pig [24], cow [25], rabbit [26]) share highly homologous, conserved sequences in this region. Thrombin cleavage resulting in similarly sized fragments also has been reported for a protein, from a canine kidney cell line, that is likely the canine homolog of OPN [51]. Together these results suggest that thrombin cleavage may be important to the function of OPN. Ashkar et al. [50] also reported a minor thrombin cleavage site 4 amino acids from the cleavage site identified by Senger by amino-terminal sequencing [10].

We found that cleavage of both GST-OPN and bovine OPN by thrombin destroyed the RGD-mediated adhesive function of the proteins. Both full-length and thrombin-cleaved OPN have been found in human serum [14] and milk [10]. Because thrombin divides OPN near the RGD sequence, Senger postulated that thrombin cleavage might alter the function of the RGD site [14]. We have confirmed this prediction with the functional studies reported here. The proximity of the RGD adhesion sequence and the RS thrombin cleavage site (9 amino acids between arginine residues) appears to be sufficient to destroy RGD-mediated adhesive interactions, likely by conformational changes in the cleaved protein. Our results show that thrombin cleavage is sufficient to eliminate RGD-mediated in vitro adhesive function of OPN. This finding suggests that thrombin could be a natural regulator of OPN function in vivo.

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