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Osteoblasts Induce Osteopontin Expression in Response to Attachment on Fibronectin: Demonstration of A Common Role for Integrin Receptors in the Signal Transduction Processes of Cell Attachment and Mechanical Stimulation

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Abstract Osteopontin is a predominant integrin binding protein of bone and its expression has been shown to be induced by mechanical stimuli within osteoblasts (Toma et al. [1997] J. Bone Miner. Res. 12:1626–1636). The present studies examined if the cell adhesion would mimic the mechano-transduction that stimulated *opn* mRNA expression and whether integrin receptors were involved in these processes. Osteopontin mRNA expression was induced three- to four-fold, 24 hours after embryonic chicken calvaria osteoblast attachment to fibronectin (FN), however no induction was observed if the cells were plated on tissue culture plastic alone. Osteopontin mRNA induction in response to cell attachment on FN was dependent on new protein synthesis and the activation of a tyrosine protein kinase(s) but unlike mechano-induction was independent of the maintenance of the cell's microfilament structure. Integrin receptor(s) were shown to be involved in mediating the signal transduction processes of both cell attachment and mechanical stimulation since incubation of osteoblasts with the integrin binding peptide RGDS partially blocked the induction of opn expression in response to both stimuli. Interestingly, incubation of the osteoblasts that were adherent on tissue culture plastic alone with the RGDS peptide lead to an induction in *opn* expression suggesting that integrin occupancy by itself was sufficient to initiate the signal transduction process that induced *opn* expression. In order to assess the role of integrin occupancy vs. focal adhesion complex formation that accompanies cell attachment, in the signal transduction process that induces *opn* expression, receptor clustering was stimulated pharmacologically with bombesin or lysophasphatidic acid in osteoblasts attached to tissue culture plastic. Neither compound in the absence of occupancy of the integrin receptors was capable of stimulating *opn* expression in attached cells, however if the cells were placed in suspension pharmacological mediation of receptor clustering and integrin occupancy were additive in their effect of inducing *opn* expression. These data demonstrate that induction of *opn* expression by mechanical stimuli and cell attachment are commonly mediated through integrin receptor(s). However, when cells are attached receptor clustering alone which accompanies focal adhesion formation was incapable of mediating signal transduction suggesting that receptor occupancy was a prerequisite to the signal transduction process that leads to the induction of *opn* mRNA expression. J. Cell. Biochem. 70:376–390.

Key words: osteopontin; integrins; mechano-transduction; tyrosine kinase

Osteopontin (OPN) is recognized as one of the major non collagenous extracellular matrix proteins belonging to the super family of cell adhesion molecules [Guo and Denhardt, 1994]. OPN has been shown to interact both with integrin receptors through its RGD motiff [Somerman et al., 1987; Oldberg et al., 1988; Gotoh et al., 1990; Ross et al., 1993] and more recent studies have shown that the carboxyl terminal portion

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of OPN has the potential to interact with a variant form of the CD44v receptor [Weber et al., 1996]. The identification of osteopontin's potential to interact both with integrin and CD44v receptors has led to major research efforts directed towards identifying the role that this molecule plays in cell matrix interactions and cell migration [Liaw et al., 1995; Weber et al., 1995] during normal and pathological processes. It has been shown to be expressed by a wide variety of cell types and has been shown to be an early response gene during both T-cell [Patarca et al., 1989] and macrophage activation [Singh et al., 1991]. OPN is an immediate early response gene to PKC activation through phorbol ester tumor promoter treatment in a wide variety of cells [Smith and Denhardt, 1987; Moore et al., 1990; Rafidi et al., 1993]. It has been identified as a serum marker of malignant cell growth [Senger et al., 1989] and has been shown to be induced by mechanical stimulation in skeletal cells [Harter et al., 1995; Kubota et al., 1993; Toma et al., 1997].

Initially however, OPN was characterized as one of the predominant noncollagenous proteins that is accumulated in the extracellular matrices (ECM) of bone tissue [Butler, 1989; Gerstenfeld et al., 1990, 1994], and the temporal and spatial expression of the *opn* gene during embryogenesis and skeletal tissue development [Nomura et al., 1988; Mckee et al., 1990] suggests that this protein plays important roles in the processes of bone metabolism through its functional roles in mediating cell adhesion, attachment and cell migration of fibroblasts, osteoblasts, and osteoclasts [Olberg et al., 1988; Gotoh et al., 1990; Flores et al., 1992; Miyauchi et al., 1991; Ross et al., 1993]. This protein is ~66 kDa and contains approximately 300 amino acids. Sequence analysis of OPN has demonstrated it to be very acidic, and identified a number of conserved functional domains within its primary structure, including its RGD recognition site for integrin-mediated cell adhesion [Butler, 1989; Miyauchi et al., 1991; Tezuka et al., 1992] and its conserved sites of serine and threonine phosphorylation [Salih et al., 1997].

Osteoblasts are anchorage dependent cells adhering to a substrate via cell adhesive molecules. Receptor ligands such as integrins bind to these proteins through RGD-dependent interactions and are known to function as signal transduction molecules in a series of critical recognition events of cell-substratum and cell-cell adhesion [Kornberg et al., 1991; Hynes, 1992 for a review]. These events are key to a variety of biological processes including cell polarity, migration, immunological recognition, and tumor cell metastasis [Ingber, 1991; Milam et al., 1991; Clover et al., 1992; Ginsberg et al., 1992; Hynes, 1992; Majda et al., 1994]. The signals transduced by cell matrix receptors such as integrins are quite complex and may be mediated through receptor clustering and/or cellular shape changes, alterations in cytoskeletal structure, specific cytoskeletal interactions with the receptors or second signal second signal transducers, as well as activation of specific second signal cascades [Juliano and Haskill, 1993]. Such changes are known to lead to specific genomic effects [Werb et al., 1989], however, the nature of many transmitted signals and the downstream effects of these signals remain unidentified. Considerable data has been accumulated, demonstrating that integrin receptors mediate some of their effects by specific tyrosine kinase(s) such as focal adhesion kinase that transduce specific signals in a variety of stimulatory mechanisms [Ingber, 1992; Schaller and Parsons, 1994].

Mechanical stimulation has been shown to play an important role in the regulation and maintenance of bone cell phenotype [Lanyon, 1984; McLeod et al., 1987] and many signaling mechanisms have been implicated in the role of transducing mechanical signals in bone tissue [Sandy et al., 1989; Watson, 1991; Brighton et al., 1991; Carvalho et al., 1993]. Previous studies from several research groups have shown that *opn* expression is responsive to mechanical stimulation [Kubota et al., 1993; Raab-Cullen et al., 1994; Harter et al., 1995; Toma et al., 1997]. However, the biological systems that transduce mechanical stimuli, which mediate specific genomic effects in osteoblasts, are still poorly understood. In previous studies from this laboratory, the mechanisms of mechanosignal transduction that mediated the expression of opn were examined. These results demonstrated that the signal transduction process that mediated *opn* expression were: a primary response through the activation of pre-existing transcriptional factors; dependent on the activation of a tyrosine kinase(s) and protein kinase A (PKA) or a PKA-like kinase; and dependent on microfilament integrity. These studies also demonstrated that mechanical stimuli activated focal adhesion kinase $pp125^{FAK}$, which specifi-

cally became associated with the cytoskeleton after mechanical perturbation [Toma et al., 1997]. Such data suggested that a member or members of the integrin receptor family through interaction with specific ligand(s) in the extracellular matrix may be a crucial component of the signal transduction pathway for mechanical stimuli. In the present investigation experiments examined whether cell adhesion to fibronectin would mimic the induction processes of *opn* expression that were seen for mechanical stimulation, and what role integrin receptors played in both these processes.

MATERIALS AND METHODS

Experimental Procedures

Materials. All tissue culture supplies, cytochalasin D, colchicine, cycloheximide, RGDS, RGES, and RFDS peptides, lysophosphatidic acid (LPA) and bombesin (BM) were from Sigma Chemical Company (St. Louis, MO). H89 genistein was from LC Laboratories (Woburn, MA). Nylon membranes for Northern blots were from Biotrans, ICN Corporation (Aurora, OH).

Cell culture. Seventeen-day embryonic chicken calvaria osteoblasts were and grown in culture as previously described [Gerstenfeld et al., 1988]. Only the cells from the third sequential digest were used for the experiments. These cells were plated at a density of 2 x 10^6 cells in 100 mm tissue culture dishes coated either with purified fibronectin (FN; 1 mg/ml) as previously described [Schaffer et al., 1994] or uncoated plates. Cultures were grown for 2 weeks until confluency in minimum essential media supplemented with 10% fetal bovine serum (FBS). At confluency, the medium was changed to BGJb supplemented with 10% FBS with the addition of 10 mM β-glycerophosphate and 12.5 µg ascorbic acid. All analyses were performed on at least three separate preparations of cells and all data is presented as a percent increase in expression over that of the controls which were determined from parallel cultures grown under identical conditions. All error bars represent the S.D. of the determinations from experiments carried out with a minimum of three separate preparations of cells.

Mechanical stimulation/Attachment assays. The mechanical stretch apparatus used for these experiments was previously described [Schaffer et al., 1994]. The design of the device imposes a verified temporal and spatial displacement profile to an optically transparent elastomeric membrane in which the strain magnitude was experimentally demonstrated to be homogeneous and isotropic (i.e., radial strain = circumferential strain = constant over the culture surface). A polyurethane membrane (a generous gift of Dow Chemical Corporation, Midland, MI) was used in the culture dishes allowing for a constant 1.3% uniform biaxial strain at 0.25 Hz to be applied for a single 2-h period. For each experiment nonstimulated controls were carried out on cultures at the same time and from the same preparation of cells grown with identical conditions to the mechanically stimulated cultures. In all experiments for mechanical deformation, determinations were carried out 6 h after the end of the 2-h period of active cellular stimulation.

Assays of cell response to attachment on fibronectin were carried by plating 2 x 10^6 cells on FN-coated (1 mg/ml) 100 mm tissue culture dishes for 24 h. The control for these studies was attachment to uncoated tissue culture surfaces. In experiments in which the cells were forced to remain in suspension, cells were trypsinized and plated on nonfibronectin-coated bacterial petri dishes to maintain the cells in a nonattached state. Under these conditions a small minority of the cells would attach to the plastic surfaces while the rest of the cells formed nonadherent clumps in the media. Cells were then treated as described below with RGDs peptide, bombensin, or both reagents and RNA was collected at 24 h after the addition of the various reagents. In the experiments in which cells were maintained in suspension the nonattached cells were separately assayed from cells that were adhered to culture surfaces.

Signal transduction studies. Signal transduction pathways that mediate the cell responses of mechanical stimulation and/or attachment/ligation were investigated by the use of specific chemical inhibitors. The final concentration for each of these compounds was: 50 µM cycloheximide, 20 µg/ml genistein, 1 µM H89, 50 µM cytochalasin D, and 1 µM colchicine. Cells were pretreated with cycloheximide and genistein for 30 min, with cytochalasin D for 1 h and with colchicine for 6 h before each of the experimental purturbations was initiated. All pharmacological treatments were continued throughout the experimental period. In all experiments, the cultures were analyzed immediately following the treatment with these compounds. Controls were separately determined

for each compound, in cultures treated identically with the various compounds but in which the cells were either not mechanically stimulated or were attached to uncoated dishes.

Peptide binding. The specificity of integrin ligation was tested by the incubation of selective peptides with osteoblasts prior to cell plating. 2 x 10^6 chicken calvaria osteoblasts were incubated in suspension with RGDS, RGES, and RFDS peptides at concentrations of 15 µg/ml, 50 µg/ml, or 100 µg/ml for 2 h with gentle shaking at 37°C. The cells which had been conjugated with peptides were plated on FN-coated (1 mg/ml) or on uncoated dishes and incubated for a further 24 h prior to the termination of the cultures. For the studies of mechanically stimulated responses the same concentrations of the RGDS peptide was used following the same protocol as described above, however mechanical stretch was initiated for a 2-h period 12 h after the cells were plated and assayed 6 h after the end of the stimulation. For all these studies no peptide was used as the controls.

Receptor clustering. Confluent osteoblast cultures were washed with DMEM and incubated with different concentrations of lysophosphatidic acid (LPA) and bombesin (BM) in fresh DMEM for 10 min at 37°C. The concentrations for BM were 10 nM and 50 nM and for LPA were 50 ng/ml and 100 ng/ml. Some cultures were also incubated with 50 µM cytochalasin D and 1 µM colchicine for 30 min prior to the termination of the cultures. Control samples did not receive either compound and were assayed at the same time and from the same preparation of cells grown under identical conditions.

Isolation and analysis of RNA. Total RNA was isolated using Tri-Reagent™ (Molecular Center, Cincinnati, OH) according to the manufacturer's instructions. RNA was resolved on 1% agarose gels containing 2.2 M formaldehyde [Toma et al., 1997] and 5 µg of total RNA was loaded per gel/lane. The cDNA encoding the complete sequence of chicken opn [Moore et al., 1990] was used to examine levels of expression of *opn* mRNA. Northern blots with P^{32} cDNA-labeled probes were carried out at 65°C in 2.5 × SSC, 50 mM Na phosphate buffer, made 100 µg/ml single stranded salmon sperm DNA, and for 18 to 24 h in a rotating hybridization oven (Robins Scientific, Sunnyvalle, CA). Autoradiograms were quantified using an LKB Ultra II scanning densitometer (LKB, Broma, Sweden) and values were normalized to 18 S ribosomal RNA obtained by hybridization of each blot to a conserved nucleotide sequence probe of 18 S ribosomal subunit (Ambion Corp., Austin, TX). All analysis were performed at least three times and all data is presented as a percentage in expression over that of the control which were determined from parallel cultures. All data were evaluated as a mean ± 2 standard deviations with a minimum of three experiments from different populations of primary cells and appropriate statistical analysis were performed.

RESULTS

The signal transduction pathways mediating *opn* expression in response to cell adhesion. Initial studies were carried out to assess whether *opn* mRNA expression would be induced by cell adhesion. As can be seen in left panel of Figure 1, *opn* mRNA expression was upregulated (two- to three-fold) in response to cell adhesion to fibronectin but *opn* mRNA levels only slightly increased after cell attachment to tissue culture plastic alone, demonstrating that attachment alone did not lead to induction of *opn* mRNA expression. In the second series of studies the temporal profile of *opn* mRNA induction in response to cell adhesion was determined. Cell adhesion lead to a maximal induction in *opn* mRNA expression 24 h after cell adhesion to fibronectin (Fig. 1, middle panel). It is also interesting to note that *opn* mRNA expression began to return to its initial levels of stimulation after 48 h and eventually returned to base line after 4 days (data not shown). A comparison of this time frame of induction to the absolute levels of *opn* expression that were seen in response to mechanical stimulation can be seen (Fig. 1 right, panel). As previously demonstrated [Toma et al., 1997] and seen in the experiments presented here *opn* mRNA induction peaked earlier (9 h vs. 24 h) and to a greater fold (four- to five-fold vs. three- to four-fold) in response to mechanical stimulation.

In order to examine the signal transduction mechanisms by which cell adhesion lead to the upregulation in *opn* RNA expression, a series of pharmacological agents capable of selectively inhibiting different second signal transduction pathways as well as disrupting specific cellular processes were used. Cycloheximide, a known inhibitor of de novo protein synthesis, blocked

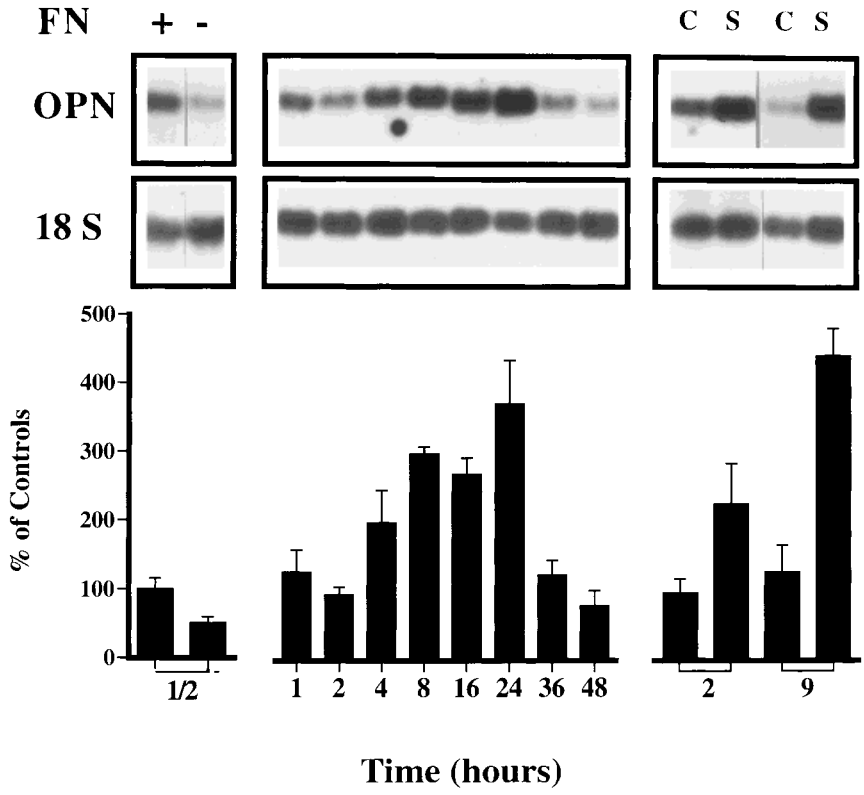

Fig. 1. Induction of osteopontin mRNA expression in response to either cellular adhesion or mechanical stimulation. **Left**: Left side of the panel depicts the Northern blot analysis of mRNA levels after plating on tissue culture dishes either fibronectin (FN) coated (+) or uncoated (-) surfaces. Osteopontin mRNA levels were examined at 30 minutes after plating. **Middle**: Middle panel depicts the time course of induction of *opn* expression after adhesion to fibronectin. Time course of osteopontin induction in response to cell adhesion. Northern blot analysis of osteopontin mRNA levels at various times after osteoblast adhesion on fibronectin. **Right**: Right side of the panel depicts the induction of *opn* mRNA after mechanical perturbation. Mechanical stimulation was carried out for a single 2-h period. Northern blot analysis of *opn* mRNA levels were then assessed at 2 and 6 h after the end of the period of mechanical perturbation. Control RNA samples are from parallel time points of cultures which were unperturbed. Graphic analysis shows the percent induction of osteopontin mRNA expression relative to the control samples. C, control; S, mechanically strained. Autoradiographs of steady state levels of *opn* mRNAs and of the 18S ribosomal RNA are separately presented. Graphic analysis shows the percent induction of *opn* mRNA expression relative to the control samples. Error bars are the S.D. of three experiments.

the induction of *opn* mRNA expression following adhesion to fibronectin. This result thereby demonstrated that the upregulation of *opn* mRNA expression was secondary to the protein synthesis of another initiating factor. Consistent with these results was the very long period of time (24 h) that was needed to achieve the maximal levels of *opn* mRNA induction by cell adhesion. Subsequently, specific inhibitors to selected second messenger systems were used. Genistein, a potent inhibitor of tyrosine kinase phosphorylation was shown to inhibit cell adhesion induced *opn* mRNA expression while it did not affect the baseline levels of *opn* mRNA expression in the absence of the stimuli (Fig. 2).

In previous studies, the integrity of the microfilament structure of the cell was shown to be necessary for the induction of osteopontin mRNA expression in response to mechanical stimulation [Toma et al., 1997]. The relationship between the biochemical processes that mediate signal transduction and the cytoskeletal structural elements of the cell were also examined in these studies. Involvement of the cytoskeleton in the transduction of the signals that mediated the induction of *opn* mRNA expression in response to adhesion was assessed by the disruption of various cytoskeletal components. Microfilaments were disrupted with cytochalasin D, whereas microtubules were disrupted with colchicine. As can be seen from these results the cytoskeleton does not appear to be important for the induction of *opn* mRNA expression in response to cell adhesion.

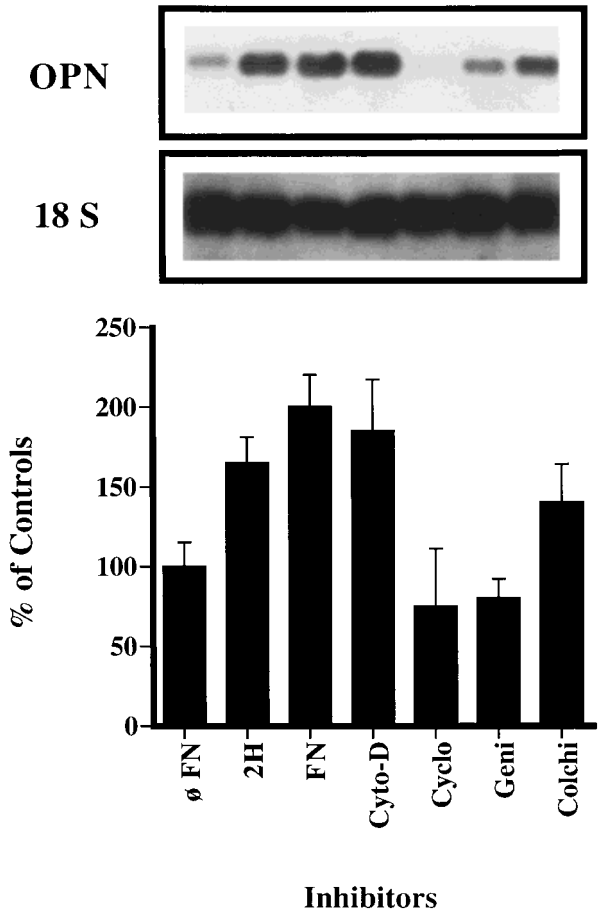

Fig. 2. Comparison of the effects of various second signal inhibitors and cytoskeletal perturbants on the induction of *opn* mRNA expression in response to cellular adhesion. Analysis of *opn* mRNAs after cell adhesion was carried out in the presence of the microfilament de-polymerization agent Cytochalasin D (Cyto-D), the protein synthesis inhibitor cycloheximide (Cyclo), the tyrosine kinase inhibitor genistein (Geni), and the microtubule depolymerization agent colchicine (Colchi). Osteopontin mRNA expression was measured at 24 h after the osteoblasts were plated on FN-coated tissue culture surfaces for the adhesion studies. Autoradiographs of steady state levels of *opn* mRNAs and of the 18S ribosomal RNA are separately presented. Graphic analysis shows the percent induction of osteopontin mRNA expression compared to controls. Error bars are the S.D. of the three experiments.

Role of integrin receptors in the induction *opn* mRNA in response to cell adhesion and mechanical stimulation. Integrins have been shown to specifically interact with the sequence RGD within the various proteins that bind to these receptors [Hynes, 1992], and this peptide has also been shown to competitively block the interaction of integrins with their various ligands [Liaw et al., 1995; Grzesik and Robey, 1994]. In order to test whether the stimulation of *opn* mRNA expression by cell adhesion to fibronectin was mediated through an integrin receptor, the RGDS peptide was used to competitively block these receptors interaction with fibronectin. Since the levels of peptide that would block interaction of the receptors with their ligands was not known three concentrations of peptide (15 µg/ml, 50 µg/ml, and 100 µg/ml) were examined. The osteoblasts were plated onto either fibronectin coated surfaces or tissue culture plastic and the levels of *opn* mRNA expression were then examined. As can be seen in Figure 3A, addition of the RGDS peptide did indeed block the induction of *opn* mRNA expression when the cells were plated on fibronectin surfaces, however a dose response to the differing concentrations of the RGDS peptide was not seen, indicating that the lowest concentrations of the peptide completely saturated the binding sites for the receptor. Interestingly, the higher concentrations of the peptide had a lesser effect than the lowest con-

centration suggesting that the interaction of the peptide with the integrin receptors was bisphasic in nature. In contrast to the ablation of the induction the *opn* mRNA expression that was seen for the addition of the peptide to the osteoblasts plated onto fibronectin coated surfaces, a strong stimulation in *opn* mRNA expression was seen when the peptide was added to the cells that were plated on uncoated tissue culture plastic surfaces. This latter result was somewhat surprising but was reproducible and quite specific for the addition of the RGDS. These results therefore suggest that the ligation of the receptor alone and not cellular adhesion per se was what mediates the signal transduction process which leads to the induction of *opn* mRNA expression. The specificity of the effects that were seen for the RGDS peptide were further validated by comparison to results obtained with the two other peptides RGES and RFDS. Both peptides are similar in their overall charge distribution however both of these peptides are believed to be nonspecific for integrin receptors. Both of the peptides at concentrations of 15 μg/ml and 50 μg/ml showed no effect in blocking the induction of *opn* mRNA expression relative to groups plated on uncoated surfaces, and neither peptide mediated the induction of *opn* mRNA levels that were seen for the cells plated on the uncoated tissue culture surfaces. It is interesting to note however that similar induction and inhibition effects were observed for *opn* mRNA expression for the RGES peptide at the highest concentrations used in these studies but not for the RFDS peptide (Fig. 3A) suggesting that the RGES peptide was indeed weakly interacting with integrin receptors.

In order to determine whether receptor occupancy by the RGDS peptide mediated its induction of *opn* expression through the activation of a tyrosine kinase, cells were plated on tissue culture plastic after interaction with varying concentrations of RGDS and RGES peptides in the presence of genistein. As can be seen in these experiments, once again the RGDS and RGES peptides stimulated the expression of *opn* mRNA with the greatest effect seen at the highest concentration of these peptides. The presence of the tyrosine inhibitor genistein did inhibit the induction of the *opn* mRNAs thereby demonstrating that comparable signal transduction mechanisms were activated by receptor occupancy as were seen for plating on fibronectin-coated surfaces (Fig. 3B).

Subsequent experiments were then directed at determining whether integrin receptors were also involved in the signal transduction process that mediated the induction of *opn* mRNA expression in response to mechanical stimulation. For these experiments only the RGDS peptide was examined (Fig. 4). These studies demonstrated that the RGDS peptide clearly was inhibitory to the mechanical stimulation of *opn* mRNA expression however at lower concentrations the effect was only partial. Such results therefore suggested that the effects of both adhesion and mechanical stimulation are separate and additive to the overall induction of the *opn* mRNA expression and titration of increasing amounts of the RGDS peptide did demonstrate complete ablation of the induction of *opn* mRNA expression.

Receptor occupancy alone is necessary for signal transduction. Cellular adhesion has been shown to bring about structural changes to a cell, including the formation of focal adhesion complexes which serve as structural locations for the interaction of cytoskeletal elements with cell surface receptors [Sastry and Horwitz, 1993]. Formation of focal adhesion complexes have also been linked with clustering of integrin receptors in the same areas and with the specific activation of tyrosine kinase second signal transducers such as focal adhesion kinase ($pp125^{FAK}$) [Schaller and Parsons, 1994]. The role of focal adhesion complex formation in the signal transduction process that induced *opn* mRNA expression in the absence of integrin receptor ligation was therefore investigated. In these studies phospholipid lysophosphatidic acid (LPA) and the neuropeptide bombesin (BM) were used to rapidly induce the formation of focal adhesions and stress fibers [Ridley and Hall, 1994]. In initial studies, several concentrations of LPA and BM were used in osteoblast cells that had been plated on both fibronectin-coated and uncoated tissue culture plastic surfaces (data not shown). For all permutations of these experiments identical results were obtained (data not shown) and only those for cells plated on uncoated surfaces, and at the highest concentrations noted in Materials and Methods are presented. Figure 5A shows that addition of LPA or BM to cells had no effect on *opn* mRNA expression. In these same experiments cells were incubated with

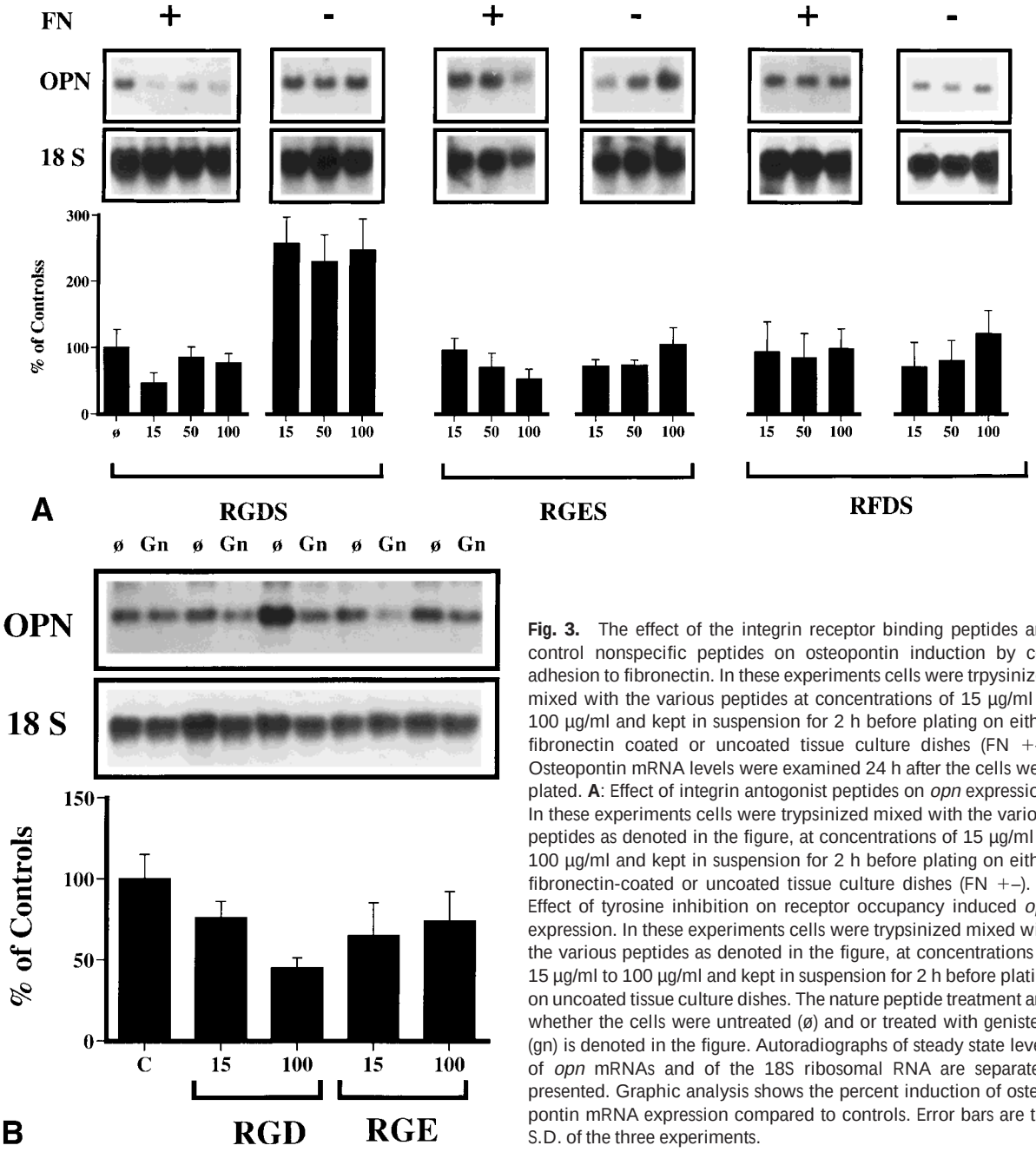

Fig. 3. The effect of the integrin receptor binding peptides and control nonspecific peptides on osteopontin induction by cell adhesion to fibronectin. In these experiments cells were trpysinized mixed with the various peptides at concentrations of 15 µg/ml to 100 µg/ml and kept in suspension for 2 h before plating on either fibronectin coated or uncoated tissue culture dishes (FN +–). Osteopontin mRNA levels were examined 24 h after the cells were plated. **A**: Effect of integrin antogonist peptides on *opn* expression. In these experiments cells were trypsinized mixed with the various peptides as denoted in the figure, at concentrations of 15 µg/ml to 100 µg/ml and kept in suspension for 2 h before plating on either fibronectin-coated or uncoated tissue culture dishes (FN +–). **B**: Effect of tyrosine inhibition on receptor occupancy induced *opn* expression. In these experiments cells were trypsinized mixed with the various peptides as denoted in the figure, at concentrations of 15 µg/ml to 100 µg/ml and kept in suspension for 2 h before plating on uncoated tissue culture dishes. The nature peptide treatment and whether the cells were untreated (ø) and or treated with genistein (gn) is denoted in the figure. Autoradiographs of steady state levels of *opn* mRNAs and of the 18S ribosomal RNA are separately presented. Graphic analysis shows the percent induction of osteopontin mRNA expression compared to controls. Error bars are the S.D. of the three experiments.

either colchicine or cytochalsin-D to further examine if in the absence of an intact cytoskeleton there would be a reduction in *opn* mRNA expression in the presence of these pharmacological agents. Incubation of either cytoskeletal depolymerization compound produced no additional effects, thus even though, the cytoskeletal elements and focal adhesion complex formation have proven to be essential in the processes that mediate the tyrosine phosphorylation of specific proteins in focal adhesion complexes, the formation of focal adhesion complexes by themselves in the absence of receptor occupancy is incapable of mediating the signal transduction process which activated *opn* mRNA expression.

In order to further discriminate receptor occupancy by itself from the structural effects of cell attachment, the osteoblasts were trypsinized and mixed with RGDS peptide, or RGDS peptide and bombesin, and maintained in suspension by plating on bacteriological petri dishes

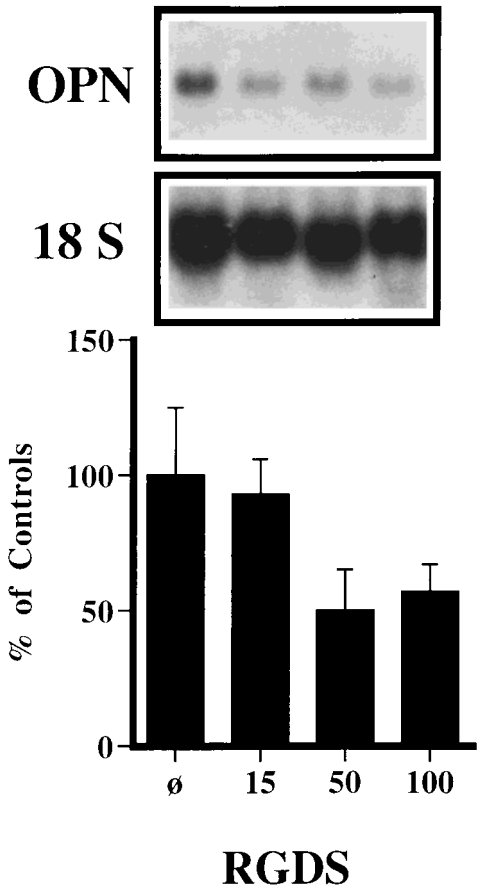

Fig. 4. The effect of the integrin receptor binding peptides on the induction of osteopontin expression in response to mechanical stimulation. In these experiments cells were trpysinized mixed with the RGDS at concentrations of 15 µg/ml to 100 µg/ml and kept in suspension for 2 h before plating on fibronectin-coated flexible membranes. Cells were allowed to attach for 24 h after which mechanical strain was applied for 2 hours and *opn* mRNA levels were examined at 6 hours after the strain had been applied. Autoradiographs of steady state levels of *opn* mRNAs and of the 18S ribosomal RNA are separately presented. Graphic analysis shows the percent induction of osteopontin mRNA expression compared to controls. Error bars are the S.D. of the three experiments.

(Fig. 5B). As can be seen in these experiments even in the absence of cellular attachment and spreading, the binding of the RGDS ligand by itself leads to a very strong induction in the *opn* mRNA expression. In these experiments, however there was clearly an additive effect if bombesin was present suggesting that some baseline level of receptor clustering further facilitates the signal transduction process which mediates the induction of *opn* expression. The additive effect that was observed for the RGDS and BM treated cells when they were placed in suspension is also consistent with the higher levels of *opn* mRNA levels that were seen for attached cells on uncoated culture surfaces.

DISCUSSION

Interactions with the extracellular matrix provides cells with both positional and regulatory signals that are important to the maintenance of a cell's phenotype. Such interactions are unique to a given tissue structure and enable cells of a tissue to respond to their external environment including mechanical stimulation. While it has hypothesized that the underlying signal transduction processes that mediate specific cellular responses to adhesion with the ECM or to mechanical stimulation may be commonly mediated in part through the cells interaction with the extracellular matrix [Ingber, 1994], there must be specific mechanisms that allows a cell to discriminate between these different types of external stimuli. Within the present studies the signal transduction processes and the role of integrin receptors that mediated *opn* expression in response to cell adhesion were examined and were compared to those seen for mechanical strain (Table 1). While previous studies have shown that focal adhesion kinase is activated in response to mechanical stimulation in osteoblasts [Toma et al., 1997], and numerous investigators have proposed that integrins are involved in the processes of mechano-transduction, the role of integrin receptors in the signal transduction of mechanical stimuli which leads to a specific genomic response has not been demonstrated. The present results therefore provide the clearest evidence to date of the direct linkage between integrin occupancy and the activation of a tyrosine kinase(s) to the generation of a specific genomic response for either cell adhesion or mechanical stimulation.

Studies examining the time frames of *opn* mRNA induction demonstrated that the peak response to cell adhesion was much slower than that seen in response to mechanical stimulation. Consistent with these different temporal patterns of induction of *opn* expression was the observation that cycloheximide inhibited cell adhesion induced expression of *opn* mRNA while having no effect on mechanically stimulated expression. These data demonstrate that mechano-transduction occurred through a direct activation of pre-existing transcription factors but *opn* induction by cell adhesion was a secondary event dependent on new protein syn-

thesis. The mechanisms of signal transduction which mediated *opn* gene expression and its relationship to the cytoskeleton in response to cell adhesion were also different. Osteopontin expression in response to cell adhesion did not change in cytochalasin-D treated cells. In contrast the induction of *opn* expression in mechanically stimulated cells was completely ablated by cytochalasin D, indicating that the signal transduction processes which mediated *opn* expression in response mechanical strain were dependent on microfilament integrity. Both the dependency of mechano-signal transduction on the cytoskeleton and the lack of the need for new protein synthesis to induce *opn* expression in response to mechanical stimulation is consistent with the concept that mechano-signal transduction is immediate in nature and may be uniquely transduced through the cells cyto-architecture [Ingber, 1991, 1994]. Finally, it is of interest to note that tyrosine kinase(s) were involved in the signal transduction processes which mediated the induction of

TABLE I. Comparison of Signal Transduction Pathways for Osteopontin mRNA Induction of Cell Adhesion vs. Mechanical Stimuli

	Adhesion	Mechanical stimulation[a]
Time after stimulation to peak expression	24 hours	9 hours
Dependence on de novo protein synthesis	yes	no
Tyrosine kinase mediated	yes	yes
PKA kinase mediated[b]	no	yes
Requires microfilament structures	no	yes
Dependence on an integrin receptor	yes	yes

[a]Data concerning mechanical stimulation is from Toma et al. 1997.
[b]Data for the role of PKA mediation in cell adhesion effects on *opn* mRNAs are not shown but were derived by analysis of the effects of H89 on *opn* expression after cell adhesion.

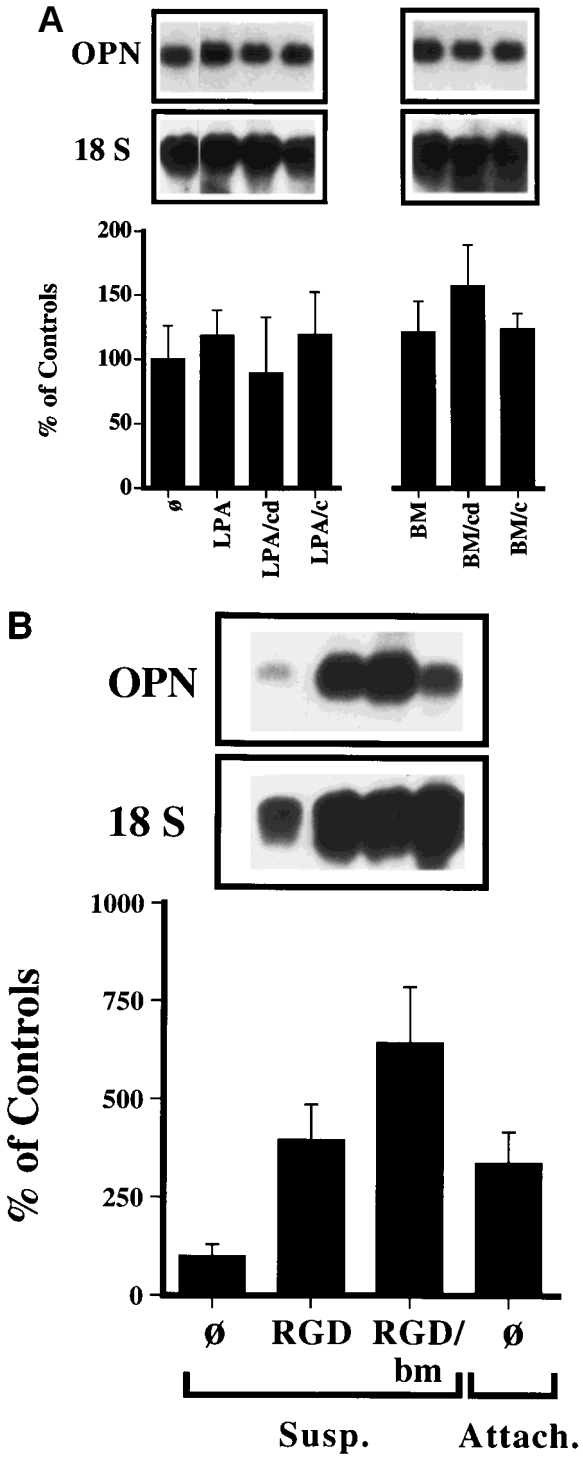

Fig. 5. Role of receptor clustering in mediating the increased expression of osteopontin in response to cell adhesion. **A**: Role of receptor clustering in attached cells. Receptor clustering was initiated pharmacologically through the use of Bombesin (BM) or Lysophosphatidic acid (LPA) in cells attached to uncoated tissue culture plastic. The effect of specific cytoskeletal elements in facilitating osteopontin induction in response to receptor clustering was examined through the use of the microfilament disrupting agent cytochalain-D (cd) or the microtubule disrupting agent colchicine (c). **B**: Role of receptor clustering and receptor occupancy in suspended cells. Control cells in suspension (Susp.); Cells were mixed with RGDS (RGD), or RGDS and bombesin(RGD/bm), and compared to cells attached to uncoated culture surfaces (Attach.). Autoradiographs of steady state levels of *opn* mRNAs and of the 18S ribosomal RNA are separately presented. Graphic analysis shows the percent induction of osteopontin mRNA expression compared to controls. Error bars are the S.D. of the three experiments.

opn expression for both cell adhesion and mechanical stimulation.

Thus the present results demonstrate that while cell adhesion to fibronectin and mechanical stimulation both induce *opn* mRNA expression through the interaction of an integrin receptor and the activation of a tyrosine kinase, aspects of the two mechanisms of signal transduction are clearly different. A number of explanations may be offered as to how a single gene may be similarly regulated by these two different stimuli. The simplest explanation may be that the occupancy or activation of different integrin receptors may correspond to different signaling mechanisms which lead to an identical genomic response. In this context, the interaction of integrins with specific matrix proteins have been shown to be involved in the detection of mechanical strain in comparison to those interactions involved in adherence and spreading [Wilson et al., 1996]. Osteoblasts have also been shown to express and use multiple receptors for cell attachment to extracellular matrix glycoproteins [Grzesik and Robey, 1994], while other studies have shown that different integrin receptors expressed by osteoblasts are used to differentially promote cellular adherence to different material substrates [Schneider and Burridge, 1994]. Alternatively it is possible that mechanical stimuli through structural changes produced in cells, alters the interactions or conformation of a given integrin receptor thereby leading to its activation of different signal transduction pathways, than would be activated by cell adhesion or receptor occupancy. Finally it may be necessary that the integrin receptors is engaged with an extracellular ligand in order to mediate mechano-signal transduction. Such a result is clearly consistent with the unique dependency of mechano-transduction on the maintenance of an intact cytoskeleton. For these latter two possibilities either identical or different receptors might be used to convey the stimuli.

The induction of *opn* mRNA expression by cell adhesion to fibronectin but not to tissue culture plastic alone, implies that the while integrin receptors are structurally involved in mediating cell attachment, this process alone and the changes in cellular structure that come about due to cell attachment are discrete events different from those that generate the specific signal transduction processes mediated through integrin receptor occupancy. Similar conclusions were first put forth by other investigators that have examined activation of specific signal transducers such as $pp125^{FAK}$ in response to integrin receptor occupancy, in the absence of the structural events of cellular adhesion that are mediated by the receptor [Miyamoto et al., 1995]. It has also been suggested that fibronectin may mediate cell adhesion not through a direct structural interaction with its receptor but through activation of specific signals secondary to receptor ligation which then promote cellular adherence [Curtis et al., 1992]. This is an intriguing suggestion, which supports the idea that adhesion and integrin ligand occupancy (RGD-dependent) are unique, separate, and self-sufficient mechanisms for activating different but possibly interrelated signal transduction pathway.

Several aspects of the results reported here are consistent with the suggestion that receptor occupancy alone is sufficient to activate signal transduction processes which mediate cellular processes. The first is the demonstration that *opn* expression was upregulated in response to integrin ligation with the RGDS peptide in the absence of plating the cells on fibronectin. In this case where cells adhere to plastic, they are presumably using other mechanisms or non-integrin receptors to mediate cell attachment. The second set of results that suggests that receptor occupancy is crucial to the activation of the signal transduction processes that lead to specific cellular responses, was the demonstration that the RGES and RFDS peptides showed very specific behavior relative to the induction of *opn* expression. The RFDS peptide at the three concentrations that were tested had almost no biological activity either in blocking *opn* induction when the cells were plated on fibronectin or for the induction of *opn* expression when the cells were plated on tissue culture plastic. In contrast RGES showed an identical behavior as RGDS but at higher concentrations, indicating that it does have specific but much weaker binding to integrin(s) receptors similar in nature to behavior to that of a weak agonist. Overall while these finding appear to be contrary to current data pertaining to how integrins interact with their ligands most of assays reported in the literature are predicated on studies of cellular responses to adherence of cells to ligands coated unto surfaces. Such studies by their nature do not separate cell responses that occur in response to cell

adhesion vs. receptor occupancy. Similarly cell adhesion has been equated to ligand occupancy with extrapolation of dose response of the antagonist peptides based on assaying adhesion. Such a measure of biological response may indeed be separate from a measure of the responses mediated by second signals that are generated solely by ligand occupancy or the combination of signals generated through adhesion and receptor occupancy. Thirdly, while many studies have shown that adhesion vs. receptor occupancy leads to the activation of the same set of second signals, few studies have examined genomic responses to these second signals. There has been a failure to identify divergencies in cellular responses that may be separately mediated through shape changes or cell spreading vs. those that are in response to the ligation of the integrin receptors alone. In the present studies marked changes in attachment inhibition following the addition of the peptides to the cultures were not detected when the cells were plated onto tissue culture plastic, clearly indicating that in the absence of a specific or preferred ligand such as fibronectin that cells use surface bindings sites that facilitate attachment independent of integrin receptor sites.

Numerous reports have demonstrated that integrin-ligation and cell adhesion stimulates the formation of membrane-associated focal adhesion complexes [Mueller et al., 1989]. Previously, Miyamoto et al. [1995] have also shown that integrin receptors may induce cellular responses to binding of a ligand or to clustering or to a combination of both effects. These data also demonstrated that while clustering alone was not sufficient to control the distribution of specific cytoskeletal proteins it was sufficient to cause integrin signaling as assessed by the activation of $pp125^{FAK}$ [Miyamoto et al., 1995]. In the studies reported here the use of LPA and bombesin, was used to induce integrin clustering alone, however in the absence of receptor occupancy no induction of *opn* expression was observed. While in previous studies $pp125^{FAK}$ has been shown to be activated by receptor clustering induced by either LPA or bombesin and by mechanical perturbation the present data suggests that $pp125^{FAK}$ is not causally associated with the signal transduction processes that mediate the induction of *opn* expression. It is possible though that there are separate events which are dependent on both receptor occupancy and attachment and these effects synergize with each other to mediate signal transduction through activation of $pp125^{FAK}$. Such an interpretation is consistent with the demonstration that there is some basal level of *opn* expression that the cells maintain when they are attached to a substrate as seen in Fig. 5. This interpretation is also consistent with the hypothesis that there are two forms of receptor configuration: one which would occur when the receptors become clustered during attachment; and a second which would exist when the receptors are both clustered and become engaged or tethered to the ECM.

Finally it is important to consider the possible biological implication of these results. The induction of the *opn* expression which itself is an integrin ligand, through integrin occupancy leads to the interesting speculation that the molecules containing integrin binding domains such as RGDS may be autoregulatory in both the maintenance of their own expression as well as providing a feedback mechanism to selectively regulate the expression of other integrin ligands by specific receptor interaction. In this context it is interesting to note that studies of other biomechanically induced genes and the simulation of their expression by RGDS in osteoblasts demonstrates both fibronectin and bone sialoprotein are uniquely and separately regulated by these stimuli. On the other hand, neither osteocalcin an osteoblast gene product that is not a ligand for integrin receptors or collagen is regulated [Carvalho, Schaffer, and Gerstenfeld, unpublished data] by these types of stimuli.

In summary, our results suggest the following: 1) *opn* expression is dependent on integrin-ligation, 2) *opn* expression is enhanced by receptor occupancy, and 3) receptor clustering alone is not sufficient to regulate *opn* expression. In sum, the results from this study demonstrate that integrin occupancy alone is sufficient to enhance *opn* expression from osteoblasts in culture and that this phenomenon may be a determinant of further cellular regulatory mechanisms. We conclude that although both adhesion and mechanical stimuli appear to take place through integrins, cell-matrix interactions involved in signaling mechanisms of mechanical stimulation differ from those of adhesion alone.

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