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Osteopontin Stimulates Gelsolin-associated Phosphoinositide Levels and Phosphatidylinositol Triphosphate-Hydroxyl Kinase

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Based on previous studies demonstrating activation of phosphatidylinositol 3-hydroxyl kinase (PI3-kinase) and stimulation of a change in cell shape, we examined the effect of osteopontin on the association of phospholipids with gelsolin, an actin-capping/severing protein. Osteopontin stimulated a rapid increase in phosphatidylinositol bisphosphate and phosphatidylinositol triphosphate levels associated with gelsolin in Triton-soluble fractions of cell lysates. The increased levels of phosphatidylinositol triphosphate associated with gelsolin were due to stimulation of PI3-kinase activity associated with gelsolin in the Triton-soluble fractions, and they were blocked by the PI3-kinase inhibitor wortmannin. Osteopontin stimulated translocation of PI3-kinase from the Triton-insoluble to Triton-soluble gelsolin. Osteopontin also decreased Triton-soluble gelsolin/actin complexes consistent with actin uncapping, and increased F-actin levels, which were also blocked by wortmannin. The osteopontin effects were mediated through binding to the $\alpha_v\beta_3$ integrin. Taken together, our studies indicate that osteopontin/$\alpha_v\beta_3$-mediated changes in gelsolin-associated phosphoinositide levels and PI3-kinase activity are related to stimulation of F-actin formation in osteoclasts.

INTRODUCTION

Osteoclasts are multinucleated giant cells responsible for bone resorption. As they attach to bone, osteoclasts become highly polarized, and distinct cellular domains are developed. A specialized area called the clear zone (Holtrop and King, 1977) is responsible for contact with bone by specific adhesion structures called podosomes (Marchisio *et al.*, 1984, 1987; Zambonin-Zallone *et al.*, 1988). Podosomes contain a bone matrix receptor that is linked to actin filaments through proteins such as vinculin, talin, and α actinin. The bone matrix receptor, integrin $\alpha_v\beta_3$, recognizes at least one of the bone matrix proteins–osteopontin–in osteoclasts (Reinholt *et al.*, 1990). Osteopontin is highly enriched at regions on bone surfaces where osteoclasts are anchored (Reinholt *et al.*, 1990). In addition, osteoclasts produce osteopontin in large quantities (Ikeda *et al.*, 1992). When peptides containing the Arg-Gly-Asp (RGD) cell adhesion sequence are added to osteoclasts as soluble proteins, the cells retract and detach (Oldberg *et al.*, 1986; Horton *et al.*, 1991; Moore *et al.*, 1991). When osteopontin is added as a soluble protein, a retraction occurs, but the cells do not detach. Rather, the cell retraction is followed by membrane ruffling and filopodia extension relating to osteoclast motility and crawling over bone surfaces. The difference between RGD peptides and osteopontin may be that osteopontin is a ligand for more than the integrin $\alpha_v\beta_3$. It also binds to CD44, another extracellular matrix receptor (Weber *et al.*, 1996). The biological significance of soluble osteopontin stimulating membrane ruffling and filopodia is unknown, but one possibility is that osteopontin is an osteoclast autocrine factor related to cell motility.

An important issue becomes the mechanism by which osteopontin stimulates changes in cell shape and the associated reorganization of the osteoclast cytoskeleton. The rounding up of the cells stimulated by osteopontin and RGD peptides is associated with an increase in the number of actin fibers visibly span-

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ning the cell diameter. The studies in this report examine whether there is an increase in F-actin production and potential mechanisms whereby actin filament formation might be stimulated. The results demonstrate dissociation of gelsolin-actin complexes that might represent "uncapping" of barbed end actin by an increase in gelsolin-associated polyphosphoinositides. A unique mechanism of polyphosphoinositide/gelsolin association is described related to osteopontin-stimulated synthesis of phosphoinositides phosphorylated in the 3-hydroxyl position. The latter effect of osteopontin is produced through stimulation of phosphatidylinositol 3-hydroxyl kinase (PI3-kinase)[1] associated with gelsolin.

MATERIALS AND METHODS

Most of the chemicals and antibodies directed toward gelsolin and α-actinin, and the phospholipid standards were purchased from Sigma Chemical (St. Louis, MO). Rainbow molecular weight protein marker and [^{32}P]orthophosphate were obtained from Amersham (Arlington Heights, IL). The protein assay reagent kit was obtained from Bio-Rad (Richmond, CA). Phosphatidylinositol (4,5)-bisphosphate (PtdIns P_2) antiserum was obtained from Advanced Magnetics (Cambridge, MA). pEZZ expression vector for osteopontin expression and IgG Sepharose were obtained from Pharmacia (Piscataway, NJ). LM609 antibody was a kind gift of Dr. David Cheresh (Scripps Clinic and Research Foundation, La Jolla, CA). Chicken osteopontin cDNA (used for expression) and osteopontin antibody (used to characterize the expressed protein) were kind gifts from Dr. Lou Gerstenfeld (Harvard Medical School, Boston, MA). The anti-profilin antibody was a kind gift from Dr. T. Pollard (Johns Hopkins University, Baltimore, MD).

Osteopontin Expression

An avian osteopontin cDNA was cloned into a protein A-containing expression vector, pEZZ. A collagenase cleavage site was introduced between the protein A and osteopontin sequences for the purpose of isolation. The expressed protein was purified from media as well as from cell pellets using IgG Sepharose chromatography. Recombinant chicken osteopontin, purified from bacteria, was used for all experiments. Adherent avian osteoclast precursor cells in serum-free media were treated with osteopontin for different time periods.

Preparation of Osteoclast Precursors

Avian osteoclast precursors were prepared as described previously (Alvarez *et al.*, 1991; Medhora *et al.*, 1993). Briefly, osteoclast precursors were isolated from bone marrow of egg-laying hens, maintained on Ca^{2+}-deficient diets. Partially purified preparations of mononuclear cells were recovered from the interface of Ficoll/Hypaque gradients and then cultured in the presence of cytosine arabinoside (5 μg/ml) to prevent proliferation of contaminating cells. After 3 days in culture, nonadherent cells were removed. Multinucleated osteoclast precursor cells formed between 3 and 6 days in culture. The multinucleated cell preparations were uniformly TRAP positive, and resorbed bone by forming resorption pits on bone slices.

[1] Abbreviations used: LM609, antibody to integrin $\alpha_v\beta_3$; OP, osteopontin; PBS, phosphate-buffered saline; PI3-kinase, phosphatidylinositol 3-hydroxyl kinase; PMSF, phenylmethylsulfonyl fluoride; PtdIns P_2, phosphatidylinositol bisphosphate; PtdIns P_3, phosphatidylinositol triphosphate.

Preparation of Lysates, Immunoprecipitation, and Western Analysis

After 4 days in culture, the osteoclast precursors were put in serum-free media for 2 h. Subsequently, each plate was treated with one of the following (osteopontin, collagen, protein A, GRGESP, or GRGDSP; 100 μg/ml) in a concentration of 25 μg/ml of media unless otherwise mentioned for 15 min at 37°C. Some cultures were pretreated either with the antibody LM609 or with wortmannin (Yano *et al.*, 1993), a specific inhibitor of PI3-kinase, before stimulation with or without osteopontin (25 μg/ml) for 15 min at 37°C. Pretreatment conditions were either LM609 (100 μg/ml) for 30 min at 37°C, or wortmannin (0.1 μM) for 10 min at 37°C. Following treatment, cells were washed three times with ice-cold phosphate-buffered saline (PBS; 136 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and added with Triton-containing lysis buffer (10 mM Tris-HCl, 50 mM NaCl, 0.5% Triton X-100, 30 mM $Na_4P_2O_7$, 50 mM NaF, 100 μM Na_3VO_4, 5 mM $ZnCl_2$, and 2 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.05). Cells were rocked on ice for 15 min and scraped off with cell scraper. Cell lysates were transferred to Eppendorf tubes. The lysates were centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was saved as a Triton-soluble fraction. The pellet was resuspended in RIPA buffer (10 mM Tris-HCL, pH 7.2, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1% aprotinin,and 2 mM PMSF), solubilized by pipetting up and down a few times and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant is the Triton-insoluble fraction. Protein contents were measured using the Bio-Rad protein assay reagent. Equal amounts of lysate proteins were precleared with protein A-Sepharose, presoaked in lysis buffer containing bovine serum albumin, and with nonimmune IgG coupled to Sepharose. The precleared supernatants were incubated with anti-gelsolin or anti-PI3-kinase antibodies and the immune complexes were adsorbed onto protein-A Sepharose beads. The beads were pelleted and washed twice with Triton X-100 containing lysis buffer, twice with PBS containing 0.5% Tween (PBS-T), 1 mg/ml ovalbumin, and 2 mM PMSF, and then finally twice with PBS-T with PMSF. The immune complexes were then eluted in electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol) and analyzed on 9% polyacrylamide gels with SDS (Lammelli, 1970). For immunoblot analysis, proteins were electrophoretically transferred to a PVDF membrane. Blots were blocked with 5% milk in PBS-T and then incubated with primary antibody for 2 h. The blots were washed three times for 10 min each with 100 ml of PBS-T and then incubated with a 1:1000 dilution of peroxidase-conjugated secondary antibody for 2 h at room temperature. After three washes for 10 min each with PBS-T, protein bands were visualized by chemiluminescence using the ECL kit.

Measurement of F-actin Content Using Rhodamine-Phalloidin Binding

Osteoclast precursor cells were cultured in 24-well culture plates for 4 days. The cells were treated with osteopontin for various time periods or with various treatments as described above. The cells were fixed with 1.5% formaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were rinsed and incubated with rhodamine-phalloidin in PBS for 30 min. The cells were washed quickly several times with PBS and extracted with absolute methanol. The fluorescence of each sample was measured using fluorimetry (Gilford FluoroIV). In control experiments, a 10-fold excess of unlabeled phalloidin was used to determine the nonspecific binding. The nonspecific binding was subtracted from the total binding to get the specific binding (Cooper, 1992).

Immunoprecipitation of Gelsolin-Actin Complexes

To immunoprecipitate gelsolin-actin complexes, avian osteoclast precursors cells were labeled with [^{35}S]methionine for 4 h in serum-free and methionine-free media. Triton-soluble and Triton-insoluble lysates were made after treatment with or without osteopontin essentially as described above. Equal amounts of lysate proteins were used for immunoprecipitation with anti-gelsolin antibody. EGTA-resistant gelsolin-actin complex (Chaponnier *et al.*, 1987; Howard *et al.*, 1990) was prepared from the immune complexes adsorbed to protein A-Sepharose. The adsorbed proteins were washed with different buffers as mentioned below. Initially, the beads were washed twice with lysis buffer containing 5 mM ATP; then three times with buffer containing 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM EGTA, and 0.3 M $MgCl_2$; and then finally, twice in 10 mM Tris-HCl, pH 7.4, and 100 mM NaCl. The Sepharose beads were boiled in electrophoresis sample buffer and analyzed on 5–15% SDS-PAGE and the radioactive bands were visualized by autoradiography.

^{32}P Labeling of Cells and TLC Analysis of Lipids

Avian osteoclast precursors cells, after 4 days in culture, were kept in serum-free (PO_4^-) media for 2 h. The cells were labeled as described (Meenakshi *et al.*, 1993) with carrier-free [^{32}P]orthophosphate for 2 h at 37°C. After labeling, the cells were washed twice with serum-free media and incubated with or without osteopontin for 15 min at 37°C. Either LM609 or wortmannin pretreatments were completed before osteopontin treatment, as described above. Lipids were extracted from gelsolin immunoprecipitates as described (Whitman *et al.*, 1985) and dried under N_2. The dried lipids were reconstituted in 100 μl of chloroform:methanol (1:1) and spotted on silica gel TLC plates pretreated with 1.2% potassium oxalate in methanol and water (2:3). The plates were developed in chloroform: methanol:acetic acid:acetone:water (40:15:13:12:7) and dried. Bands were visualized by autoradiography and quantitated by counting of scraped material in a scintillation counter. Phospholipid standards were visualized by exposure to iodine vapors.

PI3-Kinase Assay

Lysate preparations and immunoprecipitations were done essentially as described above with minor modifications. Lysates were immunoprecipitated either with anti-gelsolin or anti-phosphotyrosine antibody (PY20). The immune complexes, adsorbed to protein A-Sepharose pellets, were washed successively as follows: once with Triton-lysis buffer; twice with 0.5 M LiCl, 0.1 M Tris-HCl, pH 8.0; once with 0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH, 7.6; and finally once with kinase assay buffer (1 mM dithiothreitol, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]/NaOH, pH 7.4, at 25°C, 5 mM $MgCl_2$). The pellets were then assayed for inositol lipid kinase activity. Twenty microliters of freshly prepared lipid mix (Jackson *et al.*, 1992) was added to the beads; the mix was vortexed gently and then placed at 37°C for 5 min. Ten microliters of [γ-^{32}P]ATP (5 μCi/assay), 5 μM Na_2 ATP, 1 mM dithiothreitol, 20 mM HEPES/NaOH, pH 7.4, 5 mM $MgCl_2$ was then added. The mixture was gently vortexed and the incubation continued at 37°C for a further 15 min. Incubations were terminated by the addition of 0.425 ml of chloroform/methanol/water (5:10:2, v/v). Lipids were then extracted as described (Whitman *et al.*, 1985) and dried under N_2. The dried lipids were reconstituted in 100 μl of chloroform: methanol (1:1) and analyzed by TLC as described above.

Statistical Analysis

All comparisons were made to "Control," which refers to (−) osteopontin (i.e., vehicle-treated cultures). The raw fluorescence data for F-actin were computed to obtain means (M) and standard error of the means (SEM) for the various experimental groups. Statistical comparisons between the treatment groups were performed using analysis of variance (ANOVA) with the Bonferroni correction for multiple comparisons.

RESULTS

Osteopontin Stimulation of Gelsolin-associated Phosphatidylinositol Bisphosphate

We have previously demonstrated that osteopontin increases polyphosphoinositide synthesis in osteoclast precursors (Hruska *et al.*, 1995). Polyphosphoinositides have been reported to participate in the function of several classes of actin binding proteins (Lassing and Lindberg, 1985, 1988; Stossel, 1993, for review), and the actin cytoskeleton could participate in the change in cell shape stimulated when osteopontin is added as a soluble protein to osteoclast cultures. We analyzed osteopontin stimulation of polyphosphoinositide association with some of the candidate proteins involved in the regulation of the actin cytoskeleton such as α-actinin, gelsolin, and profilin. Immunoprecipitates were formed with antibodies to the respective candidate proteins and immunoblotted with anti-PtdIns P_2 antibody. Our results demonstrated that osteopontin stimulation of osteoclasts had no effect on PtdIns P_2 associated with α-actinin or with profilin. In contrast, osteopontin stimulated PtdIns P_2 association with Triton-soluble gelsolin (Figure 1, panel A, lane 2) as compared with untreated cells (Figure 1, panel A, lane 1). The level of PtdIns P_2 associated with Triton-insoluble gelsolin was unchanged by osteopontin treatment (Figure 1, panel A, lane 4). The levels and distribution of gelsolin in the Triton-soluble or -insoluble fractions were not affected by osteopontin as shown by the anti-gelsolin immunoblot (Figure 1, panel B, lanes 1–4).

Because osteopontin had stimulatory effects on PtdIns P_2 association with Triton-soluble gelsolin, the time course and specificity of osteopontin stimulation were analyzed in that fraction. Figure 2 shows the time-dependent effect of osteopontin on PtdIns P_2 association with Triton-soluble gelsolin. Association of PtdIns P_2 with gelsolin was maximal by 15 min of treatment and the effect persisted for 30 min before declining somewhat by 60 min (Figure 2, panel A, lanes 2–6). Return to basal levels was observed by 120 min (Figure 2, panel B). Immunoprecipitations performed with antibodies to the regulatory component of PI3-kinase, p85, with molecular size similar to that of gelsolin, were analyzed for PtdIns P_2 association, and our results demonstrated that PtdIns P_2 was not associated with p85 (Figure 2, panel A, lanes 7 and 8). Furthermore, osteopontin stimulation of PtdIns P_2 association with gelsolin was limited to the Triton-soluble component consistent with the previous figure (Figure 2, panel A, lanes 9 and 10).

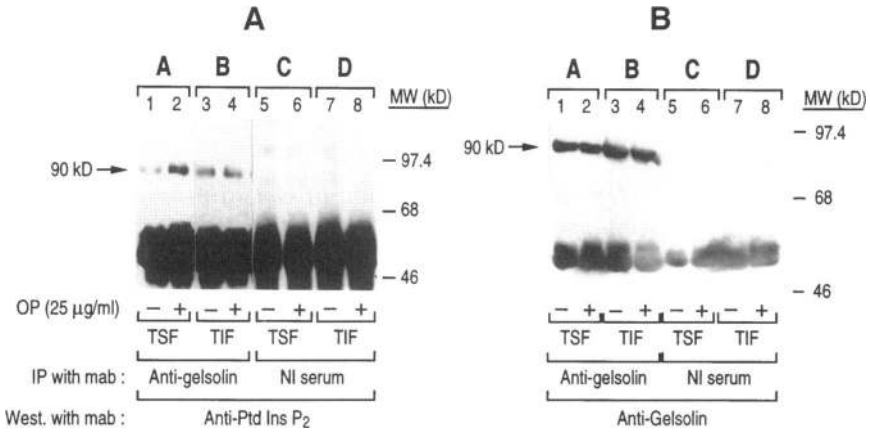


Figure 1. Effect of osteopontin treatment on gelsolin-associated PtdIns P_2. Gelsolin was immunoprecipitated from osteoclasts precursor lysates with either an anti-gelsolin monoclonal antibody (A and B) or a nonimmune serum (C and D). The immunoprecipitates were separated by SDS-PAGE, and immunoblotted using an anti-PtdIns P_2 antibody (panel A) or an anti-gelsolin antibody (panel B). The immunoprecipitates in lanes designated with (−) were obtained from control cells, while the lanes designated by (+) were obtained from osteopontin-treated cells. Triton-soluble fractions of cell lysates (TSF) (A and C) and Triton-insoluble fractions (TIF) (B and D) were immunoprecipitated and run next to each other in SDS gels.

To examine the specificity of the osteopontin stimulation of PtdIns P_2 association with gelsolin, osteoclast precursors were treated as shown below the immunoblots in Figure 3. In anti-gelsolin immunoprecipitates, we observed an increase in PtdIns P_2 associated with gelsolin in the Triton-soluble fraction of cells treated with osteopontin (Figure 3, panel A, lane 4 and panel B). Osteopontin has a GRGDS cell binding sequence that is recognized by the integrin receptor $\alpha_v\beta_3$, and GRGDSP (100 μg/ml) treatment produced an effect (Figure 3, panel A, lane 3) equivalent to osteopontin (25 μg/ml). GRGESP, with a mutated RGD, did not have any effect (Figure 3, panel A, lane 6 and Figure 3, panel B) compared with vehicle-treated cells (Figure 3, panel A, control, lane 7). To examine the role of $\alpha_v\beta_3$ in osteopontin-stimulated association of PtdIns P_2 with gelsolin, cells were treated with monoclonal antibody LM609 (anti-$\alpha_v\beta_3$,) for 30 min before osteopontin treatment. LM609 treatment alone (Figure 3, panel A, lane 5) or osteopontin stimulation of the LM609-treated cells (Figure 3, panel A, lane 2) did not increase the levels of PtdIns P_2 associated with gelsolin. The ability of LM609 to block the osteopontin effect suggests that this process is $\alpha_v\beta_3$ mediated. Immunoprecipitates formed by nonimmune serum from Triton-soluble fractions failed to demonstrate immunoprecipitation of proteins associated with PtdIns P_2 (Figure 3, panel A, lane 1).

Analysis of Lipids Associated with Gelsolin

To analyze the phospholipids associated with gelsolin, anti-gelsolin immunoprecipitates were made from cells exposed to ^{32}Pi. Lipids were extracted from the immunoprecipitates and separated by TLC. Autoradiograms of the ^{32}Pi-labeled phospholipids were developed as shown in Figure 4, A and B. Phospholipid spots were scraped from the TLC plates and radioactivity was determined by liquid scintillation counting. Data from six experiments are shown in Figure 4C. Osteopontin treatment stimulated the association of PtdIns P_2, and phosphatidylinositol (3,4,5) trisphosphate (PtdIns P_3) (Figure 4A, lane 3, and Figure 4B, lane 4) with gelsolin in the Triton-soluble fractions. The effect of osteopontin was inhibited by the anti-$\alpha_v\beta_3$ antibody LM609, in agreement with the studies shown in Figure 3. The finding of PtdIns P_3 associated with gelsolin suggested that osteopontin stimulation of PI3-kinase contributed to the increased levels of phosphoinositides associated with gelsolin.

Effect of Wortmannin on Phosphoinositide Associated with Gelsolin

Figure 4, B and C, demonstrate the effect of wortmannin, an inhibitor of PI3-kinase (Yano *et al.*, 1993), on the osteopontin-stimulated phosphorylation of phospholipids associated with gelsolin. Wortmannin pre-treatment of cells for 10 min before osteopontin resulted in a block of the osteopontin stimulation of PtdIns P_3 association with gelsolin (Figure 4B, lane 3). Wortmannin treatment appeared to diminish but not block the osteopontin stimulation of PtdIns P_2 association with gelsolin in the experiment portrayed in Figure 4B, but this observation was not consistent as shown by the group data in Figure 4C.

Association of PI3-Kinase with Gelsolin

To further examine the mechanism of osteopontin stimulation of PtdIns P_3 association with gelsolin, we analyzed whether PI3-kinase was associated with gelsolin. Lysates of osteoclast precursors were subjected to immunoprecipitation using antibodies to gelsolin. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with either anti-PtdIns P_2 antibody or anti-phosphotyrosine antibody (PY20). Consistent with our demonstrations as shown in Figure 1, panel A, lanes 1 and 2, osteopontin stimulated PtdIns P_2 association with gelsolin (Figure 5A, lane 2).

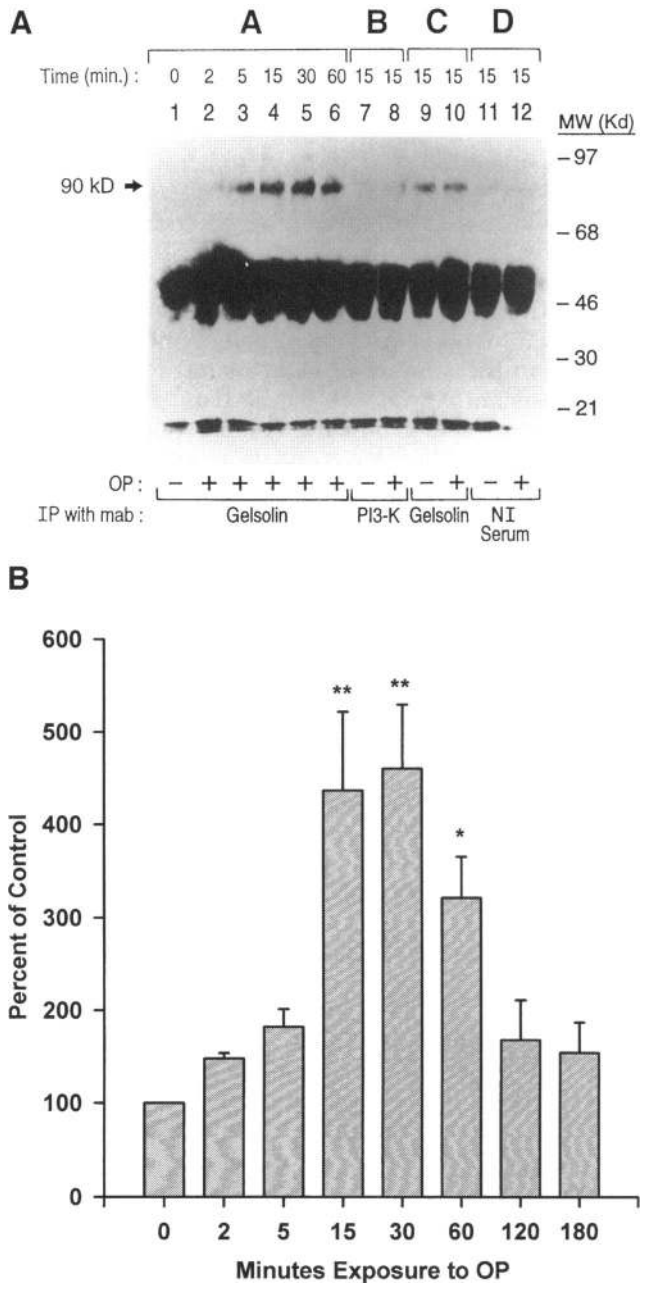


Figure 2. The time course of the osteopontin effect on gelsolin-associated PtdIns P_2. (Panel A) Triton-soluble (A, B, and D) and Triton-insoluble (C) fractions of osteoclast precursor lysates were immunoprecipitated using the anti-gelsolin antibody (A and C), or an antibody directed against the p85 regulatory subunit of PI3-kinase (B), or with beads coated with nonimmune serum (D). Immunoprecipitates were separated on SDS-PAGE and immunoblotted with an antibody to PtdIns P_2. Lanes 1 to 6 represent various durations of osteopontin treatment. Osteopontin treatment produced a rapid increase in PtdIns P_2 associated with a 90-kDa protein in the anti-gelsolin immunoprecipitates of Triton-soluble fractions. There was no stimulation of PtdIns P_2 association with p85, and there was no change in the PtdIns P_2 levels associated with gelsolin in the Triton-insoluble fractions. The nonimmune serum failed to immunoprecipitate proteins associated with PtdIns P_2. Results

The blot was stripped and reprobed with anti-phosphotyrosine antibody. Osteopontin stimulated phosphorylation of a 85 kDa protein (Figure 5B, lane 4), which was confirmed as PI3-kinase by immunoblotting analysis with polyclonal antibody to the regulatory subunit of PI3-kinase, p85. This association was correlated with a disappearance of p85 (PI3-kinase) from gelsolin association in the Triton-insoluble fraction (Chellaiah and Hruska, unpublished data). In fractions from untreated cells, p85 (PI3-kinase) was associated with gelsolin in the Triton-insoluble fraction but not in the Triton-soluble fraction.

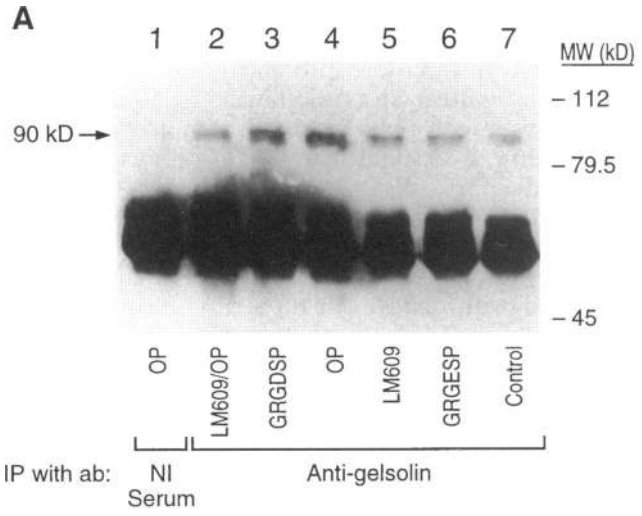
In Vitro Immunecomplex PI3-Kinase Assay

Because immunoblots of anti-gelsolin immunoprecipitates revealed that p85 was associated with gelsolin, we determined whether the p85 regulatory subunit was reflective of p110 catalytic subunit activity. PI3-kinase activity was measured in the anti-gelsolin immunoprecipitates from Triton-soluble (Figure 6, lanes 3 and 4) and -insoluble fractions (Figure 6, lanes 1 and 2) reacted with PtdIns P_2 liposomes and [γ-^{32}P]ATP as described in MATERIALS AND METHODS. The activity of the PI3-kinase paralleled the observations (Figure 5) in which the PI3-kinase was greater in anti-gelsolin immunoprecipitates of Triton-soluble fractions of cell lysates following osteopontin treatment. Anti-gelsolin immunoprecipitates of the Triton-soluble fraction from osteopontin-treated cells demonstrated osteopontin-stimulated PI3-kinase activity (Figure 6, lane 4) as compared with immunoprecipitates from untreated cells (Figure 6, lane 3). An anti-phosphotyrosine immunoprecipitate, from a Triton-soluble fraction of osteopontin-treated cells, also demonstrated PI3-kinase activity (Figure 6, lane 6).

Gelsolin-Actin Interactions: Gelsolin-Actin Ratios in EGTA-resistant Complexes

To assess gelsolin/actin complexes, osteoclasts were incubated with [^{35}S]methionine, and EGTA-resistant gelsolin/actin complexes were isolated as described by Howard *et al.*, (1990). Figure 7, A and B, shows the quantitative changes in the actin/gelsolin ratios as measured from the EGTA-resistant fraction of gelsolin bound to actin. As shown in Figure 7A, anti-gelsolin immunoprecipitates of EGTA-treated cell lysates from osteopontin-treated cells exhibited a decrease in the intensity of the 43-kDa actin band despite slightly more gelsolin having been immunoprecipitated from

(Figure 2 cont.) repeating this representative experiment were observed in three separate additional experiments. (Panel B) Densitometric scans of the experiments were expressed as percent of control (zero time) and mean ± SE values are shown. The 15-s, 30-s, and 1-h values are significantly increased; **$p < 0.01$ and *$p < 0.05$.



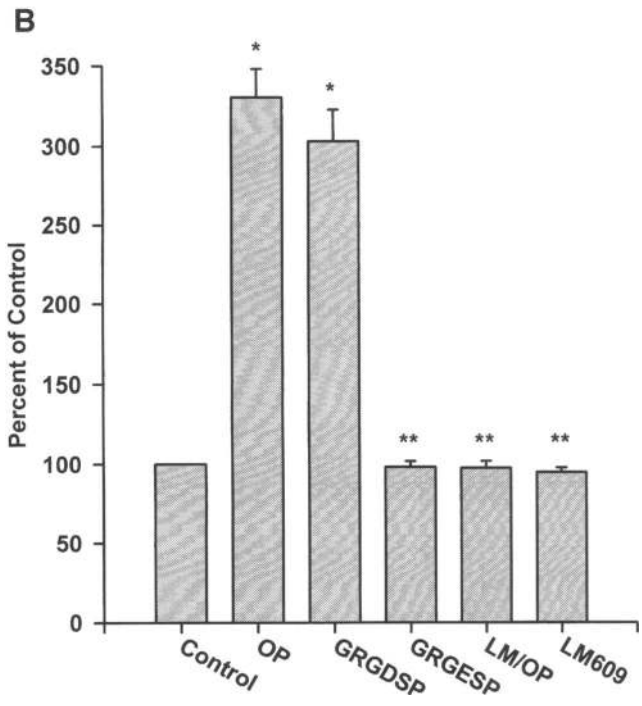


Figure 3. Analysis of the specificity of the effect of osteopontin on PtdIns P_2 association with gelsolin. (A) Triton-soluble lysates made from osteoclasts after various treatments, shown below the figure, were immunoprecipitated either with nonimmune serum (lane 1) or an anti-gelsolin antibody (lanes 2–7). The treatments were as follows: lane 2, LM609 pretreatment before osteopontin; lane 3, GRGDSP treated; lane 4, osteopontin (OP) treated; lane 5, LM609 (anti-$\alpha_v\beta_3$) alone treated; lane 6, mutant analogue of GRGDSP peptide GRGESP; and lane 7, untreated control cells. Osteopontin and GRGDSP stimulated PtdIns P_2 association with gelsolin, and the pre-treatment of osteoclasts with LM609 before osteopontin treatment blocked the osteopontin effect. The experiment is representative of three others with identical results. (B) Densitometric scans of the experiments expressed as percent of control are shown as mean ± SE. *$p < 0.001$ compared with control and **$p < 0.001$ compared with osteopontin.

the osteopontin-treated cells. The actin and gelsolin bands were excised from five other experiments and the radioactivity was determined by liquid scintillation counting. The actin/gelsolin ratio in the complex was calculated. Anti-gelsolin immunoprecipitates from the untreated cells showed an actin/gelsolin ratio of 1.30 ± 0.12 (mean ± SE, n = 5), and in immunoprecipitates from osteopontin-treated cells, the ratio was decreased to 0.65 ± 0.08 (mean ± SE, n = 5). The data shown in Figure 7B are the actin/gelsolin ratios of individual experiments along with the mean ± SE. The results demonstrated that osteopontin caused a decrease in the amount of actin bound to gelsolin in EGTA-resistant complexes. These data prompted investigation of changes in the F-actin content in osteoclasts, because they are compatible with uncapping of barbed end actin favoring polymerization.

Measurement of F-actin Content

The time course of F-actin formation following osteopontin treatment of osteoclast precursors is shown in Figure 8. The time-dependent effect of osteopontin on the increase in F-actin content was parallel to the increase PtdIns P_2 and PtdIns P_3 association with gelsolin (Figure 2). The increase in F-actin content was maximal by 15 min and the elevation persisted for 60 min. To analyze whether other treatments (i.e., GRGDS or LM609) had similar effects on F-actin levels, as compared with their effects on phosphoinositide association with gelsolin (Figure 3), cells were treated as shown in Figure 9. Treatment with GRGDSP mimicked the effect of osteopontin, whereas the inactive analogue GRGESP did not have any effect on F-actin levels. Similarly, pre-treatment with LM609 before osteopontin treatment, or pretreatment with LM609 alone, failed to increase F-actin levels in osteoclasts (Figure 9). Besides blocking the effect of osteopontin, wortmannin pretreatment decreased F-actin levels below baseline.

DISCUSSION

We had previously demonstrated osteopontin stimulation of PtdIns P_3 production and activation of PI3-kinase in avian osteoclasts (Hruska *et al.*, 1995). Our hypothesis regarding the cell shape change associated with osteopontin treatment was that the increase in phosphoinositides was occurring in a cytoskeletal domain related to regulation of the response stimulated by osteopontin. Therefore, we first screened known actin-associated cytoskeletal proteins, which associate with polyphosphoinositides (Pollard and Cooper, 1986; Yin, 1987; Forscher, 1989; Stossel, 1989; Isenberg, 1991), for an effect of osteopontin treatment. We found that osteopontin had no effect on PtdIns P_2 levels associated with α-actinin or profilin in our assays, but

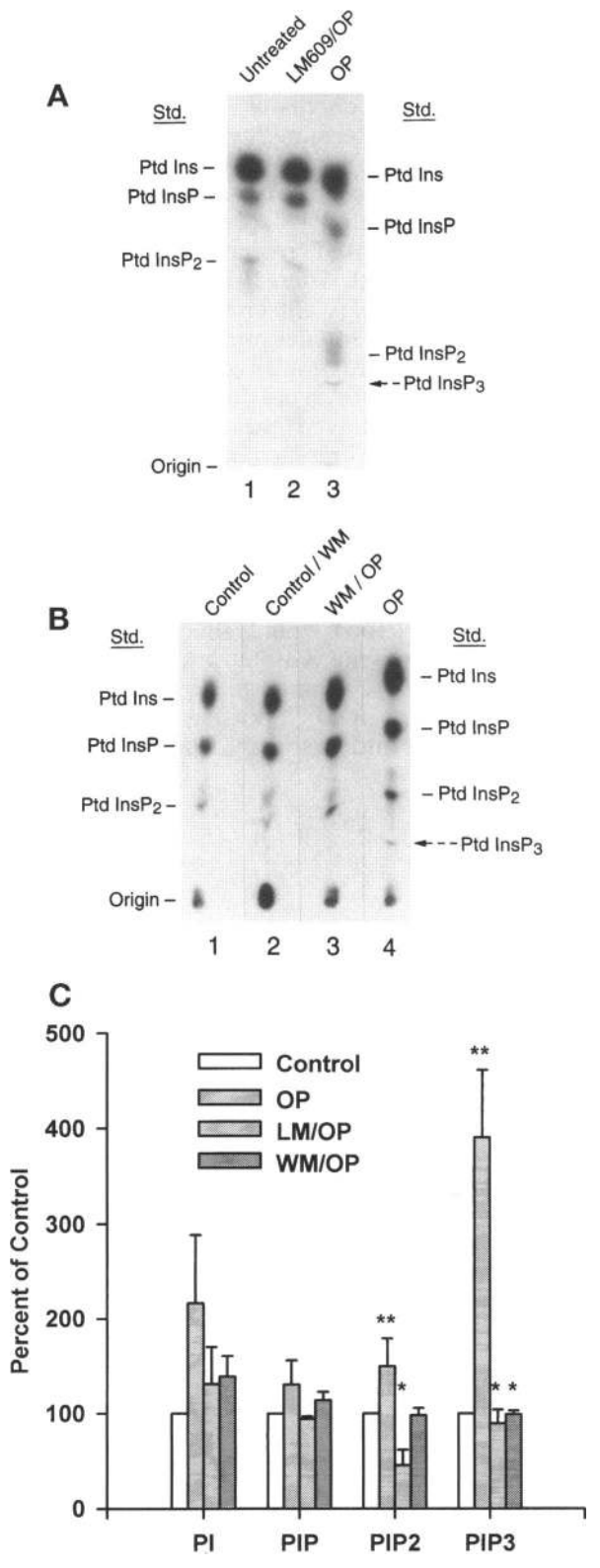


Figure 4. Analysis of the phospholipids associated with the anti-gelsolin immunoprecipitates. (A and B) TLC of the phospholipids associated with gelsolin. To analyze the phospholipids associated with gelsolin, immunoprecipitations were performed with an anti-gelsolin antibody, from Triton-soluble fractions of cell lysates, after various treatments of ^{32}Pi-labeled osteoclasts, as described in MATERIALS AND METHODS. The treatments in panels A and B are listed above the various lanes. They were osteopontin (25 μg/ml for 15 min), pretreatment with LM609 (100 μg/ml for 30 min at 37°C), or Wortmannin (0.1 μM for 10 min at 37°C). Lipids were extracted from the immunoprecipitates and analyzed by TLC as described in MATERIALS AND METHODS. The migration of phospholipid standards is indicated on the side of the panels. The migration of PtdIns P_3 is indicated by the arrow on the right side of panels A and B. Osteopontin treatment increased the levels of PtdIns P_2 and PtdIns P_3 associated with gelsolin. (C) Densitometric scans of the experiments expressed as percent of control are shown as mean ± SE (n = 4). (Control vs. osteopontin (OP), **p < 0.01; *p < 0.01 LM/OP or WM/OP vs. OP).

that phosphoinositides associated with gelsolin in the Triton-soluble fraction of cell lysates were increased. Specifically, we found increased levels of PtdIns P_2 and PtdIns P_3 associated with gelsolin, which were absent when the cells were preincubated with wortmannin, an inhibitor of PI3-kinase (Yano *et al.*, 1993). The effect of osteopontin also appeared to be mediated through the $\alpha_v\beta_3$ integrin because a blocking antibody to the integrin prevented stimulation of phosphoinositide levels associated with gelsolin.

Furthermore, osteopontin stimulated translocation of gelsolin-associated PI3-kinase in the cytoskeletal fraction of cell lysates to Triton-soluble gelsolin. Osteopontin also stimulated tyrosine phosphorylation of the p85 regulatory subunit of PI3-kinase, and increased PI3-kinase activity in anti-gelsolin immunoprecipitates, as measured by PtdIns P_3 production in immune complex phospholipid kinase assays in vitro. Similar to the association of PI3-kinase with gelsolin, PI3-kinase has been shown to associate with α-actinin in NIH 3T3 cells (Shibasaki *et al.*, 1994), but osteopontin does not affect PtdIns P_2 levels associated with α-actinin.

The above results, demonstrating an increase in phosphoinositide association with an actin capping protein, gelsolin, are compatible with a functional profile favoring actin uncapping. In agreement with this prediction, we were able to demonstrate a reduction in the actin/gelsolin ratio of EGTA-resistant actin-gelsolin complexes immunoprecipitated by anti-gelsolin antibodies. In turn, a reduction in actin/gelsolin complexes, suggesting actin uncapping, would favor barbed end actin polymerization that could participate in the cell shape change stimulated by osteopontin and RGD peptides (Horton *et al.*, 1991). This actin polymerization would require increased F-actin levels, and we were able to measure a very significant increase in F-actin stimulated by osteopontin.

Our studies first focused on the actin capping protein, gelsolin, found to be affected by osteopontin. We realize that other good candidates for a function similar to gelsolin exist, i.e., Cap 100 (Hofmann *et al.*, 1992)

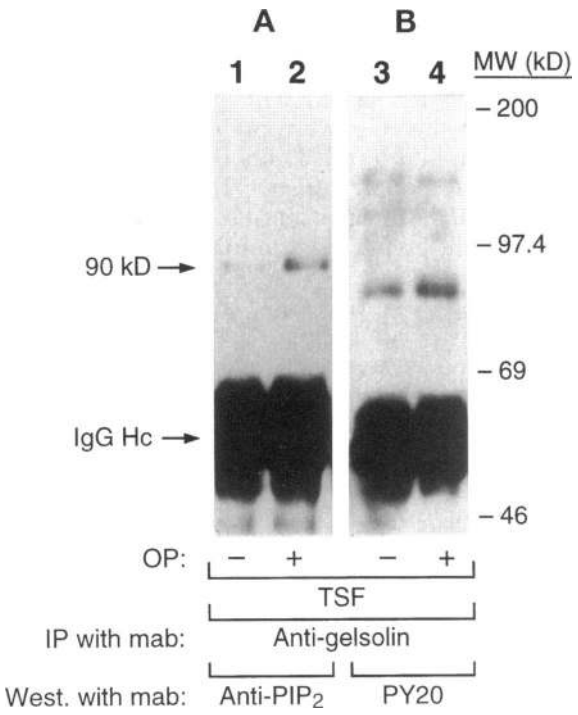


Figure 5. Osteopontin stimulation of PI3-kinase association with gelsolin. Triton-soluble fractions of osteoclast precursor lysates were immunoprecipitated with the antibody to gelsolin (A and B). The immunoprecipitates were subjected to SDS-PAGE and Western analyses were performed utilizing PtdIns 4,5 bisphosphate antibody (A, lanes 1 and 2) or anti-phosphotyrosine antibody, PY20 (B, lanes 3 and 4). Osteopontin-stimulated association of PtdIns P_2 with gelsolin in the Triton-soluble fraction (A, lane 2). The p85 associated with gelsolin in the Triton-soluble fraction was tyrosine phosphorylated as demonstrated by the antiphosphotyrosine immunoblot (B, lanes 3 and 4), and osteopontin treatment increased the level of phosphorylation (lane 4).

and Cap Z (Heiss and Cooper, 1991). The mechanism of action proposed for these proteins is that polyphosphoinositides may prevent their association with actin monomers and facilitate F-actin formation (Janmey and Stossel, 1987; Stossel, 1989).

While our data indicate that the PtdIns P_3 species of phosphoinositides contribute to the increased levels associated with gelsolin, they may not be the sole source. Chong *et al.* (1994) have suggested that liganding of integrins by matrix activates phosphatidylinositol 4-phosphate 5-kinase. Our data suggest that the increase in polyphosphoinositides associated with gelsolin derive mainly from PtdIns P_3-OH kinase. Our studies demonstrate osteopontin activation of PI3-kinase associated with Triton-soluble gelsolin. This activation of PI3-kinase is different from the translocation of the enzyme to the Triton-insoluble cytoskeleton fraction during stimuli that switch off actin assembly (Payrastre *et al.*, 1991; Zhang *et al.*, 1992). Thus, again our results are compatible with osteopontin stimulation of actin polymerization by uncapping of barbed ends.

Although we did not analyze the association of the p110 catalytic component of the PI3-kinase heterodimer, we did determine, by using phospholipid kinase assays in vitro, that anti-gelsolin immunoprecipitates had PI 3-kinase activity. Furthermore, p85 is tyrosine phosphorylated when it is associated with Triton-soluble gelsolin. Tyrosine phosphorylation of p85 is often observed when PI3-kinase is activated, although its functional role in enzyme activity remains to be fully clarified (Downes and Carter, 1991).

Our data do not prove a direct association of PI3-kinase with gelsolin or actin. Enzymatic assays and Western analyses showed that $pp60^{c\text{-}src}$ and other phosphoproteins also interact with gelsolin (our unpublished observations). This would suggest that linker proteins may serve to bring PI3-kinase into association with gelsolin. The mechanisms of PI3-kinase association with gelsolin require analysis, along with the mechanism of translocation from the Triton-insoluble cytoskeleton, where several investigators have found the enzyme (Painter and Ginsberg, 1982; Grondin *et al.*, 1991; Kouns *et al.*, 1992). In thrombin-activated platelets, $pp60^{c\text{-}src}$ kinase is found in the actin-rich fraction and has been described to interact

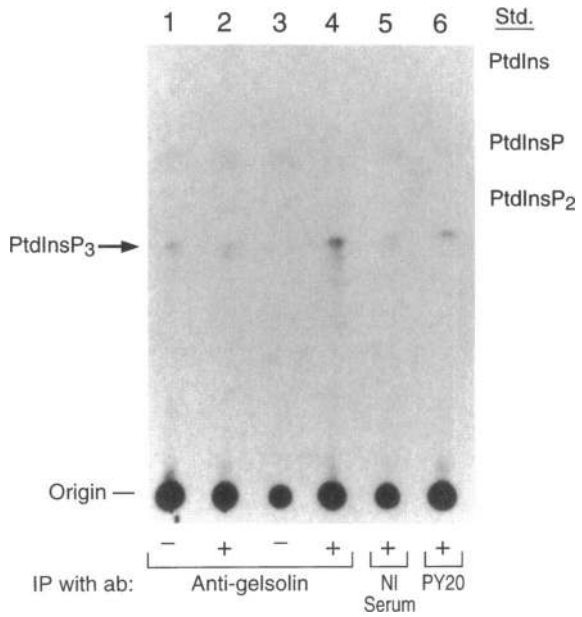
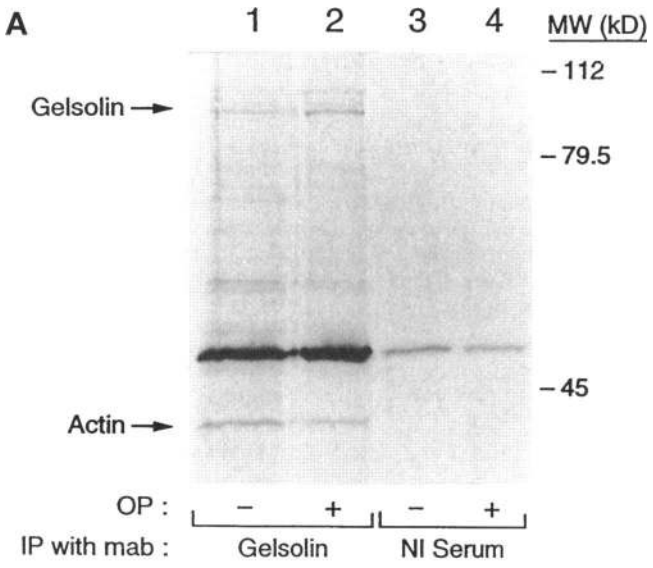


Figure 6. Effect of osteopontin on the PI3-kinase activity observed in the gelsolin immunoprecipitates. Triton-soluble and Triton-insoluble fractions of osteoclast precursor lysates were immunoprecipitated with the antibody to gelsolin and subjected to the in vitro immune complex phospholipid kinase assay (see MATERIALS AND METHODS). Lanes 1 and 2 are Triton-insoluble fractions and lanes 3–6 are Triton-soluble fractions. (–) represents untreated cells and (+) represents osteopontin-treated cells. These results are representative of four separate experiments.



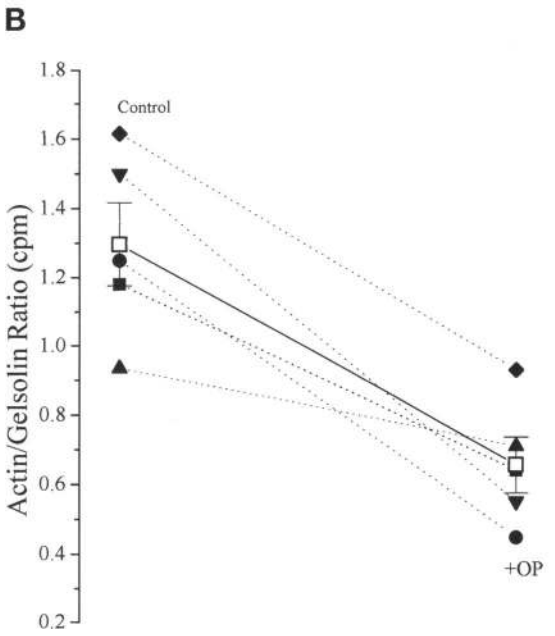


Figure 7. The effect of osteopontin on actin:gelsolin 1:1 EGTA-resistant complexes immunoabsorbed by anti-gelsolin antibody from Triton-soluble supernatants. (A) Shown are SDS-PAGE autoradiography of [^{35}S]methionine-labeled proteins immunoprecipitated (IP), from Triton-soluble supernatants of osteoclast precursors, by anti-gelsolin antibody (lanes 1 and 2) and by nonimmune serum (lanes 3 and 4). The gelsolin and actin bands are marked by arrows. (B) Shown are the values of 1:1 EGTA-resistant actin/gelsolin complex from five different experiments. The ratio is expressed from the measurement of the incorporation of radio activity in actin and gelsolin bands (cpm in actin to cpm in gelsolin). Control and osteopontin-treated pairs for each experiment are connected. The mean ± SE for five experiments is plotted with error bars (1.30 ± 0.12 for control and 0.65 ± 0.08 for osteopontin-treated cells).

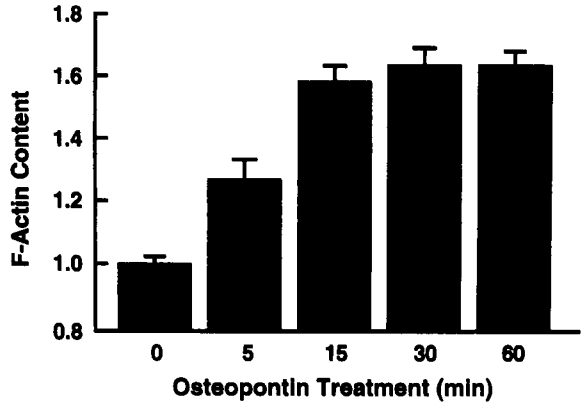


Figure 8. The effect of osteopontin stimulation on F-actin content of osteoclast precursors. F-actin levels were determined by rhodamine-phalloidin binding in osteoclast precursors, exposed to osteopontin (25 μg/ml) or vehicle for the indicated times at 37°C as described in MATERIALS AND METHODS. The treated cells were rapidly washed and subjected to methanol extraction. F-actin contents of the 0-min cells were assigned a value of 1.0 and all other values were expressed relative to the 0-s values. The F-actin content of the vehicle-treated cells was constant for 60 min. Values plotted are mean ± SEM from three experiments.

weakly with PI3-kinase in the Triton X-100–soluble fraction (Gutkind *et al.*, 1990). Thus, pp60$^{c\text{-}src}$ could be responsible for the association of PI3-kinase with gelsolin or vice versa.

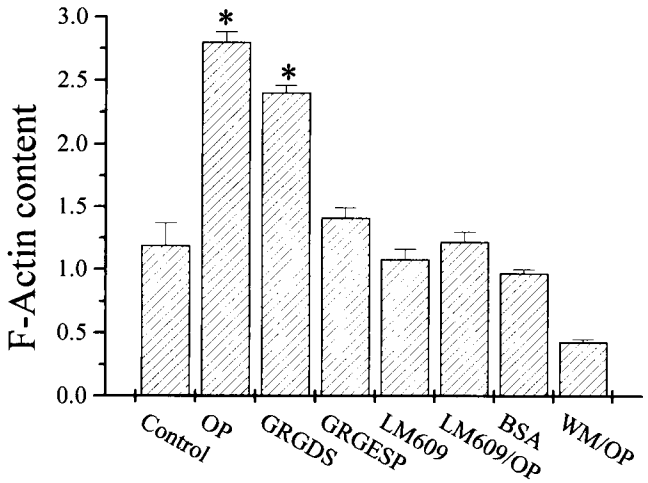


Figure 9. Quantification of the F-actin content in osteoclasts after various treatments. Cells were grown in 24-well tissue culture plates and the F-actin content was measured as described in MATERIALS AND METHODS. Binding of rhodamine phalloidin was determined by measuring the fluorescence increase, caused by binding with actin. Fluorescence data are mean ± SEM of three experiments for the various experimental groups. Osteopontin and GRGDS increased the F-actin content of the cells (*$p < 0.01$ compared with control). LM609 or pretreatment with LM609 before osteopontin stimulation did not have any effect. Wortmannin pretreatment, before osteopontin treatment, significantly reduced the F-actin levels below that of the control cells.

In summary, our results demonstrate that soluble osteopontin, binding to the osteoclast precursor $\alpha_v\beta_3$ integrin, stimulates phosphoinositide association with Triton-soluble gelsolin in part through activation of PI3-kinase. The finding of the osteopontin stimulation of PtdIns P_3 association with Triton-soluble gelsolin suggests a function of this phospholipid in cytoskeletal regulation. These studies indicate a new area of study related to integrin-based signal transduction, phospholipid synthesis, and cytoskeletal organization.

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