Research article

Polyphosphoinositides-dependent regulation of the osteoclast actin cytoskeleton and bone resorption

Rajat S Biswas[1], De Anna Baker[1], Keith A Hruska[2] and Meenakshi A Chellaiah*[1]

Address: [1]Department of Biomedical Sciences, University of Maryland, Baltimore, Maryland 21201, USA and [2]Department of Pediatrics, Washington University, St. Louis, MO 63110, USA

Email: Rajat S Biswas - rsb001@dental.umaryland.edu; De Anna Baker - dbaker8@umbc.edu; Keith A Hruska - Hruska_K@kids.wustl.edu; Meenakshi A Chellaiah* - mac001@dental.umaryland.edu

* Corresponding author

Abstract

Background: Gelsolin, an actin capping protein of osteoclast podosomes, has a unique function in regulating assembly and disassembly of the podosome actin filament. Previously, we have reported that osteopontin (OPN) binding to integrin $\alpha_v\beta_3$ increased the levels of gelsolin-associated polyphosphoinositides, podosome assembly/disassembly, and actin filament formation. The present study was undertaken to identify the possible role of polyphosphoinositides and phosphoinositides binding domains (PBDs) of gelsolin in the osteoclast cytoskeletal structural organization and osteoclast function.

Results: Transduction of TAT/full-length gelsolin and PBDs containing gelsolin peptides into osteoclasts demonstrated: 1) F-actin enriched patches; 2) disruption of actin ring; 3) an increase in the association polyphosphoinositides (PPIs) with the transduced peptides containing PBDs. The above-mentioned effects were more pronounced with gelsolin peptide containing 2 tandem repeats of PBDs (PBD (2)). Binding of PPIs to the transduced peptides has resulted in reduced levels of PPIs association with the endogenous gelsolin, and thereby disrupted the actin remodeling processes in terms of podosome organization in the clear zone area and actin ring formation. These peptides also exhibited a dominant negative effect in the formation of WASP-Arp2/3 complex indicating the role of phosphoinositides in WASP activation. The TAT-PBD gelsolin peptides transduced osteoclasts are functionally defective in terms of motility and bone resorption.

Conclusions: Taken together, these data demonstrate that transduction of PBD gelsolin peptides into osteoclasts produced a dominant negative effect on actin assembly, motility, and bone resorption. These findings indicate that phosphoinositide-mediated signaling mechanisms regulate osteoclast cytoskeleton, podosome assembly/disassembly, actin ring formation and bone resorption activity of osteoclasts.

Background

Phosphoinositides are involved in modulating a variety of actin regulatory proteins [1] as well as promoting filament cross-linking to form stable, bundled actin fibers [2]. Phosphoinositide kinases comprise a unique family of enzymes that catalyze the phosphorylations of phosphatidylinositol and its phosphorylated metabolites to produce seven phosphoinositides [3] with PtdIns 4,5 P2 being the most active one [4]. PtdIns 4,5 P2 (PtdIns P_2) regulates several actin-binding proteins, including gelsolin, profilin, α-actinin, and vinculin to promote actin polymerization [4-7]. A number of phosphoinositide binding motifs have been identified in several cytoskeletal as well as structural proteins. Phosphoinositides binding with these domains are highly dynamic, and rapidly reversible [8-10]. Interaction of phosphoinositides with the specific binding domains helps to coordinate the assembly of one to several signaling molecules. These molecules regulate cellular signal transduction processes involving stress fiber and focal adhesion formation [11,12]. Our previous studies have provided insights into the roles of phosphoinositides in gelsolin function, in the regulation of actin reorganization, and podosome assembly/disassembly in avian and mouse osteoclasts [13-16].

Cell shape changes and motility are directly related to structural dynamics of actin cytoskeleton and function of actin binding proteins. Actin binding proteins comprise a large family of proteins with distinct properties such as severing, capping, cross linking, and nucleation. Actin filament remodeling is a critical element of cell motility [17]. Besides cell motility, actin binding proteins and actin remodeling play crucial roles in several cellular responses, including, cell differentiation, secretion, and cell shape changes [18-21]. Gelsolin is one of the actin binding proteins that regulates actin assembly and disassembly. By a variety of mechanisms gelsolin controls the length of actin filaments *in vitro*, as well as cell shape changes and motility *in vivo* [22]. Gelsolin severs assembled actin filaments, caps the fast growing plus ends, and promotes growth of actin filament by creating nucleation sites [23-25]. Binding of PtdIns P_2 to gelsolin causes the release of gelsolin from the actin filament end (uncapping), providing a site for rapid monomer addition [6,26,27]. We have demonstrated similar gelsolin uncapping actions by PtdIns P_2 in osteoclast podosome assembly [13,15].

Osteoclasts are characterized by unique cell adhesion structures found in highly motile cells called podosomes. This type of cell adhesion structures is a property also shared by several types of cancer cells and monocytes and/or macrophages [28-31]. When the osteoclasts are cultured on glass surfaces, multiple rows of podosomes are localized in the area corresponding to the sealing zone of osteoclasts [15,29,30,32]. A higher rate of osteoclast motility is brought about by accelerated podosome assembly and disassembly [33,34]. Podosomes consist of an F-actin core surrounded by the actin-binding proteins vinculin, talin, gelsolin, and α-actinin [16,32,35].

We have shown previously that osteopontin (OPN) binding to integrin $\alpha_v\beta_3$ stimulates the levels of gelsolin-associated polyphosphoinositides such as PtdIns 3,4 P2, PtdIns 4,5 P2 (PtdIns P2), and PtdIns 3,4,5 P3 (PtdIns P3) in osteoclasts. The increase of PtdIns P2 levels associated with gelsolin uncaps actin oligomers leading to an increase in F-actin content and actin filament formation [13-15]. Gelsolin deficiency blocks podosome assembly and $\alpha_v\beta_3$-stimulated signaling related to motility in gelsolin null osteoclasts [15]. Gelsolin deficient osteoclasts, macrophages, and fibroblasts are hypo motile and are partially disabled [15,36].

A substantial body of studies on the structure-function analysis *in vitro* has defined distinct domains of gelsolin responsible for severing and capping of actin filaments as well as phosphoinositides binding. Gelsolin contains 1–6 repeats of a 120–130 amino acid segment [20]. The 1–6 segments of gelsolin exist as two tandem homologous halves, each of which contains a three fold segmental repeat (segments S1-S3 and S4-S6, respectively [27,37,38]. The amino- and carboxyl-halves are connected by a linker, which is cleaved by caspase-3 *in vivo* [39,40] and by many other proteases *in vitro*. The F-actin binding domains reside within amino acid residues 161– 171 [27] and the two-phosphoinositide binding domains (PBDs) have been identified in the S1 and S2 segments of gelsolin. The amino acid sequences of PBDs (1 and 2) in S1 and S2 segment of gelsolin are: YFKSGLKYKKG-GVASGF (PBD1; 133 to 149 aa residues) and KHVVPNEV-VVQRLFQVKGRR (PBD2; 150 to169 aa residues), respectively [37]. In addition to these two PBDs, a third site in the C-terminal part of gelsolin was identified recently using fluorescent phosphoinositide derivatives [41]. This site resides in amino acid residues 620–634 [42].

The sequence QRLFQVKGRR in PBD2 has been shown to compete with intact gelsolin for binding polyphosphoinositides when introduced into fibroblasts, platelets, and neutrophils. It also blocked cell motility and actin assembly in these cells [43-45]. Since gelsolin has a unique function in regulating assembly and disassembly of the podosome actin filament [13,14], we addressed the question whether phosphoinositides and actin binding peptides of gelsolin would be able to block actin assembly and bone resorption in osteoclasts. In this study, we characterized the effects of gelsolin peptides containing PBDs on cytoskeletal organization and osteoclast function.

Transduction of gelsolin PBD peptides into osteoclasts produced a dominant-negative effect on actin assembly, actin ring formation, and bone resorption.

Results
Expression and purification of recombinant peptides
A schematic diagram of gelsolin constructs used in this study is shown in Fig. 1 (Panel A). Cloning was performed as described in the Methods section. Full-length gelsolin and its fragments were purified as TAT-fusion peptides using Ni-NTA column. Western analysis of the purified peptides was performed with an antibody to HA (Panel B). In an 8% SDS-PAGE (lanes 1–3), the purified 98.5 kDa full-length gelsolin (FL-GSN; lane 1), 43 kDa carboxyl terminal (COOH-GSN; lane 2) and 36 kDa middle gelsolin peptides (Mid-GSN; lane 3) are shown. In a 15% SDS-PAGE (lanes 4–7), the purified HIV-TAT fused 26 kDa amino terminal (NH-GSN; lane 4), the 18.5 kDa two tandem repeats of PBD (PBD (2); lane 5), the 13.5 kDa PBD (lane 6) gelsolin peptides and the 6–8 kDa TAT-HA vector peptide (lane 7) are shown. The NH-terminal fragment (NH-GSN) contains the first PBD (133–149 amino acid (aa) residues) and 2 aa residues (150KH151) of the second PBD. Mid-GSN fragment (aa 256–491) does not contain any PBDs but contains actin binding domain (ABD) 3 and part of ABD 4. The COOH-terminal fragment (aa 491-aa 755) contains the actin binding domains (ABDs) 5 and 6 in addition to the 17-aminoacid residue of ABD 4, which is missing in Mid-GSN fragment. The COOH-terminal domain also contains the recently identified third PBD, which resides within amino acid residues 620–634. The amino terminal PBDs 1 and 2 reside in amino acid residues 133–169. The PBD and the TAT sequence together generate a polypeptide with MW 13.5 kDa constituting PBD. The 2 tandem repeats of PBD (PBD (2)) and the TAT sequence together generate a polypeptide with MW 18.5 kDa constituting PBD (2).

Phosphoinositides associate with the transduced gelsolin peptides containing PBD
To identify the association of phosphoinositides with PBDs of gelsolin, TAT-HA fusion peptides of gelsolin, as indicated in Fig. 2 (Panel A), were transduced into osteoclasts generated from mice for 45 min. at 37°C. Triton-soluble lysates were prepared for immunoprecipitation and Western analyses. Western analysis of HA immunoprecipitates with an anti-PtdIns P2 antibody is shown in Panel A. The anti-PtdIns P2 antibody recognized the following transduced peptides containing PBD: FL-GSN (lane 2), NH-GSN (lane 5), COOH-GSN (lane 7), PBD (lane 8) and PBD (2) (lane 9). Neither the Mid-GSN (lane 6) nor the control peptides (TAT-HA, lane 3; and HSV-TK, lane 4) had any detectable association with PtdIns P2. Immunoprecipitation with non-immune serum is shown in lane 1. Immunoblots of SDS gels containing the various

gelsolin peptides prior to transduction were negative for PtdIns P2 (data not shown).

To further analyze the phospholipids associated with the transduced peptides, lipids associated with anti-HA immunoprecipitates were extracted from osteoclasts exposed to ^{32}Pi and subjected to TLC analysis (Panel B). Binding of PtdIns P2 and PtdIns P3 with FL-GSN (lane 1), NH-GSN (lane 2), COOH-GSN (lane 3), PBD (2) (lane 4), and PBD (lane 5) were observed. Negligible amounts of phosphoinositides were associated with either Mid-GSN (lane 6) or TAT-HA (lane 7) peptides. A significant increase in the binding of PtdIns P2 and PtdIns P3 was observed with the PBD (2) (lane 4). The presence of an additional PBD in the transduced protein enhances its affinity to PtdIns P2 and PtdIns P3. Immunoprecipitation with non-immune serum is shown in lane 8.

Osteopontin stimulates phosphoinositides association with the transduced peptides containing PBD
We next examined the effects of OPN on PtdIns P2 association with the transduced peptides. Anti-HA immunoprecipitates made from lysates of osteoclasts treated as indicated in Fig. 3 were immunoblotted with an anti-PtdIns P2 antibody. OPN stimulated PtdIns P2 association with the following transduced peptides: FL-GSN (lane 2), COOH-GSN (lane 5), NH-GSN (lane 7) and the gelsolin peptide containing 2 tandem repeats of PBD (2) (lane 9). PBS-treated osteoclasts were used as control (lanes 1, 4, 6, and 8). Neither PBS (lane 10) nor OPN (lane 11) had any effect on the association PtdIns P2 with the Mid-GSN fragment. Immunoprecipitation with non-immune serum is shown in lanes 3 and 12. Western analysis with anti-HA (Fig. 3, panel B) demonstrates the levels of transduced peptides immunoprecipitated in each lane shown in Fig. 3 (Panel A). These experiments (Figs. 2 and 3) demonstrate that the transduced FL-Gsn and its fragments containing PBD can associate with PtdIns P2 and PtdIns P3.

Transduction of PBD containing gelsolin peptides decreases the association of polyphosphoinositides with endogenous gelsolin
We then addressed the question whether increasing the intracellular levels of gelsolin peptides affected the function of endogenous gelsolin (Fig. 4). Osteoclast lysates, which were immunodepleted for the transduced peptides by immunoprecipitation with an anti-HA antibody, were subjected to a second immunoprecipitation with an anti-gelsolin antibody. One half of the gelsolin immunoprecipitates were used for Western analysis with anti-PtdIns P2 antibody (Fig. 4, panel A) and the second half were used for Western analysis with anti-gelsolin antibody to detect the levels of gelsolin protein immunoprecipitated (Fig. 4, panel B). Immunoprecipitations made with lysates
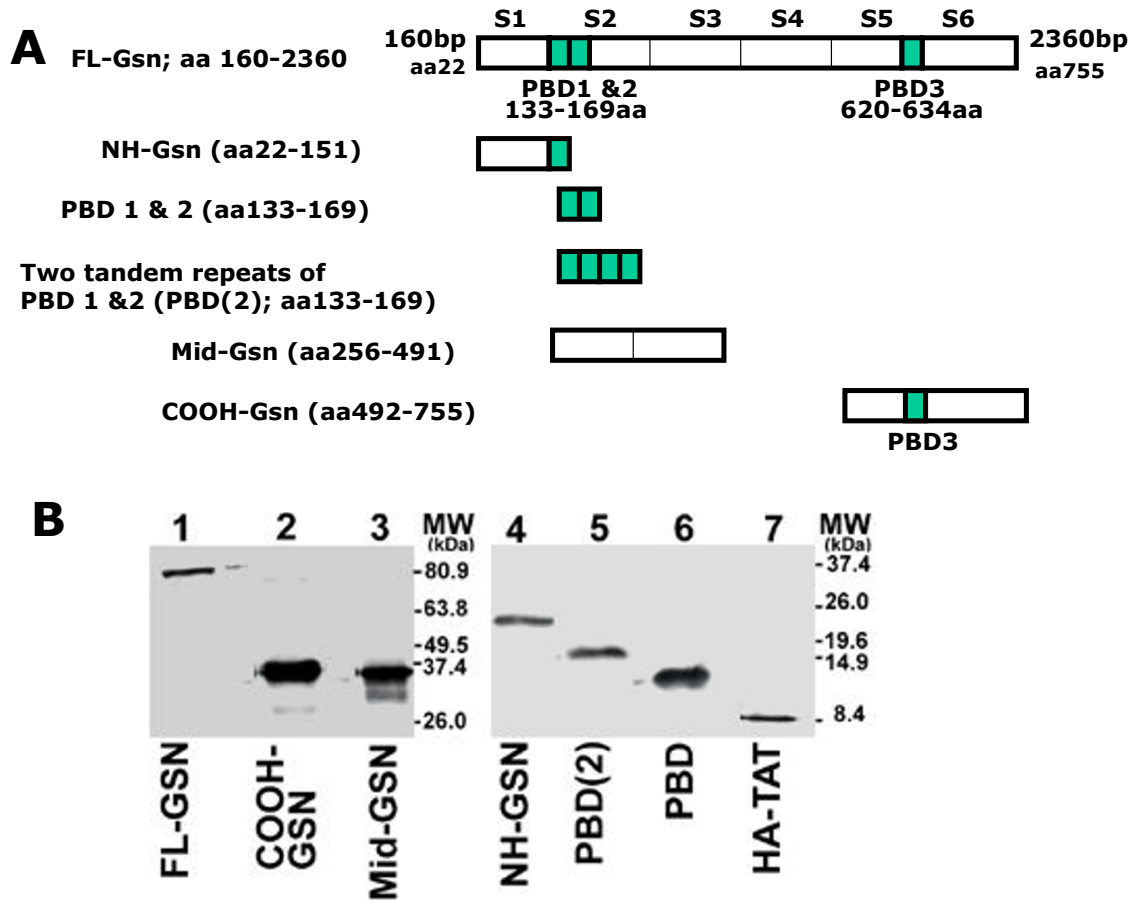
Figure 1
Panel A. Schematic diagram demonstrating various gelsolin constructs generated in HIV-TAT-expression vector. Actin binding domain (S1-S6) and phosphoinositide binding domains (PBDs) are indicated in full-length gelsolin (FL-Gsn). Cloning was performed as described in the Methods Section. Phosphoinositides binding domains 1, 2, and 3 are shown in green color. Two-tandem repeats of first and second phosphoinositides binding domains (aa 133–169) were also cloned. This is represented as PBD (2) in the text. **Panel B. Western analysis of the purified peptides using a HA antibody.** Purified TAT-fused gelsolin peptides were subjected to 8% (lanes 1–3) and 15% SDS-PAGE (lanes 4–7). The blots were subjected to Western analysis with an antibody to HA. The results shown are representative of three independent experiments.

prepared from PBS- and OPN-treated osteoclasts were used as positive control (Fig. 4; Panel A, lanes 1 and 2). OPN stimulated PtdIns P2 association with gelsolin in untransduced (lane 2), Hsv-TK (non-specific control, lane 4), and Mid-GSN fragment (lane 6) transduced osteoclasts as compared with the respective PBS-treated controls (lanes 1, 3, and 5). A decrease in the basal level of PtdIns P2 association with endogenous gelsolin was observed in PBS-treated NH-GSN (lane 7), PBD- (lane 12), PBD (2) (lane 14), and FL-GSN (lane 16) peptides

transduced osteoclasts. OPN stimulation had minimal effect on the increase in PtdIns P2 association with endogenous gelsolin in osteoclasts transduced with the above-mentioned peptides (lanes 8, 13, 15, and 17) as compared with the OPN-alone treated control osteoclasts (lane 2). In osteoclasts transduced with TAT/FL-GSN protein, more PtdIns P2 association was observed with the transduced protein (Lane 17, indicated as TAT-gelsolin by an arrow) than the endogenous gelsolin (indicated as gelsolin) in osteoclasts treated with PBS (lane 16) and OPN (lane 17).
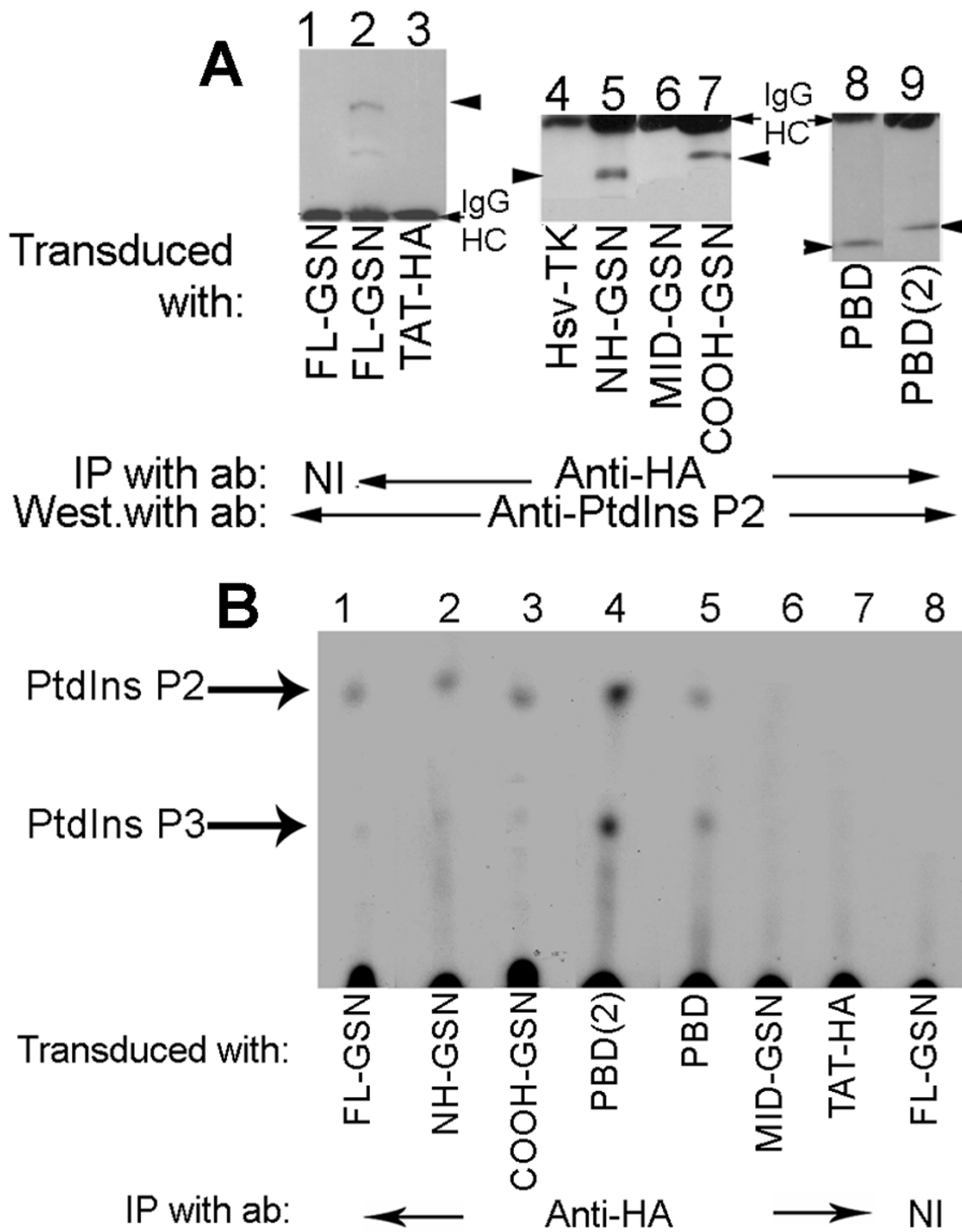
Figure 2
Analysis of PtdIns P$_2$ association with the transduced protein. Panel A. Western analysis with an anti-PtdIns P2 anti-body: Treatments are as indicated below the figure. Lysates were immunoprecipitated with an antibody to HA (lanes 2–9) or non-immune serum (NI; lane 1). The immunoprecipitates were subjected to 8% SDS-PAGE (lanes 1 to 3) or 15% SDS-PAGE (lanes 4 to 9) and Western analyzed with an antibody to PtdIns P2. PtdIns P2 binding to the transduced peptides is indicated with arrowheads. Arrows indicate IgG heavy chain (IgG HC). The results shown are representative of three independent experiments. **Panel B.** TLC analysis of the phospholipids associated with the anti-HA immunoprecipitates: ^{32}P-labeled osteo-clasts were subjected to various treatments as indicated in the figure. Osteoclast lysates made from these cells were subjected to immunoprecipitation with an antibody to HA. ^{32}P-labeled phosphoinositides associated with anti-HA immunoprecipitates were analyzed by TLC. Arrows on the left side of the figure indicate the migration of PtdIns P2 and PtdIns P3. The results shown are representative of three independent osteoclast preparations and experiments.
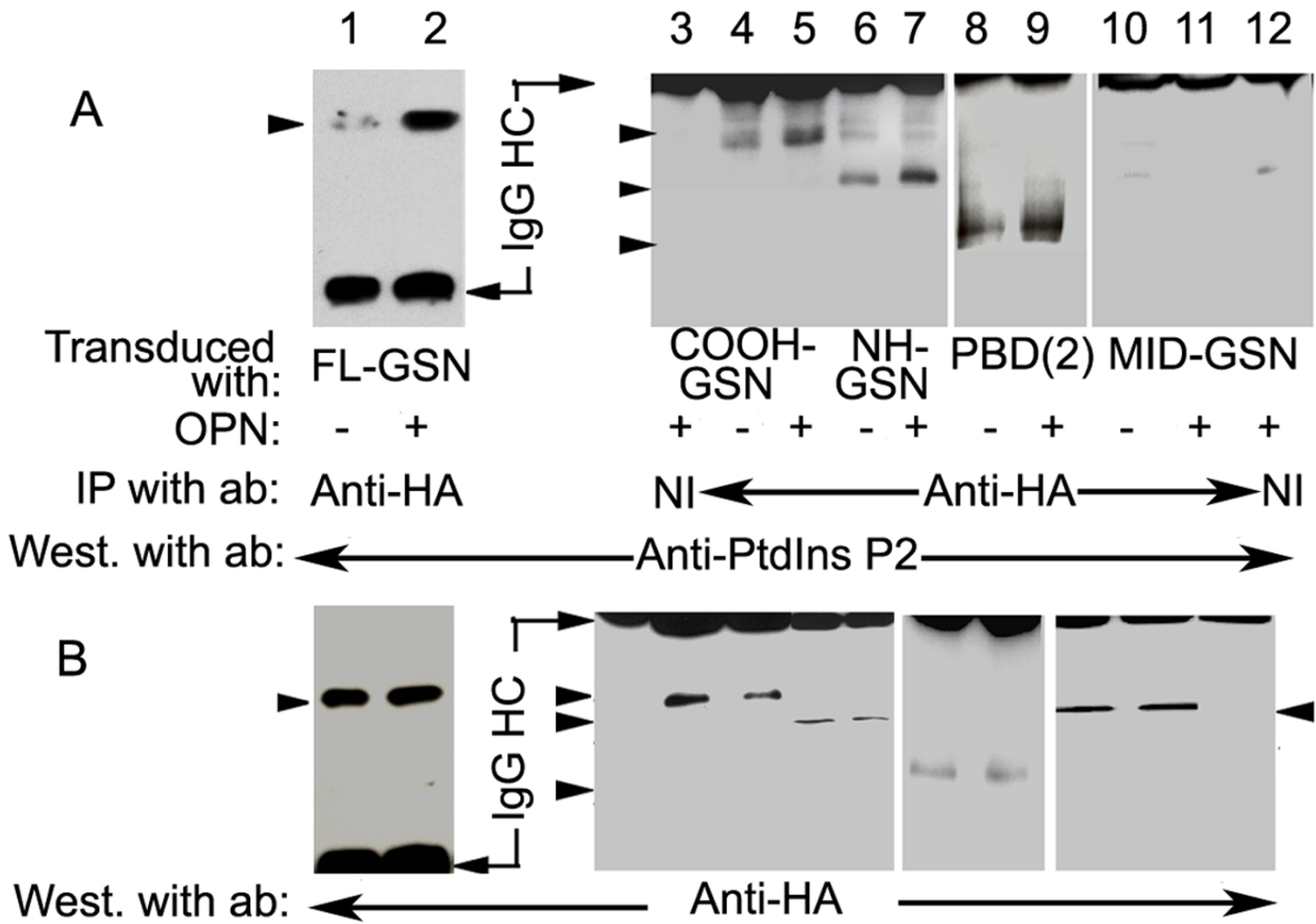
Figure 3
The effects of OPN or TAT-HA-protein transduction on PtdIns P₂ association with the transduced peptides
Osteoclasts were transduced with the indicated gelsolin peptides and treated with PBS (-) or OPN (+) as shown below the Figure. Lysates were immunoprecipitated with an antibody to HA (lanes 1, 2, 4–11) or non-immune serum (NI; lanes 3 & 12). The immunoprecipitates were subjected to 8% SDS-PAGE (lanes 1 and 2) or 15% SDS-PAGE (lanes 3–12). Panel A: Western analysis with an antibody to PtdIns P2. Panel B: Western analysis with an antibody to HA. The immunoblot shown in panel A was stripped and blotted with a HA-antibody to detect the levels of transduced proteins immunoprecipitated in each lane. Arrowheads point to transduced proteins associated with PtdIns P2. Arrows indicate IgG heavy chain (IgG HC). The results represent one of three experiments performed from three separate osteoclast preparations.

IgG heavy chain (IgG HC) is indicated by an arrow. The levels of gelsolin protein immunoprecipitated are demonstrated by Western analysis of the second half of the immunoprecipitates with an anti-gelsolin antibody (Fig. 4, panel B). The transduced TAT/FL-GSN gelsolin was also recognized by the gelsolin antibody in osteoclasts treated with PBS and OPN (lanes 16 and 17). Although the transduced level of PBD (2) is lower than FL-GSN, it exhibits either equal or more blocking effects in the association of PtdIns P2 with endogenous gelsolin (lanes 14 and 15).

Transduction of PBD containing gelsolin peptides decreases the interaction of PI3-Kinase with endogenous gelsolin

We have demonstrated that OPN stimulated tyrosine phosphorylation of the p85 regulatory subunit of PI3-kinase associated with gelsolin, and increased its activity as measured by PtdIns P3 production in immune complex phospholipid kinase assay *in vitro* [13,46]. Our recent studies have defined unique biochemical properties of gelsolin and have demonstrated strong phosphoinositides-mediated interaction of PI 3-Kinase with gelsolin [16]. Hence, the possibility that the increased levels of
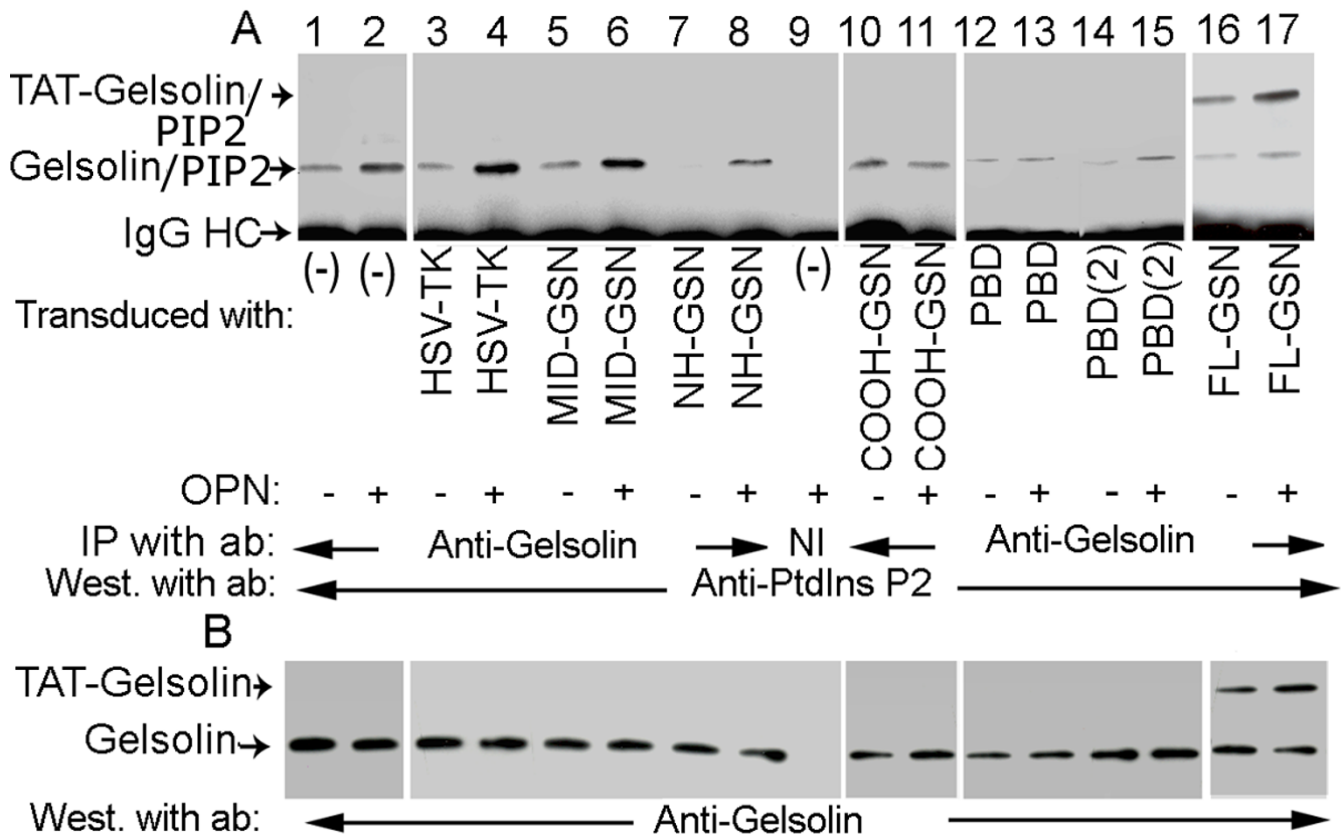
Figure 4
The effects of transduction of TAT-fused gelsolin proteins on PtdIns P_2 association with endogenous gelsolin
Osteoclasts were transduced with TAT-peptides and treated with PBS (-) or OPN (+) as indicated below the figure. Lysates were immunoprecipitated with a monoclonal antibody to gelsolin (lanes 1–8; 10–17) or non-immune serum (NI; lane 9). **Panel A**: Western analysis with an antibody to PtdIns P2. Arrows point to endogenous gelsolin (lanes 1–8 and 10–17) and the copre-cipitated transduced TAT-gelsolin protein (lanes 16 and 17) associated with PtdIns P2. IgG heavy chain is also indicated by an arrow. **Panel B**: Western analysis with an antibody to gelsolin. The level of gelsolin in each lane is shown. Arrows indicate endogenous (lanes 1–8 and 10–17) and TAT-gelsolin (lanes 16 and 17) proteins. The results represent one of three experiments performed from three separate osteoclast preparations.

PtdIns P3 associated with the transduced PBD (2) could be due to interaction of activated p85 regulatory subunit. To investigate this possibility, osteoclast lysates were subjected to immunoprecipitation with an anti-HA antibody. To have an internal control for immunoprecipitation, an antibody to actin was also added to each immunoprecipitate (Fig. B, lanes 1–6). One half of the immunoprecipitates used for Western analysis with anti-PI3-kinase antibody (Fig. 5, panel A) and the second half were used for Western analysis with anti-actin antibody (Fig. 5, panel B). Coprecipitation of PI3-kinase was observed with HA-immunoprecipitates made from osteoclasts transduced with PBD (Panel A, lane 3), PBD (2) (lane 4), FL-Gsn (lane 5) and NH-Gsn (lane 6) peptides. PI 3-kinase was not detected in the HA-immunoprecipitates made

from osteoclasts transduced with Mid-Gsn peptide (lane 2), which does not contain PBD. This suggests that the association of PI3 kinase with other peptides containing PBDs is specific and indeed is mediated through the associated phosphoinositides. Detection of more PI3-K levels in FL-Gsn and PBD (2) transduced osteoclasts (lanes 4 and 5) may be due to the presence of multiple phosphoinositide binding sites in these peptides. Western analysis with an actin antibody (Fig. B) demonstrated equal amount of actin in all the lanes. Gelsolin plays a key role in the recruitment of signaling proteins such as, c-Src, PI 3-Kinase, p130[Cas], FAK, integrin $\alpha_v\beta_3$, vinculin, talin and paxillin to the podosome signaling complex through phosphoinositide-protein interactions [16]. We failed to
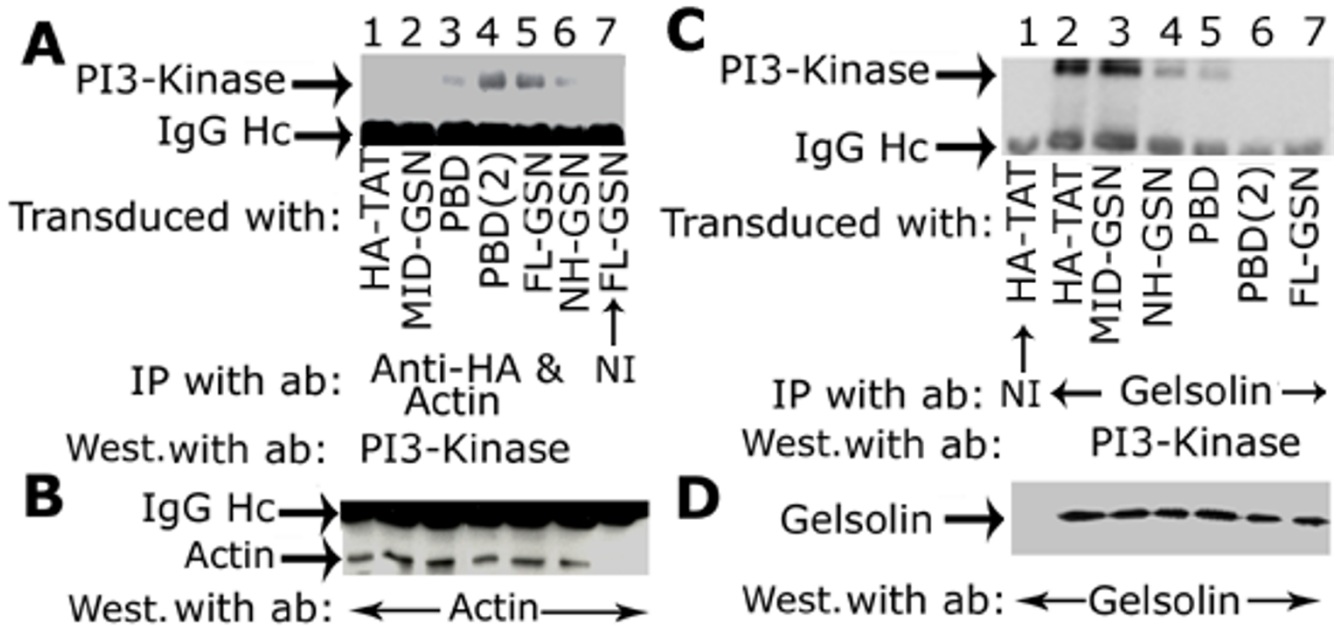
Figure 5
Analysis of association of PI3-kinase with transduced TAT-HA peptides Osteoclasts were transduced with TAT-peptides as indicated below the panels A and C. **Panel A:** Western analysis using an antibody to PI3-kinase. Lysates were immunoprecipitated with antibodies to HA and actin (lanes 1–6). Actin antibody was used as an internal control for immunoprecipitation. Immunoprecipitation with a non-immune serum (NI) is shown in lane 7. **Panel B:** Western analysis with an actin antibody. **Panel C**. Detection of association of PI3-kinase with endogenous gelsolin. Lysates were immunoprecipitated with a monoclonal antibody to gelsolin (lanes 2–7) or non-immune serum (NI; lane 1). **Panel D:** Western analysis using an antibody to gelsolin. To detect the levels of gelsolin, the immunoblot shown in panel C was stripped and blotted with an antibody to gelsolin. PI3-kinase, actin, gelsolin, and IgG heavy chain (IgG HC) protein bands are indicated by arrows. The results shown are representative of three independent osteoclast preparations and experiments.

detect association of Src, FAK or other signaling molecules complexed with these transduced proteins.

The ability of the transduced peptides containing PBD to interfere the binding of PI3-Kinase with endogenous gelsolin was investigated by Western analysis of anti-gelsolin immunoprecipitates with an antibody to PI3-kinase (Fig. 5, panels C and D). Osteoclasts transduced with vector protein, HA-TAT (Panel C, lane 2), Mid-Gsn (lane 3) exhibited association of PI3-kinase with gelsolin. A significant decrease in the interaction of PI3-kinase was observed in osteoclasts transduced with NH-Gsn (lane 4) and PBD (lane 5) peptide transduced osteoclasts. Very minimal or no interaction of PI3-kinase with endogenous gelsolin was observed in osteoclasts transduced with peptides containing multiple phosphoinositides binding domains, such as PBD (2) (lane 6) and FL-Gsn (Lane 7). The decrease or failure in association of PI3-kinase with the endogenous gelsolin (Panel C) was parallel to the increase in association of PI3-kinase with the transduced peptides (Fig. A). Immunoprecipitation with a non-

immune serum is shown in lane 1. Panel D shows the levels of gelsolin in each immunoprecipitate as control for comparable loadings. These observations confirm the previously demonstrated specific interaction of PI3-kinase with gelsolin [16].

Transduction of PBD containing gelsolin peptides disrupts actin ring and peripheral dot-like podosome structures
We have previously demonstrated that increased association of phosphoinositides with gelsolin was accompanied with actin cytoskeletal reorganization [13,14]. Therefore, actin organization in response to transduction of various peptides was analyzed by staining the osteoclasts with rhodamine phalloidin (Fig. 6). The localization of transduced peptides was identified by immunostaining the cells with an antibody to HA and viewing under confocal microscopy (Fig. 6). As demonstrated earlier [14], F-actin enriched dot-like podosomal organization with actin ring at the periphery was observed in osteoclasts treated with either PBS (untransduced; A) or transduced with TAT-vector protein (B) and Mid-GSN fragment (E) (indicated by
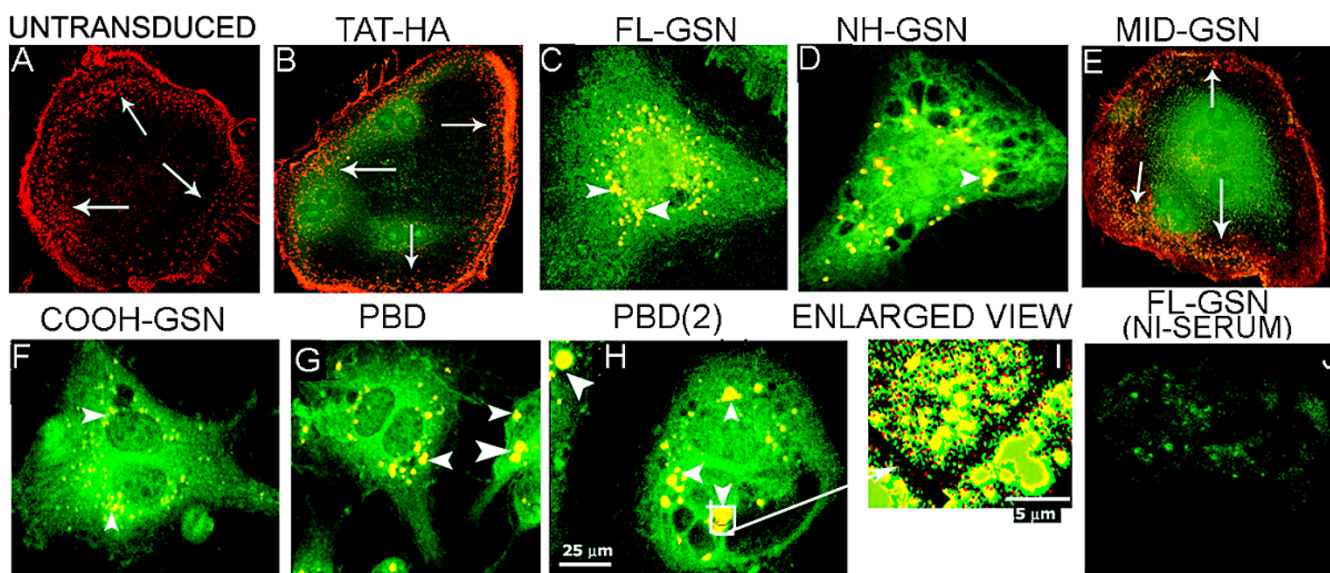
Figure 6
The effects of TAT-HA proteins transduction on actin distribution Confocal microscopy images of mouse osteoclasts stained for actin (red) and HA-protein (green) are shown. An actin enriched patch marked by a white square in Fig. H is magnified in Fig. I. Yellow color indicates the colocalization of transduced peptides with actin. Immunostaining with a non-immune (NI) serum is shown in Panel J. Scale bars- 25 μm (Panels A-H and J) and 5 μm (Panel. I). Arrowheads indicate patches enriched in transduced peptides and actin (Panels C, D, F, G, and H). Arrows indicate typical podosome structures in panels A, B, and E. The results shown are representative of three independent osteoclast preparations and experiments.

arrows). Colocalization of the transduced peptides with F-actin was not observed in these osteoclasts. Co-localization of the transduced peptides with actin (yellow color) was observed in punctate dot-like and patchy structures in osteoclasts transduced with FL-GSN (Fig. C), NH-GSN (Fig. D), COOH-GSN (Fig. F), PBD (Fig. G), and PBD (2) (Fig. H) peptides. These structures were not observed in the clear zone area of osteoclasts (compare C, D, F, and G to A, B, and E). Moreover, the patchy structures (indicated by arrow heads in Figs. C, D, F, G. and H) comprise numerous punctate dot-like structures. A magnified view of a patch (indicated by white square in Fig. H) is shown in Fig. I. In addition to the above changes, transduction of these peptides also caused dissolution of the peripheral actin ring-like structures in these osteoclasts. The transduced gelsolin peptides were localized diffusely throughout the cytoplasm as well as in vesicles (Fig. 6; C-H).

Osteopontin has no effect on the organization of actin ring and podosomes in osteoclasts transduced with various gelsolin peptides containing PBD

Next, we analyzed the effects of OPN on the distribution of podosomes (Fig. 7). Consistent with our previous observations [14], OPN stimulated F-actin rich podosomes formation throughout the surface of osteoclasts

transduced with TAT-HA (B) and Mid-GSN (D) peptides. Podosome organization in the clear zone area is shown in PBS-treated TAT-HA protein transduced control osteoclast (A). Similar to the observation shown in Fig. 6, osteoclasts transduced with FL-GSN (C) or PBDs (E and F) demonstrated changes in the organization of F-actin enriched punctate structures, which was accompanied by a loss of peripheral actin ring-like structure in these osteoclasts (C, E, and F).

Transduction of PBD containing gelsolin peptides increases the F-actin content but has no effect on endogenous gelsolin–actin ratios

Transduction of PBD containing TAT-Gsn fragments as well as FL-Gsn decreases the association of phosphoinositides to endogenous gelsolin (Fig. 4). Subsequently, we tested the hypothesis that increasing the gelsolin peptide levels by transduction would block the ability of OPN to stimulate a change in endogenous gelsolin/actin ratios. Osteoclast lysates, which were immunodepleted for the transduced peptides by immunoprecipitation with an anti-HA antibody, were subjected to a second immunoprecipitation with an anti-gelsolin antibody. We immunoprecipitated the EGTA-resistant gelsolin-actin complexes from lysates made from
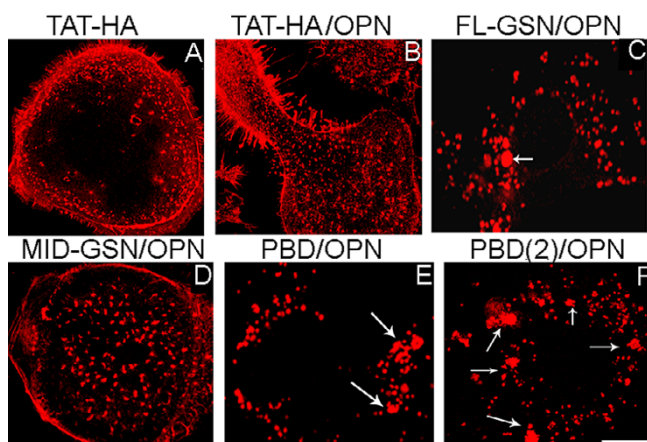
Figure 7
The effects of TAT-HA-protein transduction and osteopontin on actin distribution Confocal microscopy images of mouse osteoclasts stained for actin are shown. Scale bar- 25 μm. Arrows point to patches enriched in F-actin. The results represent one of three experiments performed.

osteoclasts transduced with the indicated proteins shown in Fig. 8. Consistent with our previous observation [13], OPN treatment of osteoclasts transduced with HA-TAT (Fig. 8, lane 2) and Mid-Gsn (lane 6) fragment exhibited a decrease in gelsolin/actin ratios as compared to the PBS-treated controls (lanes 1 and 5). The gelsolin/actin ratio was similar in both PBS- and OPN-treated osteoclasts transduced with FL-Gsn (lanes 3 and 4) and its PBD (2) (lanes 7 and 8), COOH-Gsn (lanes 10 and 11) and NH-Gsn (lanes 12 and 13) fragments. Neither actin nor gelsolin was observed in the non-immune serum immunoprecipitation made from lysates of OPN-treated osteoclasts transduced with PBD (2) fragment (lane 9). The actin and gelsolin bands were scanned and the actin/gelsolin ratio in the complex was calculated. Table 1 shows the quantitative changes in the actin-gelsolin ratios as measured from the EGTA-resistant fraction of endogenous gelsolin bound to actin. Consistent with our previous observations, anti-gelsolin immunoprecipitates of EGTA-treated cell lysates from OPN-treated cells demonstrated a decrease in the amount of actin bound to gelsolin. Osteoclasts from PBS-treated or transduced with HA/TAT (vector protein) demonstrated an actin-gelsolin ratio of 1.45 ± 0.15, and 1.33 ± 0.09, respectively. A decrease in actin/gelsolin ratio was observed in osteoclasts untransduced or transduced with HA-TAT and TAT-Mid-Gsn and stimulated with OPN (Table 1). This decrease was not observed in osteoclasts transduced with FL-GSN as well as its fragments NH-GSN, COOH-GSN, and PBD and treated with OPN. Thus, transduction of PBD containing gelsolin peptides blocked the effects of OPN to decrease gelsolin/actin ratios in osteoclasts, which we interpret to mean that the stimulation of actin uncapping by OPN was abrogated.

Although there were no changes in endogenous gelsolin/actin ratio, the increase in the F-actin enriched structures in osteoclasts transduced with various TAT-gelsolin fusion proteins containing PBD (Fig. 6) prompted subsequent investigations on the levels of F-actin content in these osteoclasts (Table 1). Consistent with our previous observations, OPN stimulated F-actin content in osteoclasts prepared from wild type (WT) mice [13]. An increase in F-actin content was detected in WT osteoclasts transduced with FL-GSN, NH-GSN, COOH-GSN, and PBD proteins. Even though, there is a small increase in the patchy F-actin enriched structures in OPN-treated osteoclasts transduced with FL-GSN or PBD containing peptides (Fig. 6C,6E and 6F), no significant additional effect of OPN in increasing the F-actin content was observed in these osteoclasts. In Gsn-null osteoclasts, neither transduction nor OPN treatment had any effect on F-actin content as demonstrated earlier (data not shown; [15]).

Transduction of PBD containing gelsolin peptides decreases bone resorption activity of osteoclasts
Actin ring formation is a prerequisite for efficient bone resorption. Hence, we analyzed the effects of transduction of TAT/FL-GSN and gelsolin peptides on osteoclast bone resorption activity (Fig. 9). Consistent with our previous observations [14,15], OPN stimulated bone resorption and the formation of multiple overlapping resorption pits in untransduced (B), Mid-GSN (H), and Hsv-TK (P) transduced osteoclasts as compared to PBS-treated respective controls (A, G, and O). Transduction of FL-GSN (C), NH-GSN (E), COOH-GSN (I), PBD (K), and PBD (2) fragment (M) exhibited smaller or equal size of pits compared to control osteoclasts (A and O). OPN treatment had no effect in increasing the pit area in these osteoclasts (D, F, J, L, and N). Quantitating resorption pits on multiple dentine slices per treatment demonstrates a significant increase in pit area in osteoclasts untransduced, transduced with mid-Gsn or HSV-TK and treated with OPN (Fig. 10). This was not observed in osteoclasts transduced with gelsolin peptides containing PBD and treated with OPN (D, F, J, L, and N).

Transduction of PBD containing gelsolin peptides reduces the association of phosphoinositides with WASP and Ezrin proteins
We analyzed OPN stimulation of PtdIns P2 association with some of the candidate proteins involved in the regulation of actin cytoskeleton such as, α-actinin, gelsolin, and profilin. Our results demonstrated that OPN stimulation of osteoclasts had no effect on PtdIns P2 association with vinculin, α-actinin, and profilin [13]. Recent studies
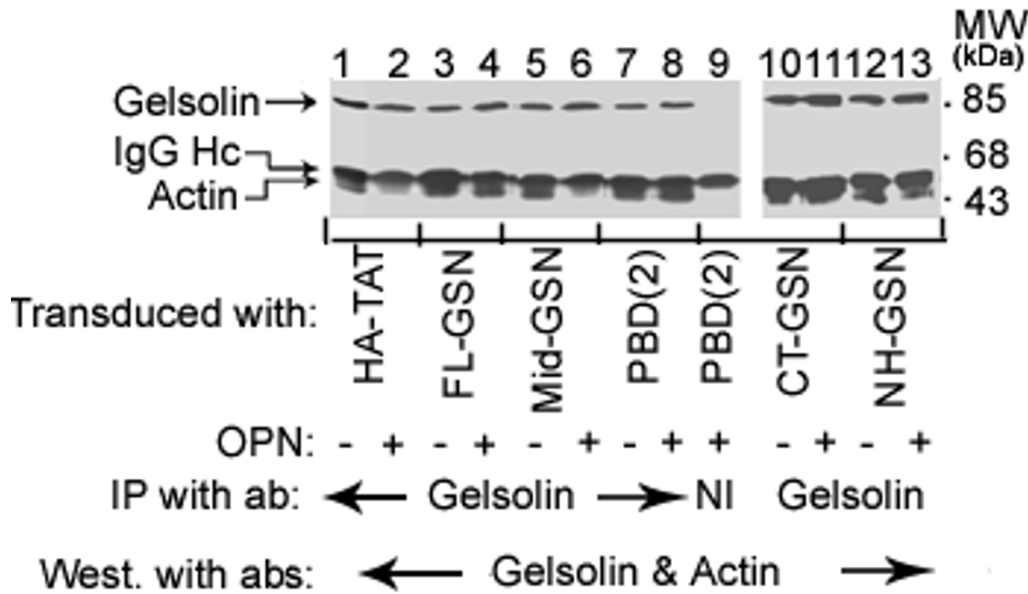
Figure 8
Analysis of the effects of transduction of PBD containing gelsolin peptides and osteopontin on endogenous gelsolin-actin ratio Osteoclasts were transduced with indicated TAT-proteins and treated with OPN (denoted as (+)) or PBS (denoted as (-)). Equal amount of lysates were immunoprecipitated with anti-gelsolin antibody. The EGTA-resistant gelsolin/actin complex was subjected to SDS-PAGE as described in the Methods section. Immunoprecipitation with a non-immune serum (NI) is shown in lane 9. Western analysis was performed with antibodies to gelsolin and actin. Gelsolin, actin, and IgG heavy chain (IgG Hc) bands are indicated by arrows. The quantitative changes in the actin/gelsolin ratio were determined by scanning the gelsolin and actin bands. The mean ± SEM for three experiments is provided in Table 1.

Table 1: The effects of HA-TAT-protein transductions and OPN treatment on gelsolin/actin ratio and F-actin content of osteoclasts. F-actin content was measured by rhodamine-phalloidin binding in osteoclasts treated as indicated in the table. About 4–5 wells were used for each treatment. The experiment was repeated three times. The data presented are mean ± SE for three experiments.

	Gelsolin/actin ratio		F-actin content	
OCs transduced with	PBS	OPN	PBS	OPN
Untransduced	1.45 ± 0.15	0.82 ± 0.18••	1.0	3.5 ± 0.09***, *
Tat/FL-Gsn	1.3 ± 0.15	1.23 ± 0.08	2.5 ± 0.32 *	3.2 ± 0.81*
Tat/NH-Gsn	1.32 ± 0.13	1.35 ± 0.15	1.95 ± 0.05*	2.6 ± 0.06*
Tat/Mid-Gsn	1.38± 0.18	0.71 ± 0.06••	1.20 ± 0.11	3.1 ± 0.33 **, °°°
Tat/PBD	1.42 ± 0.16	1.37 ± 0.14	2.17 ± 0.22*	2.2 ± 0.2 *
Tat/PBD(2)	1.36 ± 0.11	1.44 ± 0.21	2.19 ± 0.10*	2.6 ± 0.23*
Tat/COOH-Gsn	1.35 ± 0.12	1.41 ± 0.14	2.11 ± 0.11*	2.8 ± 0.09*
HA-TAT	1.33± 0.09	0.73 ± 0.07••	1.092 ± 0.02	3.6 ± 0.15 °°°

••p < 001-OPN, Mid-Gsn/OPN, and HA-TAT/OPN-treated vs. respective PBS-treated osteoclasts °°°p < 0.0001 Mid-Gsn/OPN and HA-TAT/OPN-treated vs. HA-TAT/PBS-treated osteoclasts *p < 0.01-PBS, OPN-treated TAT-fusion proteins transduced osteoclasts vs. PBS-treated untransduced (-) and HA-TAT transduced control osteoclasts **p < 001-OPN-treated Mid-GSN transduced osteoclasts vs. PBS-treated Mid-Gsn transduced osteoclasts *** p < 0.0001-OPN-treated osteoclasts vs. untransduced PBS-treated (-) osteoclasts

have demonstrated that podosomes are not formed in macrophages harboring mutations in the Wiscott-Aldrich Syndrome protein (WASP) [47]. PtdIns P2 association has been shown to activate WASP [48] and ERM proteins [49,50]. Here we have demonstrated that in addition to gelsolin, OPN stimulation of PtdIns P2 association with ezrin (Fig. 11, lane 1) and WASP (lane 5) was observed. The levels of PtdIns P2 associated with WASP (lane 3) and ezrin (lane 7) were reduced in osteoclasts transduced with TAT-PBD (2) peptide and treated with OPN as compared
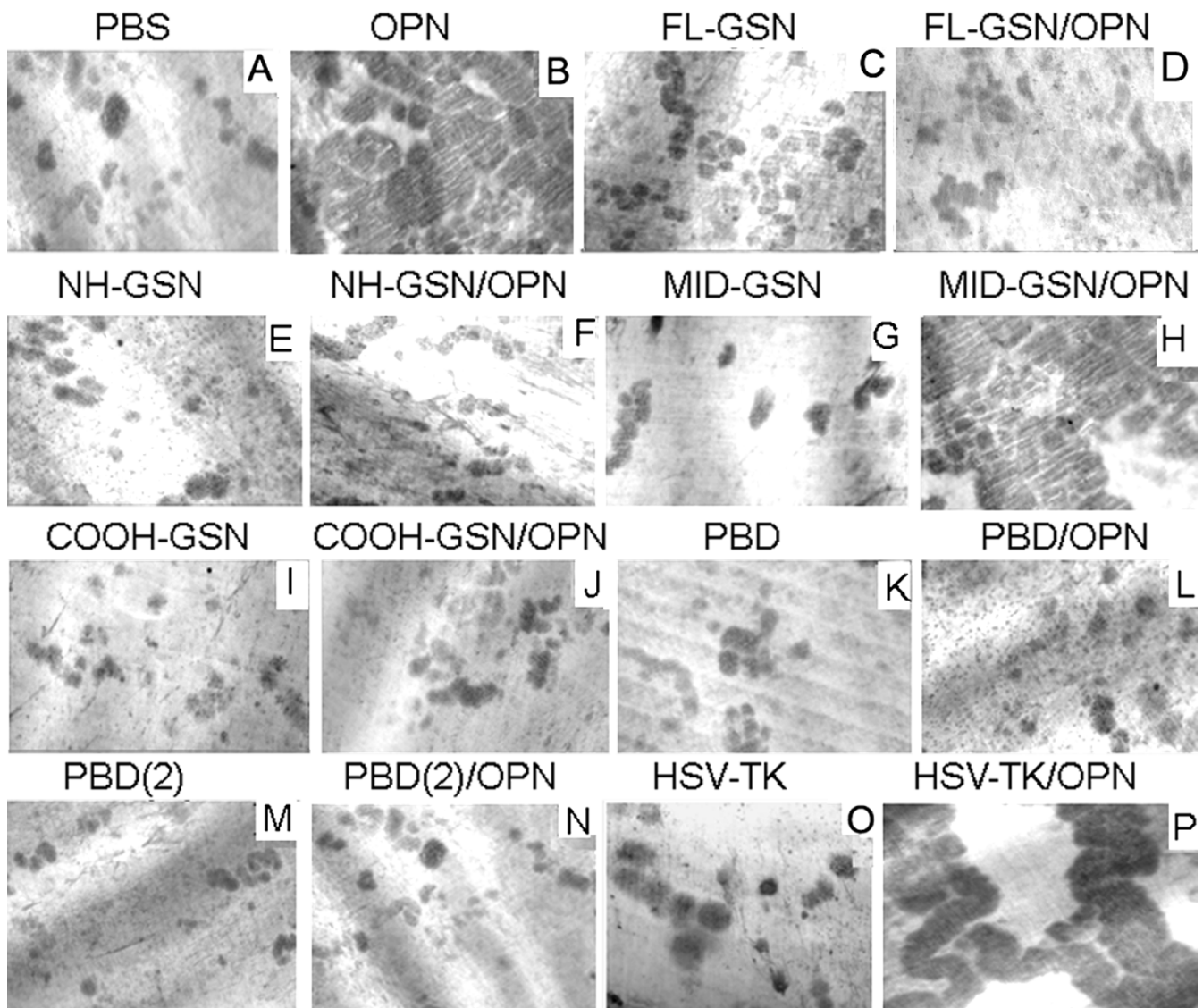
Figure 9
The effects of TAT-HA-protein transduction and osteopontin on bone resorption Osteoclasts were transduced or treated with indicated proteins. Panels A-P: Pits were viewed under 40× objective in a phase contrast microscope and photographed. Each treatment was performed in triplicates. The experiment was repeated three times with three independent osteoclast preparations. The results represent one of the three experiments performed.

to OPN alone treated osteoclasts (lanes 1 and 5). Immunoprecipitation with non-immune serum (NI) is shown in lanes 4 and 8.

Transduction of PBD containing gelsolin peptides reduces the interaction of WASP with Arp3 protein
Arp2/3 complex plays a key role in actin polymerization and contractile ring (actin ring) formation in yeast cells [51]. Arp2/3-WASP complex is essential for the structures-

like podosomes [52] and lamellipodia [53]. Therefore, we hypothesized that WASP-Arp2/3 complex formation would be blocked in osteoclasts transduced with gelsolin fragments containing PBDs. Our observations have supported this hypothesis and demonstrated that transduction of gelsolin fragments containing PBD indeed blocked the Arp2/3–WASP complex formation (Fig. 12). Lysates made from osteoclasts transduced with FL-GSN and PBD (2) were subjected to immunoprecipitation with anti-
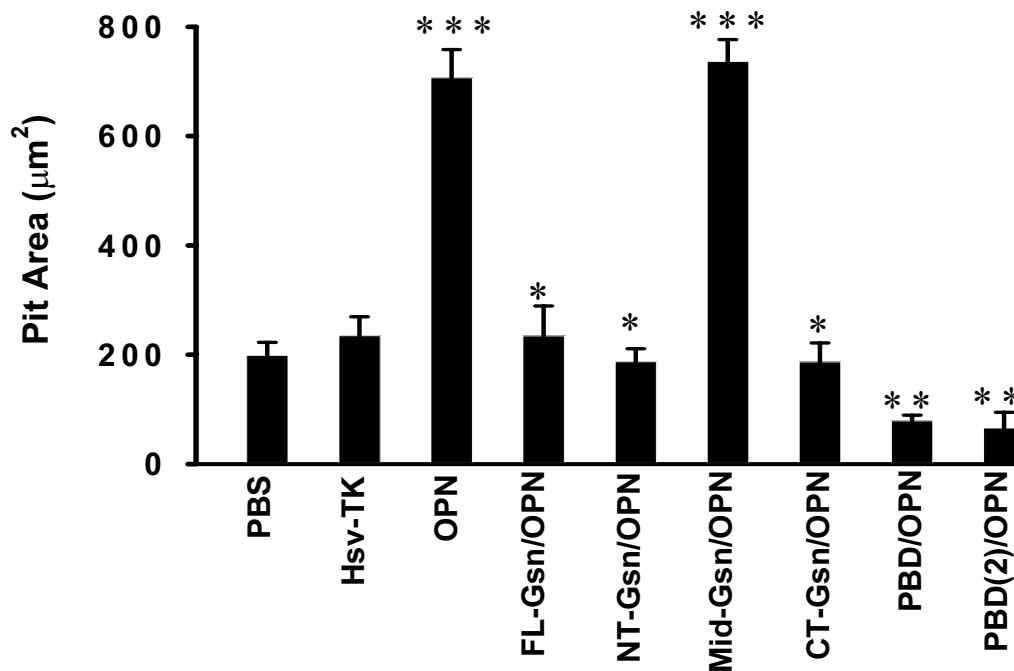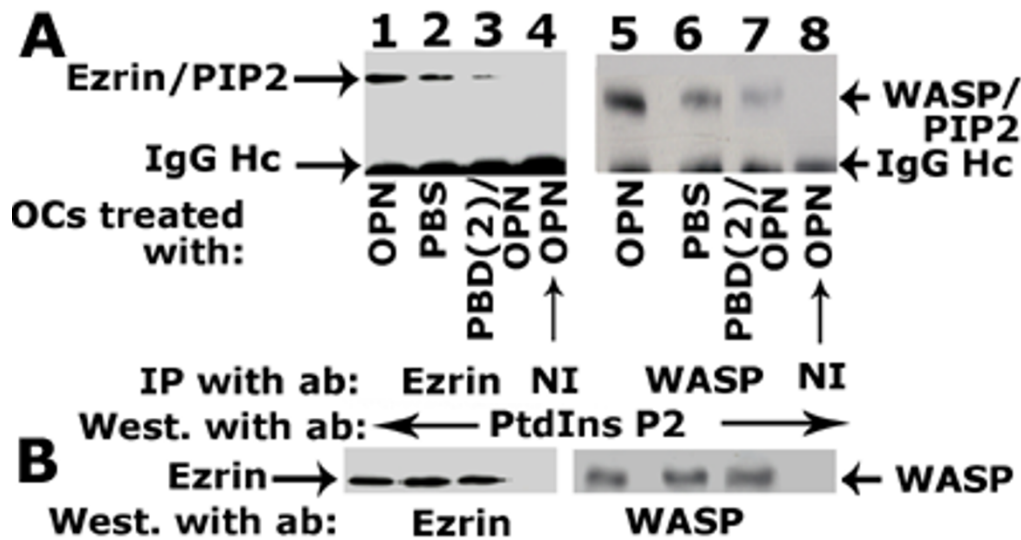
Figure 10
Measurement of pit area. About 25 to 30 pits/slice and three slices from each experiment were scanned. Data shown are mean ± SEM of three experiments. *** p < 0.0001-PBS-treated or Hsv-TK transduced osteoclasts vs. OPN-treated osteoclasts **p < 0.001-OPN alone or Mid-Gsn/OPN-treated osteoclasts vs. PBD (2)/OPN-treated osteoclasts. *p < 0.01 OPN-treated, Mid-Gsn/OPN-treated osteoclasts vs. FL-Gsn/OPN, NH-Gsn/OPN, CT-Gsn/OPN, PBD/OPN-treated osteoclasts.

Arp3 antibody and Western analyzed with WASP antibody. Coprecipitation of WASP with Arp3 was observed in PBS-treated osteoclasts either untransduced (lane 8) or transduced with Mid-Gsn fragment (lane 7). This interaction was more in OPN-treated osteoclasts (lanes 7 and 9). Neither basal level association of Arp3 with WASP nor OPN stimulation was observed in osteoclasts transduced with either FL-Gsn (lanes 2 and 3) or tandem repeats of PBD (lanes 4 and 5). Panel B demonstrates the levels of Arp3 in each immunoprecipitates. Arp3 immunoprecipitates from mid-Gsn transduced osteoclasts exhibited a decrease in the intensity of Arp 3 levels in PBS- (Panel B, lane 6) and OPN- (lane 7) osteoclasts despite more WASP having been coprecipitated (Panel A, lanes 6 and 7) as compared to FL-GSN (lanes 2 and 3) or PBD-transduced (lanes 4 and 5) osteoclasts. Immunoprecipitation with non-immune serum is shown in lane 1 (Panels A and B).

Discussion
On the basis of our previous reports demonstrating the role of gelsolin in osteoclast cytoskeletal reorganization and cell shape changes, we proposed the hypothesis that gelsolin-polyphosphoinositides association was critical to osteoclast function. The results presented here demonstrate that transduced gelsolin peptides, which contain PBDs, associated with polyphosphoinositides, PI3-kinase and actin. Furthermore, the levels of associated polyphosphoinositides were regulated by OPN. The transduced PBD containing peptides caused randomly distributed podosome-like structures. However, the transduced peptides were not targeted to sites of podosome organization in the sealing zone, and they did not substitute or enhance endogenous gelsolin function. Rather, they served dominant negative functions by decreasing polyphosphoinositides binding with endogenous gelsolin, and causing loss of peak stimulation of polyphosphoinositides/gelsolin association in response to OPN. The results regarding osteoclast cytoskeleton were a loss of functional podosomes and actin ring. In cell function terms, the results were bone resorption inhibition, hypomotility, and loss of stimulation in response to osteopontin. The finding that limiting polyphosphoinositides associated with gelsolin disables podosome assembly and organization of the actin ring demonstrates that polyphosphoinositides-mediated signaling is critical to osteoclast cytoskeletal organization, OPN/$\alpha_v\beta_3$ signaling, and cell function.

Figure 11
Transduction of PBD containing gelsolin peptides reduces the association of phosphoinositides WASP and Ezrin proteins Panel A: Western analysis with an antibody to PtdIns P2. Osteoclasts were treated as indicated in the figure. The lysates were immunoprecipitated with either ezrin (lanes 1–3) or WASP (lanes 5–7). Immunoprecipitation with non-immune serum is shown in lanes 4 and 8. Arrows point to IgG HC as well as PtdIns P2 (PIP2) associated ezrin and WASP proteins. **Panel B:** Western analysis with an antibody to ezrin or WASP. Arrows indicate ezrin and WASP proteins. The results represent one of three experiments performed.

We have previously demonstrated that gelsolin is a high affinity barbed-end-capping protein in osteoclasts [13]. Actin polymerization is increased in osteoclasts treated with OPN. Osteoclasts dependent on actin filaments with free barbed ends, for podosome organization and actin ring formation. Here, we have demonstrated that osteoclasts transduced with PBD containing gelsolin peptides failed to exhibit dissociation of endogenous gelsolin from actin filaments in OPN-treated osteoclasts. Therefore, actin/gelsolin (endogenous) ratio was found to be unaffected in both PBS and OPN-treated osteoclasts. Transduction of PBD containing peptides disrupted regulated dissociation of gelsolin from actin filaments as well as actin polymerization as shown in Fig. 8. Binding of phosphoinositides has previously been shown in segment 2, which can induce dissociation of actin-gelsolin complexes (uncapping) [23-25]. The mechanism by which gelsolin regulates actin polymerization is not completely understood. Deletion of S2-3 segments containing the first half PBD domains demonstrated a deleterious effect on cells. Cells exhibited an abnormal shape and complete disruption of the actin cytoskeleton [54]. Introduction of synthetic cell permeant peptide derived from the polyphosphoinositides binding region was shown to disassemble actin filament structures and blocked motility in fibroblasts, platelets, neutrophils, and

melanoma cells [43]. These studies are compatible with our results (Figs. 6 and 7) demonstrating loss of the actin ring at the periphery as well as formation of actin-enriched abnormal large patchy structures and larger size podosomes-like structures randomly throughout the ventral surface of the cell.

Increasing the PBD content in osteoclasts by transduction enhances local accumulation of phosphoinositides. The transduced peptides have varied numbers of PBD i.e. one in NH-GSN, 2 in PBD, three in FL-GSN, and four in PBD (2). The effects of phospholipid were more pronounced with peptide containing PBD (2). The interaction is rank ordered as NH-Gsn = PBD < FL-GSN < PBD (2) by the amount of polyphosphoinositides (PtdIns P2 and -P3) binding (Fig. 2; panel B). Also, binding of transduced PBD peptides with F-actin is evident from the staining of osteoclasts with rhodamine phalloidin. Co-localization of the transduced peptides with F-actin was observed in patchy as well as dot-like podosome structures. Recently, Akisaka et al have demonstrated that structures exhibiting patchy podosomes demonstrated colocalization of F-and G-actin [55]. In the present study, binding of transduced PBD peptides with F-actin is evident from the staining of osteoclasts with rhodamine phalloidin. Also, amino acid residues 168RRV170 in PBD 2 are predicted to prevent
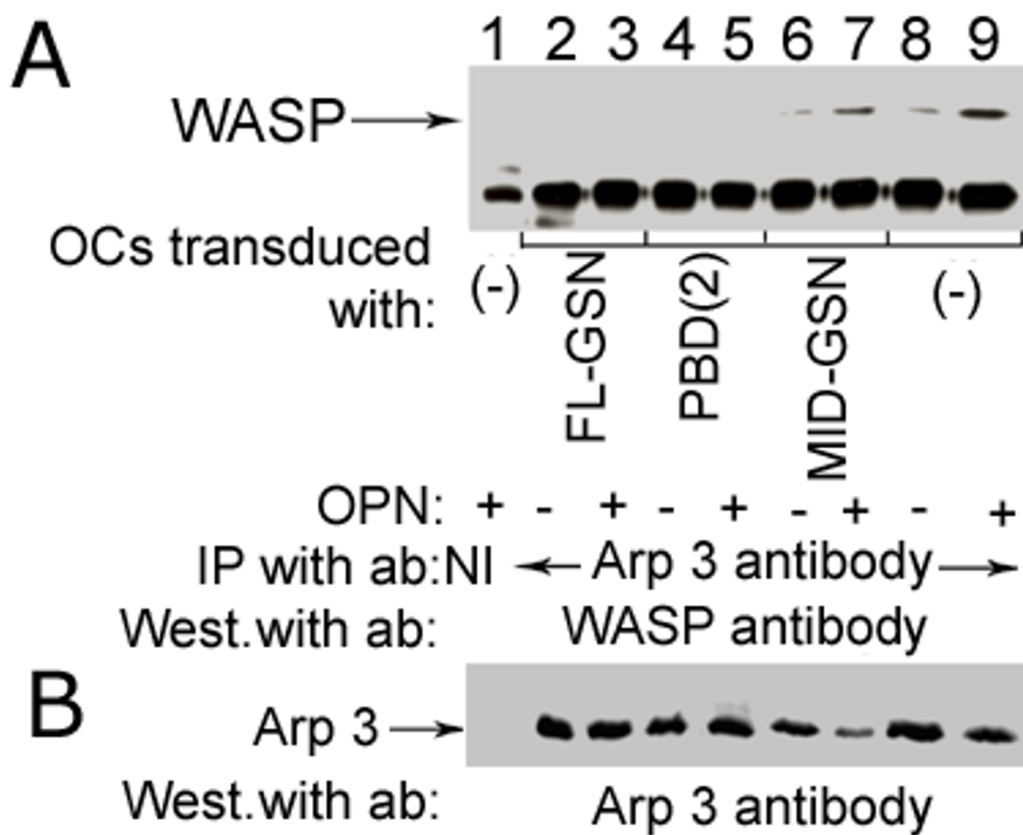
Figure 12
Transduction of PBD containing gelsolin peptides reduces the interaction of WASP with Arp3 protein Osteoclasts were transduced with indicated TAT-proteins and treated with OPN (denoted as (+)) or PBS (denoted as (-)). Equal amount of lysates were immunoprecipitated with anti-Arp3 antibody (lanes 2–9) and non-immune serum (NI, lane 1). The immunoprecipitates were subjected to SDS-PAGE. **Panel A.** Western analysis with a WASP antibody. Arrow points to WASP protein. **Panel B.** Western analysis with an Arp3 antibody. The immunoblot shown in panel A was stripped and blotted with an Arp 3 antibody to detect the levels of Arp 3 in each immunoprecipitates. The results represent one of three experiments performed.

gelsolin domain 2 from binding to monomeric actin (G-actin). PBD (2) present in the amino acid residues 161–169 has the predicted 168Arg-Arg169 (RR) amino sequences, which will block gelsolin domain 2 from binding to monomeric actin. It appears that the patchy structures in PBD transduced osteoclasts is not due to accumulation of G-actin. Hence these patchy may be due to the accumulation of F-actin as well as a decrease in F-actin turnover rate.

We have documented two important roles of phosphoinositides associated with gelsolin in osteoclasts; those are a) Uncapping of actin by PtdIns P2 binding to gelsolin [13]. b) Unique mechanism of PtdIns P3-SH2 mediated signal generating complex formation in osteoclast podosomes [16]. Does transduction of PBD affect PI3-K associ-

ated with endogenous gelsolin? An important insight into the role of phosphoinositides results from the finding that PtdIns P3 associates directly with the SH2 domain of kinase and that PtdIns P3 also disrupts the association of PI3-K with tyrosine phosphorylated proteins by binding to the SH2 domains of the p85 subunit [56]. We have previously demonstrated that SH2 domains of Src and PI3-kinase also bind PtdIns P2, albeit more weakly than their binding to PtdIns P3. Since PtdIns P2 is a substrate for PI3-kinase, the increase in the formation and association of PtdIns P3 with the transduced PBD containing peptides is mediated through preferential interaction of PI3-kinase. Here, we demonstrate that transduction of PBD containing gelsolin fragments have a dominant negative effect in the aspect of limiting the availability of phosphoinositides to the endogenous gelsolin as shown

in Fig. 4. Since the transduced PBD peptides limit the availability of PtdIns P2 to endogenous gelsolin, it limits PI3-kinase interaction as well. The increase in PtdIns P3 levels associated with the transduced PBD containing fragments (Fig. 2B) indicates that the associated PI3-kinase is active. The signaling complex formation in the podosomes could be the direct means for assessing the active nature of the podosomes required for osteoclast function. Even though, PI3-kinase was co-precipitated with the transduced peptides, we do not observe co-precipitation of Src, FAK or other signaling molecules (Chellaiah, unpublished observations [2]) indicating that the patchy podosome-like actin enriched structures do not exhibit normal organization of podosome with respect to signaling complex formation. This also confirms our previously demonstrated observation of PI3-kinase as an immediate interacting protein with gelsolin [16].

OPN was shown to increase the PtdIns P2 levels associated specifically with gelsolin and not with other proteins that bind to phosphoinositides such as, vinculin, α-actinin, or profilin [13]. Phosphoinositides have also been identified as key regulator of members of two families of proteins, ERM and WASP [48,57-59]. PtdIns P2 and Rho GTPases have been shown to be the upstream signaling molecules in WASP-Arp2/3 complex formation [48,60-63] as well as in the activation of ERM proteins [49].We have previously demonstrated that ERM activation is required for its interaction with CD44 receptor and surface expression of CD44 [64]. WASP has been shown to recruit the Arp2/3 complex into podosomes [65]. Podosomes are not formed in cells null for WASP [66] or harboring mutations in the WASP [65]. More recently, Calle et al., have demonstrated that WASP-null osteoclasts are markedly depleted of podosomes. On bone surfaces, osteoclasts fail to form actin rings at sealing zones [67]. We have shown here that OPN stimulated WASP-Arp2/3 complex formation. Transduction of PBD of gelsolin blocked this complex formation. WASP distribution was observed in podosomes and actin ring of osteoclasts. Osteoclasts transduced with PBD failed to demonstrate WASP in the newly formed podosomes or in the actin ring area (unpublished observations). These observations demonstrate that transduction of PBD containing gelsolin fragment exhibit dominant negative effect not only on gelsolin function but also on other phosphoinositides binding proteins such as N-WASP and ezrin.

Cells exhibiting podosomes have been shown to be highly motile. Osteoclasts are uniquely motile in that they continue to resorb bone beneath one area of the cell undersurface while another area of cell is reorganizing and moving [68]. The result is the production of complex multiple overlapping resorption pits as shown in Fig. 9 (Figs. B, H, and P). The rapidity of podosome assembly

and disassembly enables this feature of coordinating cell function with motility [33,69]. Osteoclasts from gelsolin null mice (Gsn-/-) are hypomotile due to retarded remodeling of the actin cytoskeleton [15]. Despite distinct podosome structures are not present in Gsn-/- osteoclasts, actin ring was clearly observed. In Gsn-/- osteoclasts, we did not observe any redundancies within the gelsolin-mediated signaling pathway in podosomes assembly [15]. The presence of actin ring in Gsn-/- osteoclasts indicates the function of WASP in the formation of actin ring. Actin ring formation is required for bone resorption but is not required for motility. With OPN stimulation of αvβ3 integrin, podosomes assembly proceeds by gelsolin/actin dissociation. We fail to observe dissociation of endogenous gelsolin from actin in osteoclasts transduced with PBD containing gelsolin peptides. The dominant negative effect of the transduced proteins, limiting polyphosphoinositides association with endogenous gelsolin was the most likely mechanism causing failure of new podosome assembly. While gelsolin content is a factor in controlling the motility, our results demonstrate that its appropriate targeting to the sealing zone is critically involved in osteoclast cytoskeletal reorganization, cell shape changes, and cell motility. In addition, the nucleating, severing and actin binding functions of gelsolin are responsible for variations between motile and nonmotile status of a cell [19]. Further analyses of the roles of phosphoinositides as well as WASP-Arp2/3 complex formation in podosomes assembly and actin ring formation will give insight into the molecular mechanisms involved in these processes. The data presented here suggests additional role of phosphoinositides in actin ring formation, leading to a critical avenue for future research.

Conclusions

Microinjection or transfection of 'VCA' and 'CA' domains of N-WASP into cells of interest has been shown to function as a constitutively active and dominant negative fragments, respectively in Arp2/3 complex activation [2]. Here, we have demonstrated permeable peptides containing PBD, which has dominant negative effect on the formation of podosomes and actin ring in mouse osteoclasts. These biologically active permeable PBD containing peptides function as inhibitors of motility and bone resorption activity *in vitro* in osteoclasts. Regardless of the type of receptors or signaling pathways, down stream molecules involved in actin cytoskeleton and motility are the same. Therefore, these peptides can be used as a tool to delineate phosphoinositides-mediated signaling orchestrating motility of various cell types involved in inflammatory responses, angiogenesis, normal development, osteoporosis, and cancer metastasis.

Methods
Materials
PtdIns P2 antisera was purchased from Advanced Magnetics (Cambridge, MA) and Echelon Research Laboratories Inc. (Salt Lake City UT). Monoclonal antibodies to gelsolin and hemagglutinin (HA) antibodies, non-immune mouse IgG, and rabbit IgG, Protein A-Sepharose, phospholipids standard, and all the other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies to PI3-kinase and Src were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and Oncogene Research Products, Boston, MA), respectively. Rainbow molecular weight marker and [^{32}P] orthophosphate were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Protein estimation reagent, molecular weight standards for proteins, and PAGE reagents were bought from Bio-Rad (Hercules, CA).

Generation of murine osteoclasts *in vitro*
Mouse osteoclasts were generated *in vitro* using mouse bone marrow cells as described previously [15]. Cells were cultured with the appropriate concentrations of mCSF1 (10 ng/ml; R & D systems) and RANKL (55–75 ng/ml). After 3 days in culture, media were replaced with fresh cytokines. Multinucleated osteoclasts were seen from day 4 and 90–95% TRAP-positive osteoclasts were observed from day 5 onward.

Cloning of gelsolin fragments into TAT-HA vector
Bacterial expression of constructs coding various HIV-TAT fusion peptides of gelsolin were generated by in-frame insertion of full-length human plasma gelsolin cDNA [37], as well as its fragments (Fig. 1, Panel A) into a vector, pTAT-HA. pTAT-HA vector has an N-terminal 6-histidine leader followed by the 11 aa TAT-protein transduction domain flanked by glycine residues, a hemagglutinin (HA) tag, and a polylinker [46,70]. The clone encoding the full-length gelsolin (FL-GSN) included a single open reading frame of about 754 amino acids with nucleotide sequences from 160 to 2357 bp. Two primers were made that enabled the amplification of FL-GSN from sequences 160–2357 bp was: 5'CC AAC AGC ATG GTG GTG GAA C 3' (160–180 bp) and 5'CCG CGG CTCAGC CAT GGC CCT GTC (2334–2357 bp). The following fragments were generated by restriction digestion of full-length gelsolin DNA with kpn 1 enzyme: NH-terminal fragment 169–551 bp; middle fragment: 861–1569 bp; COOH-terminal fragment: 1569 to 2357 bp. These DNA fragments were purified and cloned into the kpn1 site of HIV-TAT vector. Phosphoinositides binding domains (PBDs) 1 and 2 were created by cloning PCR generated fragments using the following primers: 493–519 bp; 5' ACT TCA AGT CTG GCC TGA AGT ACA AGA-3' and 585–602 bp; 5'-ACG CCG CCC TTT GAC CTG3'. The PCR products (FL-GSN and PBDs) were cloned into PCR 2.1 vector. The FL-GSN, and

the PBDs of gelsolin were digested with EcoR1 enzyme, purified, and cloned into TAT-HA vector (Fig. 1B). The sequences of all the clones were confirmed for reading frame by DNA sequencing.

Expression and purification of HA-TAT peptides
Escherischia coli-BL21 (Invitrogen, Carlsbed, CA) was used to transform the HIV-Tat containing gelsolin and its fragments. Bacteria were grown overnight in LB medium containing 100 μg/ml ampicillin. The cultures were induced with 1 mM IPTG for 3–4 h. Cells were sonicated and protein was purified using Ni-NTA column as described [46,70]. Herplex simplex virus thymidine kinase (Hsv-TK) protein (42 kDa) and TAT-HA vector (6–8 kDa) peptides were used as non-specific protein controls. The purified proteins were subjected to SDS-PAGE and Western analysis with a hemagglutinin (HA) antibody to HA-tag present in the TAT-fusion protein. Western analysis with a mono- and polyclonal antibody to gelsolin was also performed. The sequences of all the clones were confirmed for reading frame by DNA sequencing.

TAT-transduction into osteoclasts and preparation of lysates
After 4 or 5 days in culture, osteoclasts were either transduced with TAT-gelsolin peptides or treated with OPN to a final concentration of 25 μg per ml for 15 min. at 37°C as described [49]. For transduction, TAT peptides were added to cells to a final concentration of 100 nM in serum-free medium, after osteoclasts were kept in serum-free α-MEM for 2 h. Dose and time-dependent uptake of peptides were determined. Maximum uptake was observed between 20–60 min. We chose a 45 min. time period for the incubation with TAT peptides. After treatments with TAT peptides or OPN, lysates were made using Triton containing lysis buffer (10 mM Tris-HCl, pH 7.05; 50 mM NaCl; 0.5% Triton X-100; 30 mM sodium pyrophosphate; 5 mM NaF; 0.1 mM Na$_3$VO$_4$; 5 mM ZnCl$_2$; and 2 mM PMSF). Protein contents were measured using the Bio-Rad protein assay reagent kit.

Immunoprecipitation and Western analysis
Equal amounts of lysate proteins were precleared with protein A-sepharose, presoaked in lysis buffer containing BSA, and with non-immune IgG coupled to sepharose. The precleared supernatants were incubated with either HA or gelsolin antibodies and the immune complexes were adsorbed onto protein A-sepharose beads. The beads were pelleted and washed three times for 5 min. each with ice-cold PBS. The immune complexes were then eluted by in electrophoresis sample buffer and analyzed on 8% or 15% SDS-PAGE depending on the molecular weight of the proteins of interest. The proteins were transferred to a PVDF membrane for Western analyses. Blots were blocked

with 10% milk in PBS containing 0.5% Tween (PBS-T) for 2–3 h and then incubated with 1:100 dilution of primary antibody of interest (antibody to PtdIns P2, actin, gelsolin, or PI3-kinase) for 2–3 h. After three washes for 10 min. each with PBS-T, the blot was incubated with a 1:1000 dilution of peroxidase-conjugated species-specific respective 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 (Pierce) [13-15].

^{32}P labeling of cells and TLC analysis of phosphoinositides

After 4 days in culture, osteoclasts were labeled with carrier-free [^{32}P] orthophosphate for 2-h at 37°C as previously described [15]. After labeling, cells were washed twice with serum-free medium and incubated with OPN (25 µg/ml) for 15 min. or the indicated gelsolin peptides in Fig. 5 for 45 min. at 37°C. Osteoclasts were washed with cold PBS and lysates were made using Triton containing lysis buffer. Equal amounts of lysate proteins were immunoprecipitated with the HA-antibody or non-immune serum. Lipids were extracted from the immunoprecipitates as described and dried in the speed vacuum concentrator. 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 as described and lipid spots were visualized by autoradiography. Identification of the radioactive spots was carried out by analyzing the migration of non-radioactive standards as previously described [13].

Bone resorption assay

Whale dentine slices (1.5 cm^2 across and 0.75 mm thick) were cut and processed as described previously [15]. Osteoclasts were transduced with the indicated TAT peptides (100 nM) in Fig. 9 or treated with OPN (10 µg/ml) as described [46]. Cells were scraped gently after incubating with the cell stripper solution (Cellgro by Media tech, Inc.,) for 30 min. [71] and the osteoclast suspension (2 × 10^4 cells) was added to each well. After 2-h of adherence, the culture media were replaced with α-MEM containing either mouse OPN alone (10 µg/ml) or TAT peptides with and without OPN. After 24-h, the medium was replaced with fresh medium containing the respective treatments. Following incubation for 48-h, the slices were washed 7–10 times with water and stained with acid hematoxylin (Sigma, St. Louis, MO) for 6 min. After washing with water several times, the stained pits were imaged under 40× objective in a Zeiss inverted-phase contrast microscope fitted with a SPOT camera (Diagnostic Instruments, Inc., Alexandria, VA, USA). The images were processed by the Adobe Photoshop software program (Adobe Systems, Inc.,). The area of pit was measured from the free-hand traced perimeter using the Scantalytics software

(Scantalytics Inc., IP Lab, Fairfax, VA). Pit area was measured in 40× magnification. About 25 to 30 pits/slice and three slices from each experiment were scanned. Experiments were repeated three times with three independent osteoclast preparations. Each treatment was performed in triplicates. Statistical analysis was performed by analysis of variance (ANOVA) with the Bonferroni corrections as described below.

Immunostaining

After 5d in culture, osteoclasts grown on cover slides were kept in serum-free medium for 2-h and treated with TAT-fusion peptides or OPN as described above. Cells were fixed and immunostained with a monoclonal antibody to HA (1:100 dilution) as described [15]. F-actin was visualized by co-staining the cells with rhodamine phalloidin (1:100 dilution) as described [14]. Osteoclasts were photographed with a BIO-RAD confocal laser-scanning microscope. Confocal images were processed by the Adobe Photoshop software program (Adobe Systems, Inc.,).

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 subjected to various treatments as indicated in Table 1 and washed three times with PBS. Cells were fixed with 1.5% formaldehyde in PBS for 15 min. and permeablized with 0.1% Triton X-100 in PBS for 5 min. The cells were rinsed three times for 5 min. each with PBS and incubated with rhodamine phalloidin (1:100 dilution of 1 mg/ml stock; v/v) for 30–45 min. at 37°C. The cells were washed quickly several times with PBS and extracted with absolute methanol. The fluorescence of each sample was measured using fluorimetry (Bio-Rad). The non-specific binding in the control experiments was subtracted from the total binding to get the specific binding in each sample [72]. Experiments were repeated three times with three independent osteoclast preparations. Three to four wells were used for each treatment. Statistical comparisons were performed as mentioned in data analysis.

Immunoprecipitation of gelsolin/actin complexes

Equal amounts of lysate proteins were used for immunoprecipitation with anti-gelsolin antibody. EGTA-resistant gelsolin-actin complex was prepared from the immune complexes absorbed to protein A sepharose as described [73,74]. The absorbed 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 8% SDS-PAGE. The proteins were transferred to a PVDF membrane for immunoblot analyses. Blots were blocked with 10% milk in PBS containing 0.5% Tween (PBS-T) for 2–3 h and then incubated with 1:100 dilution of gelsolin and actin monoclonal antibodies for 2–3 h. After three washes for 10 min. each with PBS-T, the blot was incubated with a 1:1000 dilution of peroxidase-conjugated mouse 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 (Pierce). The quantitative changes in the EGTA-resistant fraction of actin/gelsolin ratio were determined by scanning the gelsolin and actin bands using the Un-Scan It gel digitizing software (Silk Scientific Inc. Orem, Utah). Statistical comparisons were performed as mentioned in data analysis.

Data analysis

All comparisons were made as "% control", which refers to vehicle-treated cells. The other treatment groups in each experiment were normalized to each control value. Data presented are mean ± SEM of experiments done at different times normalized to intra-experimental control values. For statistical comparisons, analysis of variance (ANOVA) was used with the Bonferroni corrections (Instat for IBM, version 2.0; Graphpad software).

Abbreviations

PtdIns P3, Phosphatidylinositol 3,4,5 trisphosphate; PtdIns P2, Phosphatidylinositol 4,5 bisphosphate PI 3-kinase, Phosphatidylinositol 3-kinase; PBD, phosphoinositide binding domain; FAK, focal adhesion kinase; HA, Hemagglutinin; Osteopontin, OPN; $\alpha_v\beta_3$, vitronectin receptor; F-actin, filamentous actin; G-actin, monomeric globular actin protein.

Authors' contributions

RSB carried out the construction of the TAT-gelsolin expression vector and biochemical studies. DAB assisted RSB in cloning experiments and osteoclast preparations. KAH offered critical comments and participated in drafting the manuscript. MAC designed the experiments, performed bone resorption assays and confocal microscopy analysis of the immunostained cells as well as drafted the manuscript. All authors read and approved the final version of the manuscript.

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