Regulation of Actin Ring Formation by Rho GTPases in Osteoclasts*

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Actin ring formation is a prerequisite for osteoclast bone resorption. Although gelsolin null osteoclasts failed to exhibit podosomes, actin ring was observed in these osteoclasts. Wiscott-Aldrich syndrome protein (WASP) was observed in the actin ring of gelsolin null osteoclast. Osteoclasts stimulated with osteopontin simulated the effects of Rho and Cdc42 in phosphatidylinositol 4,5-bisphosphate (PIP2) association with WASP as well as formation of podosomes, peripheral microfilopodia-like structures, and actin ring. To explore the potential functions of Rho and Cdc42, TAT-mediated delivery of Rho proteins into osteoclasts was performed. Although Rho and Cdc42 are required for actin ring formation, transduction of either one of the proteins alone is insufficient for this process. Addition of osteopontin to osteoclasts transduced with Cdc42Val12 or transduction of osteoclasts with both RhoVal14 and Cdc42Val12 augments the formation of WASP-Arp2/3 complex and actin ring. Neomycin, an antibiotic, blocked the effects of osteopontin or TAT-RhoVal14 on PIP2 interaction with WASP. WASP distribution was found to be cytosolic in these osteoclasts. Depletion of WASP by short interfering RNA-mediated gene silencing blocked actin polymerization as well as actin ring formation in osteoclasts. These results suggest that Rho-mediated PIP2 interaction with WASP may contribute to the activation and membrane targeting of WASP. Subsequent interaction of Cdc42 and Arp2/3 with WASP may enhance cortical actin polymerization in the process of actin ring formation in osteoclasts.

Phosphoinositides are involved in modulating a variety of actin regulatory proteins (1) as well as promoting filament cross-linking to form stable and bundled actin fibers (2). Phosphoinositides have been identified to have a major role in gelsolin function, in the regulation of actin organization, and podosome assembly/disassembly in both mouse and avian osteoclasts (3–5). The sequence QRLFQVKGR in the second phosphoinositides-binding domain (PBD) of gelsolin has been shown to compete with the function of endogenous gelsolin domains for binding phosphoinositides when introduced into fibroblasts, platelets, and neutrophils. These cells exhibited a block in actin assembly as well as motility (6–8). We have used these peptides to delineate phosphoinositides-mediated signaling in actin reorganization, podosomes assembly/disassembly, and bone resorption. Transduction of PBD peptides of gelsolin into osteoclasts produced clusters of podosomes and disrupted the formation of the actin ring. Hence, these osteoclasts were hypomotile and less resorptive (9). Actin ring formation is critical for osteoclast bone resorption but is not required for motility. Transduction of PBD of gelsolin not only blocked the interaction of PIP2 with endogenous gelsolin but also with WASP and ezrin proteins (9).

WASP has been identified as a phosphoinositide-binding protein that regulates actin ring organization in podosomes and lamellipodia (10–12). WASP and N-WASP are both activated by the combination of PIP2 and Cdc42. However, they have different responses when treated with either alone. Although there are differences in the response, PIP2 has a regulatory role in the activation of both WASP and N-WASP (13). Structural and biochemical studies have shown that coordinated binding of PIP2 and Cdc42-GTP causes the activation of WASP (14, 15), which, in turn, stimulates the actin-nucleating function of the Arp2/3 complex (2, 15–18). Arp2 and -3 mediate nucleation and cross-linking of actin filaments in vitro. It has a role in protrusion as well as cortical actin remodeling (19).

Podosome assembly at the leading edge of polarized cells is critically dependent upon WASP, because macrophages from patients with Wiscott-Aldrich syndrome fail to assemble podosomes (20). Macrophages from patients with Wiscott-Aldrich syndrome exhibited defects in actin structure formation because of the inability of cells to localize the actin-nucleating Arp2/3 complex (11). N-WASP plays a role in actin ring formation in fission yeast (21, 22). More recently, it was identified that osteoclasts from WASP-null mice are markedly depleted of podosomes and failed to exhibit actin rings at sealing zones. Complementation of WASP-null osteoclasts with an enhanced green fluorescent protein-WASP fusion protein restores normal cytoarchitecture (23). Osteoclasts transduced with PBD of gelsolin failed to demonstrate WASP in the newly formed podosomes or in the actin ring area (9). Although podosomes are absent in gelsolin null (Gsn−/−) osteoclasts, the actin ring was observed. WASP distribution was observed in the actin ring of Gsn+/−-osteoclasts (24). Hence, these osteoclasts were able to resorb bone, but the resorption pits were simple due to the deficiency of podosomes and the resultant hypomotile nature of osteoclasts (5).

Although distinct pathways and signaling molecules have been described to play roles in the organization of actin ring (25–29), the actual target molecule(s) involved in actin ring formation remain unknown. Hence, in the present study, our objective is to determine the intracellular pathways and the mechanisms by which the WASP-mediated actin ring organization is induced in response to OPN/avβ3 signaling in osteoclasts. An aminoglycoside antibiotic, neomycin, which binds to phosphoinositides, was shown to affect the activation of proteins such as phospholipase D, Ezrin, Radixin, Moesin proteins (ERM), and sodium (Na) hydrogen exchanger as well as binding of PIP2 to...
Role of PIP_2 and Cdc42 in Actin Ring Formation

gelsolin (30–34). We have used neomycin to determine the role of PIP_2- and TAT-mediated delivery of Rho GTPases, such as Rho and Cdc42 in the activation of WASP, as well as cortical actin remodeling in the formation of the actin ring in osteoclasts. Results presented here show that activation and membrane targeting of WASP requires Rho GTPase-dependent PIP_2 interaction with WASP. An increase in PIP_2 5-kinase activity was observed in osteoclasts treated with OPN or transduced with TAT-Rho Val14. Actin ring formation is enhanced by interaction of Cdc42 with the Arp2/3 complex. A cooperative interaction between Rho and Cdc42 has been shown to be required in the process of actin ring formation in osteoclasts.

MATERIALS AND METHODS

Reagents—Horseradish peroxidase-conjugated secondary antibodies for immunoblotting, rainbow molecular weight marker, and [32P]orthophosphate were obtained from Amersham Biosciences. PtdIns P2 antisera was purchased from Advanced Magnetics (Cambridge, MA) and Echelon Research Laboratories Inc. (Salt Lake City, UT). GAPDH antibody was obtained from Abcam Inc. (Cambridge, MA). Neomycin sulfate, rhodamine phalloidin, protein A-Sepharose, monoclonal antibodies to HA, phospholipid standards, phosphatidylinositol 3-kinase inhibitors such as LY294002 [20(4-morphodinyl)-8-phenyl-1(4fj)-benzopyran-4-one] and wort- mannin and all the other chemicals were purchased from Sigma. Protein estimation reagent, molecular weight standards for proteins, and polyacrylamide gel reagents were bought from Bio-Rad. Cy2- or Cy3-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Antibodies to Cdc42, WASP, Arp2, and Arp3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GST-fused WASP/Cdc42 binding domain (WASP-CBD) coupled to glutathione-Sepharose was bought from Cytoskeleton. Inc. (Denver CO).

Preparation of Osteoclast Precursors—Osteoclasts were generated in vitro using the mouse bone marrow cells. Cells isolated from five mice were cultured into 100-mm dishes with 20 ml of α-MEM supplemented with 10% fetal bovine serum (α-10). After culture for 24 h, nonadhered cells were layered on histopaque –1077 (Sigma) and centrifuged at 350 × g for 15 min at room temperature. The cell layer between the histopaque and the medium was removed and washed with α-10 medium at 2000 rpm for 7 min at room temperature. Cells were resuspended in α-10 medium and cultured with the appropriate concentrations of mCSF-1 (10 ng/ml) and osteoprotegerin ligand (55–75 ng/ml). After 3 days in culture, medium was replaced with fresh cytokines. The multinucleated osteoclasts were seen from day 4 onward.

Transduction of TAT-fused Proteins, Treatment of Osteoclasts with OPN, and Lysate Preparation—HA-TAT fusion proteins containing Rho GTPases (Rho and Cdc42 in constitutively active and dominant negative form) were purified as described previously (35–37). GST-C3 was purified as described earlier (38). Herpes simplex virus-thymidine kinase (42 kDa) and HA-TAT vector (6–8 kDa) proteins were used as a nonspecific and vector controls, respectively. Dose- and time-dependent effects of Rho GTPase were determined. Osteoclasts were kept in nonspecific and vector controls, respectively. Dose- and time-dependent PIP_2 interaction with WASP. An increase in PIP_2 5-kinase activity was observed in osteoclasts treated with OPN or transduced with TAT-Rho Val14. Actin ring formation is enhanced by interaction of Cdc42 with the Arp2/3 complex. A cooperative interaction between Rho and Cdc42 has been shown to be required in the process of actin ring formation in osteoclasts.

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phosphoinositide standards (Sigma) were used to identify the 32P-labeled phosphoinositide(s) associated with WASP.

**In Vitro Phospholipid Kinase Assay for PI4P 5-Kinase**—*In vitro* phospholipid kinase assay was performed with Triton-soluble lysate (about 200 μg of lysate protein) made from osteoclasts subjected to various treatments as shown in Fig. 4. Some lysates were pretreated with PI3-kinase inhibitors such as WM (100 nM) or LY294002 (10 μM) prior to *in vitro* kinase assay for 45 min on ice. *In vitro* kinase assay was performed with minor modifications as described (3). Substrates such as PI4P and phosphatidylinosine were used. About 0.16 μmol of total phospholipid (80 nmol each) was used for the assay. Phospholipid was dried from a stock solution in chloroform and then sonicated in kinase assay buffer containing 1% cholate. Phospholipid micelles were added to lysates and incubated in a water bath at 37 °C for 5 min. Ten microliters of solution containing 20 mM Hepes/NaOH, pH 7.4, 5 mM MgCl2, 5 μM ATP, 1 mM DTT, and 5 μCi of [γ-32P]ATP was then added. The mixture was vortexed gently, and the incubation was continued for 30 min at 37 °C (3, 40). Incubation was terminated by the addition of 400 μl of chloroform/methanol/water (5:10:2, v/v). Lipids were then extracted and analyzed by TLC as described above and previously (3).

**Treatment of Osteoclasts with Neomycin**—After 4 days in culture, osteoclasts were kept in serum-free (PO4-) medium for 2 h. The cells were labeled with [32P]PO4 for 2 h in PO4- and serum-free media for 2 h at 37 °C as described previously (3). After labeling, the cells were washed twice with the same serum-free medium and subsequently with the cell permeabilization buffer (120 mM KCl, 30 mM NaCl, 10 mM Hepes, pH 7.2, 10 mM EGTA, 10 mM MgCl2). Freshly prepared 5 mM DTT, 1 mM neomycin was added to the osteoclasts and incubated for 3–5 min. Resealing was achieved by the addition of α-MEM containing 10% fetal bovine serum for 10 min. Cells were washed extensively (3 to 4 times) with α-MEM containing 1% serum and 2% BSA and incubated for different time periods as shown in Fig. 2 to determine the PIP2 association with WASP. First, the time- and dose-dependent effects of neomycin on OPN-induced PIP2 interaction with WASP were performed (data not shown). Cell viability was assessed by trypan blue exclusion. Cell viability was not affected with 1 mM neomycin for 2–3 days. Therefore, 1 mM neomycin was used for the experiments shown in Figs. 4–6. Subsequent to neomycin treatment, osteoclasts were either treated with OPN or transduced with TAT-proteins. Additionally, control cells in the absence of neomycin were also permeabilized with streptolysin O and treated with PBS or OPN (25 μg/ml) as described above. After various treatments, Triton-insoluble lysates were subjected to immunoprecipitation with WASP. The associated phospholipids were extracted and subjected to TLC analysis (Fig. 2) as described previously (3) and above. Unlabeled osteoclasts subjected to various treatments and neomycin as described above were also used for *in vitro* actin polymerization assay, immunostaining, GST-pull down assay, and Western analyses (Figs. 3–6, 8 and 9).

**Cdc42 Activation Assay**—Cdc42 activation assay was performed using the GST-fused WASP-Cdc42 binding domain (CBD) coupled protein beads (catalog number WS03, Cytoskeleton, Inc., Denver, CO). The assay was performed as directed by the manufacturers’ guidelines using the reagents provided in the Cdc42 activation assay biochem kit (BK034; Cytoskeleton, Inc.). Triton-insoluble lysates (200 μg of protein) made from osteoclasts subjected to various treatments as indicated in Fig. 6a were incubated with WASP-CBD bound beads and subjected to SDS-PAGE. Subsequently, immunoblotting with an antibody to Cdc42 (provided in the kit) was performed. Cdc42-GTP molecule pull down with WASP-CBD peptide was visualized by chemiluminescence using the ECL kit (Pierce) (3–5). Total lysate (about 100 μg of protein) not subjected to either immunoprecipitation or GST-pull down assay was also probed with anti-Cdc42 antibody to determine total cellular levels of Cdc42.

**Immunocytochemistry**—Osteoclast precursors (105 cell/coverslips) were seeded on coverslips or osteologic disc for 4–5 days. At day 5, osteoclasts were fixed with 3% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min as described previously. Background fluorescence was blocked by incubating cells with either 5% horse serum or 5% BSA in PBS for 30–45 min at 4 °C. The cells were washed and incubated with primary antibodies of interest (WASP, Arp2, or Arp3, and PIP2; 1:100 dilution) in the blocking solution for 2 h at 4 °C. The primary antibodies were detected with either Cy2- or Cy3-conjugated secondary antibodies. Actin was visualized using rhodamine phalloidin (1:100 dilution; Sigma) as described previously (4, 35). Negative controls were performed with nonimmune mouse and goat sera for the double stainings. The cells were washed and mounted on a slide in a mounting solution (Vector Laboratories) and sealed with nail polish. Immunostained osteoclasts were photographed with a Bio-Rad confocal laser-scanning microscope. Images were stored in TIF image format and processed by the Adobe Photoshop software program (Adobe System Inc., Mountain View, CA).

**Pyrene Actin Polymerization Assay**—Actin polymerization assay was performed using the lysates made from PBS, OPN-, and neomycin/OPN-treated osteoclasts. 100–150 μg (10–15 μl) cytosol and 25 nM Arp2/3 complex as well as ATP and polymerization buffer provided in the kit were added to the assay mixture. 2.0 μM unlabeled and 0.4 μM pyrene-labeled G-actin was used for the assay. 50 nM purified Arp2/3 and WASP proteins were used in assays with no cytosol. The assay was performed in a total volume of 100 μl. Purified Arp2/3 complex (RP01-A) and WASP-VCA (VCG-03) proteins as well as actin polymerization kit (BK003) were purchased from Cytoskeleton, Inc. GST-fused full-length WASP (FL-WASP) was purified using pGEX/FL-WASP construct by following the method as described previously (41). *In vitro* actin polymerization assay was performed essentially by following the manufacturer’s instructions (Cytoskeleton Inc.) and as described previously (15, 42). Cytosol or purified WASP proteins (FL-WASP or WASP-VCA) were preincubated with Arp2/3 complex for 15 min to facilitate WASP-Arp2/3 interaction. Lipid vesicle was prepared essentially as described by Rohatgi et al. (43), added (100 μM total lipid) to the incubation mixture containing purified proteins (FL-WASP or WASP-VCA), and incubated for 15 min at 4 °C. Similarly for neomycin treatment, purified FL-WASP was treated with neomycin (1 mM) for 15 min at 4 °C prior to the addition of either PIP2 vesicle or Arp2/3 complex. PIP2 vesicles (100 μM) and Arp2/3 complex (50 nM) were added sequentially to the incubation mix with 15 min of incubation at 4 °C after each addition. All polymerization assays contained 1 μM unlabeled G-actin and 0.3 μM pyrene-labeled G-actin and 0.2 mM ATP (15, 42) in 100 μl of actin polymerization buffer provided in the actin polymerization kit (BK003). Actin polymerization was measured for 10–15 min at 30-s to 1-min intervals at the excitation of 350 nm (with bandwidth of 20 nm) and emission at the 405 nm in a luminescence spectrofluorometer (Fluoroscan Ascent Lab Systems type 374; software version 2.4.1) at room temperature. Data collected were analyzed and plotted with Microsoft excel (Fig. 9A). F-actin content was measured as described previously (3, 4). Statistical significance was calculated as mentioned below.

**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 the means ± S.E. of experiments done at different times normalized to
intra-experimental control values. For statistical comparisons, analysis of variance was used with the Bonferroni corrections (Instat for IBM, version 2.0; GraphPad software).

RESULTS

Analysis of Interaction of PIP₂ with WASP in Osteoclasts

We have demonstrated previously that OPN/αvβ3-stimulated PIP₂ association with gelsolin mediates actin polymerization and actin filament reorganization in both avian and mouse osteoclasts (3, 5, 9). Subsequently, the effect of OPN on PIP₂ association with WASP (Fig. 1, A–D) was explored. One-half of the WASP immunoprecipitates were immunoblotted with an antibody to PIP₂ (Fig. 1A). OPN stimulation of PIP₂ association with WASP was observed (Fig. 1A, lane 3). The ability of C₃ exoenzyme to block OPN-induced PIP₂ interaction with WASP (Fig. 1A, lane 2) suggests the role of Rho GTase in this process. The level of WASP in each lane was determined by immunoblotting with a WASP antibody as shown in Fig. 1A. The second half of the immunoprecipitates was used for immunoblotting with an antibody to Arp2 (Fig. 1C). Coprecipitation of Arp2 (43 kDa) with WASP was more in OPN-treated osteoclasts (Fig. 1C, lane 3), and this is reduced in osteoclasts treated with C₃ transferase prior to OPN stimulation (lane 2). Immunoprecipitation with nonimmune serum is shown in lane 4 (Fig. 1, A–C). In contrast to the previous observations shown in the interaction of PIP₂ with gelsolin in Triton-soluble fraction, WASP interaction with PIP₂ in response to OPN was observed mostly in the Triton-insoluble fraction. Very small PIP₂ interaction was observed in the Triton-soluble fraction (data not shown). However, Arp2/3 interaction with WASP was found only in the Triton-insoluble fraction (Fig. 1C).

To determine the profile of phospholipids (PI, PIP, PIP₂, or PIP₃) associated with WASP, osteoclasts were labeled with [³²P]PO₄, and lipids associated with WASP were subjected to TLC analysis (Fig. 1D). We have demonstrated previously the binding of PI, PIP, PIP₂, and PIP₃ with Triton-soluble gelsolin (3). Only PIP₂ interaction was observed in the WASP immunoprecipitates, and this interaction is increased in OPN-treated osteoclasts (Fig. 1D, lane 3). Consistent with the immunoblotting analysis shown in Fig. 1A, C₃ pretreatment significantly decreases OPN-induced PIP₂ interaction with WASP (Fig. 1D, lane 4). Furthermore, OPN stimulation of PIP₂ association with WASP was limited to the Triton-insoluble fraction of the osteoclast lysate. Absence of PIP₃ in the TLC analysis implicates the selective role of PIP₂ in the activation process of WASP. The above observations also identify the perceptible role of Rho GTase in the interaction of PIP₂ with WASP and WASP-Arp2/3 complex formation.

Next, the OPN effect in the formation of WASP/Arp3 complex formation was also confirmed by immunostaining osteoclasts with antibodies to WASP (Fig. 1, E and F, red) and Arp3 (green). Osteoclasts
detected in the immunoblotting analysis for the reason that the molecular mass of HA-TAT is 8–10 kDa. (immunoprecipitate to determine the loading levels (Fig. 3, scans of PIP2 interaction with WASP were expressed as percent of control in Fig. 3, top panel) of osteoclasts was observed. Distribution of WASP (white arrows) by Arp3 (yellow color; indicated by arrows) in the plasma membrane (hence referred to as actin ring) of osteoclasts was observed. Distribution of WASP (R, top) and Arp3 (G, bottom) are separately shown in the right panels of Fig. 1, E and F.

The immunofluorescence localization of WASP with PIP2, in resorbing osteoclasts plated on CaPO4-coated osteologic quartz discs is shown in Fig. 1H. These osteoclasts were not kept in serum-free medium or subjected to any treatment. Localization of WASP (Fig. 1H, red) and PIP2 (green) was determined by immunostaining with the respective antibodies. Multiple resorption areas exhibiting ring-like structure were observed. Colocalization (Fig. 1H, yellow) of WASP and PIP2 (indicated by arrows) was observed in the actin ring area. Fig. 1H, asterisks, indicates resorption pits observed underneath these osteoclasts. Immunostaining with nonimmune serum is shown in Fig. 1J.

The Effects of Various Treatments on PIP2 Interaction with WASP

Determination of the Levels of Transduced Rho Proteins in Osteoclasts—To elucidate the potential role of Rho GTPase in the interaction of PIP2 with WASP, osteoclasts were transduced with TAT-fused Rho GTPases. The following TAT fusion proteins were used: RhoVal14, RhoAsn19, Cdc42Val12, Cdc42Asn17, herpes simplex virus-thymidine kinase (negative control protein), and HA-TAT (vector control protein). The uptake of TAT-Rho fusion proteins was determined by Western analysis using an antibody to HA. About 500 μg of osteoclast lysate protein was used for Western analysis (Fig. 2). An increase in Rho uptake was observed in a time-dependent manner. The uptake reaches maximal levels at 40–60 min and decreases after 2 h. But the protein appears to be stable for up to 6 h and becomes reduced from 12 h onward in the osteoclasts. Fig. 2C demonstrates the uptake of TAT-Rho GTPases (approximate molecular mass of 30 kDa) such as Cdc42Val12 (lane 2), Cdc42Asn17 (lane 3), RhoVal14 (lane 4), and RhoAsn19 (lane 5) after transduction for 45 min. Lysate made from HA-TAT protein (8–10 kDa)-transduced osteoclasts was used in Fig. 2C, lane 1. Loading was normalized to the cellular levels of GAPDH (Fig. 2, B and D).

The Effects of Rho GTPases on the Interaction of PIP2 with WASP—Osteoclasts subjected to various treatments were immunoprecipitated with a WASP antibody and immunoblotted with an antibody to PIP2, as shown in Fig. 3, A and D. An antibody to GAPDH was added to each immunoprecipitate to determine the loading levels (Fig. 3, B and E). The effects of Rho and Cdc42 proteins are shown in Fig. 3, A and D, respectively. These results also demonstrate the effect of OPN. Densitometric scans of PIP2 interaction with WASP were expressed as percent of control in Fig. 3G. Osteoclasts treated with OPN (Fig. 3A, lane 2; Fig. 3D, lane 5) and transduced with RhoVal14 (Fig. 3A, lane 6) demonstrated an increase in PIP2 association with WASP. OPN or RhoVal14-induced PIP2 interaction was blocked in osteoclasts pretreated with C3 transferase (Fig. 3A, lanes 3 and 7). Basal level PIP2 interaction with WASP was observed in osteoclasts treated with PBS, RhoAsn19, and HA-TAT (Fig. 3A, lanes 1, 4, and 8). To compare the results quantitatively, densitometric scanning of three different blots was performed. The binding efficiency was determined (mean ± S.E.; n = 3) after normalizing the WASP-associated PIP2 levels to GAPDH levels. The data were provided...
as a histogram in Fig. 3G. An average of 3–4-fold increase in PIP2 interaction with WASP was observed in OPN-treated and TAT-RhoVal14-transduced osteoclasts.

Cdc42 was shown to activate N-WASP and WASP in combination with PI3K (14, 17). Hence, we have analyzed the effects of constitutively active Cdc42Val12 and dominant negative Cdc42Asn17 on PIP2 association with WASP (Fig. 3D). Although transduction of Cdc42Val12 increases PIP2 interaction with WASP (Fig. 3D, lane 4), the level was lower than that observed in OPN-treated (Fig. 3D, lane 5; Fig. 3A, lane 2) or RhoVal14-transduced osteoclasts (Fig. 3A, lane 6). However, OPN treatment of osteoclasts transduced with Cdc42Val12 increases PIP2 association with WASP (Fig. 3D, lane 6). An average of 2.5–3-fold increase was observed in osteoclasts transduced with Cdc42Val12 and subsequently treated with OPN (Fig. 3G). Osteoclasts transduced with RhoAsn19 prior to transduction with Cdc42Val12 had no effect in PIP2 interaction with WASP. Similarly, OPN treatment could not release the inhibitory effect mediated by RhoAsn19 in the interaction of PIP2 with WASP (Fig. 3A, lane 5). But a significant increase in PIP2 association with WASP was observed in osteoclasts first transduced with Cdc42Asn17 and subsequently with RhoVal14 (Fig. 3D, lane 7). These results strongly suggest the role of Rho GTPase in PIP2 interaction with WASP.

**TLC Analyses of Phosphoinositides**

**In Vitro Phospholipid Kinase Assay Analysis**—As there is an increase in PIP associations with WASP in osteoclasts transduced with RhoVal14 or treated with OPN, we proceeded to determine whether there was an increase in PI4P 5-kinase activity in these osteoclasts. Rho GTTPase was identified as an upstream regulator of PI4P 5-kinase and PI3-kinase (35, 44–46). The direct role of Rho in the activation of PI4P 5-kinase was determined by in vitro phospholipid kinase assay analysis. Triton-soluble lysate from osteoclasts subjected to various treatments as indicated in Fig. 4A was used for this analysis. Because both PI3-kinase and PI 5-kinase are activated by Rho or OPN treatment (35), some lysates were pretreated with PI 3-kinase inhibitors such as wortmannin (WM) or LY29004 prior to in vitro kinase assay in the presence of PI4P as substrate and [γ-32P]ATP. Labeled lipids were extracted and subjected to TLC analysis. The products generated by either PI 3-kinase (e.g. PI(3,4)P2 and PI(3,4,5)P3 (PIP3)) or PI 5-kinase (PI(4,5)P2 (PIP2)) was determined by TLC analysis. Lysates made from osteoclasts treated with OPN (Fig. 4A, lanes 2, and 8) or transduced with TAT-RhoVal14 (lanes 4 and 9) exhibited an increase in the levels of PIP2 through activation of PI 5-kinase as compared with PBS-treated (lane 1) or Herpes simplex virus-thymidine kinase-transduced (lane 3) osteoclasts. Very negligible or no increase in the PIP2 levels was observed in osteoclasts transduced with either TAT-Cdc42Val12 (Fig. 4A, lane 7) or RhoAsn19 (lane 5), respectively. Pretreatment of osteoclasts with C3 prior to transduction with TAT-RhoVal14 blocked Rho-induced PI 5-kinase activity as well as the synthesis of PIP2 (Fig. 4A, lane 6). Lysates untreated with either WM or LY290042 exhibited both PIP2 and PIP3 (Fig. 4A, lanes 10–14). Formation of PI(3,4)P2 was not observed in these assays despite the formation of PIP3. Formation of both PIP2 and PIP3 in osteoclasts treated with OPN or transduced with TAT-RhoVal14 indicates the activation of PI 5-kinase and PI 3-kinase, respectively. Lysates treated with either WM (Fig. 4A, lanes 1–7) or LY290042 (Fig. 4A, lanes 8 and 9) failed to exhibit PI 3-kinase products PI(3,4)P2 or PI(3,4,5)P3 (PIP3). PI 5-kinase mediated accumulation of PI(4,5)P2 (PIP2) was unaffected by these inhibitors (Fig. 4A, lanes 1–9). The above observations suggest that OPN-mediated increase in PIP2 interaction with WASP occurs through Rho GTTPase-mediated pathway. Rho plays a key role in the activation of PI 5-kinase and PI 3-kinase, which are involved in the formation of both PIP2 and PIP3, respectively, in osteoclasts.

**Analysis of Interaction of Phospholipids with WASP in Vivo by TLC Analysis**—We have shown interaction of PIP2 with WASP in Fig. 1. In order to determine the role of PIP3 in the activation of WASP, we have used neomycin, which has been shown to competitively inhibit PIP2 interaction with ezrin, sodium (Na+) hydrogen exchanger, and gelsolin (31–34). Determination of interaction of PIP2 with WASP in response to various treatments was determined by TLC analysis (Fig. 4B). Densitometric scans of PIP2 spots of three experiments were expressed as

![FIGURE 4. The effects of Rho transduction on PI4P 5-kinase activity and PtdIns P2 association with WASP. A, in vitro kinase assay analysis. Triton-insoluble fraction of lysates made from osteoclasts (OCs) subjected to various treatments as indicated below each lane was used for in vitro phospholipid kinase assay. Lysates were either untreated (lanes 10–13) or treated with PI 3-kinase inhibitors such as wortmannin (WM, lanes 1–7) and LY290042 (LY, lanes 8 and 9) prior to in vitro kinase assay. Assay was performed in the presence of [γ-32P]ATP as well as PI4P and phosphatidylserine as substrates. After kinase reactions, 32P-labeled lipids were extracted and subjected to thin layer chromatography. B, TLC analysis of the phospholipids associated with WASP immunoprecipitates. 32P-Labeled osteoclasts were subjected to various treatments as indicated in the figure. Lysates made from these osteoclasts were immunoprecipitated (IP) with either WASP antibody (ab) (lanes 1–12) or nonimmune serum (NI; lane 13). 32P-Labeled phosphoinositides associated with immunoprecipitates were extracted and analyzed by TLC as described under "Materials and Methods." An autoradiogram of thin layer chromatography is shown in A and B. Arrows indicate the migrated PIP2 (A and B) and PIP3 (A; lanes 10–14). The results shown are representative of three independent osteoclast preparations and experiments. C, densitometric scans of three experiments (mean ± S.E.; n = 3) is expressed as the percent changes in PIP2 association with WASP. *, p < 0.0001 versus PBS or HA-TAT-transduced osteoclasts; †, p < 0.05 versus HA-TAT transduced osteoclasts; XXX, p < 0.0001; ‡, p < 0.001 versus RhoVal14-transduced and OPN-treated and osteoclasts; ‡, p < 0.01 versus OPN-treated osteoclasts.
percent of control in Fig. 4C. An increase in PIP2 association with WASP was observed in OPN-treated (Fig. 4B, lane 2) or TAT-RhoVal14-transduced (lane 8) osteoclasts. Neomycin at a dose of 1 mg/l decreased OPN-induced association of PIP2 with WASP in a time-dependent manner (Fig. 4B, lanes 3–6). Similarly, RhoVal14 transduction of osteoclasts preincubated with neomycin had no effect in increasing PIP2 interaction with WASP (Fig. 4B, lane 7). As shown in Fig. 4B, a minimal increase in PIP2 association with WASP was observed (lane 11) in TAT-Cdc42Val12-transduced osteoclasts, and this interaction is increased by treatment with OPN (lane 12). The increase was found to be equal to the levels observed in OPN-treated (Fig. 4B, lane 2) or TAT-RhoVal14-transduced (lane 8) osteoclasts. The levels of PIP2 associated with WASP in HA-TAT (Fig. 4B, lane 9) and TAT-RhoAn19 (lane 10)-transduced osteoclasts are equal to the basal level association observed in PBS-treated osteoclasts (Fig. 4B, lane 1). Immunoprecipitation with a non-immune serum is shown in Fig. 4B, lane 13. Observations shown in Figs. 1–4 elucidate the upstream regulatory role of the Rho GTPase in the interaction of PIP2 with WASP.

Immunostaining Analysis of the Effects of Neomycin on the Membrane Targeting of WASP Protein

Recombinant WASP, expressed in Escherichia coli, was observed to bind strongly to Cdc42, weakly to Rac, and not at all to Rho (47). Although, Rho was not identified as a direct WASP binding partner, observations shown in Figs. 1–4 indicate that WASP interaction with PIP2 as well as its membrane targeting is Rho-dependent. To clarify further the roles of Rho GTPase and PIP2 in detail in the activation and membrane targeting of WASP, immunostaining analysis was performed using an antibody to WASP (Fig. 5a, green) and rhodamine phalloidin for actin (Fig. 5a, red). Osteoclasts transduced with constitutively active RhoVal14 increases clusters of podosomes formation as well as colocalization of WASP and actin in the podosomes (Fig. 5a, indicated by arrows). Although OPN-treated osteoclasts exhibit numerous podosomes throughout the subsurface of osteoclasts (Fig. 5a, panel D), podosome clusters as observed in TAT-RhoVal14-transduced osteoclasts (Fig. 5a, panel A) were not observed. OPN treatment resulted in the formation of numerous lateral microfibrillar-like extensions (Fig. 5a, panel D) from the plasma membrane. Podosomes, actin ring, as well as the lateral microfibrillar protrusions from the plasma membrane exhibit colocalization of WASP and actin in OPN-treated osteoclasts (Fig. 5a, panel D). Even though TAT-RhoVal14 transduction increases podosome cluster formation, the extent of actin ring formation, as well as colocalization of WASP/actin in the actin ring area (Fig. 5a, panel A, indicated by wiggly arrows), is smaller than that of OPN-treated osteoclasts. Microfibrillar-like extensions from the cell periphery were absent in these osteoclasts (Fig. 5a, panel A). Formation of numerous podosomes and peripheral filopodia-like structures suggests the activation of both Rho and Cdc42 in OPN-treated osteoclasts. A time-dependent inhibition of clusters of podosomes formation as well as colocalization of WASP and actin in the actin ring was observed in osteoclasts treated with neomycin for 45 min (Fig. 5a, panels B and E) and 2 h (Fig. 5a, panels C and F). Transduction of constitutively active RhoVal14 or stimulation with OPN had no effect on the formation of podosomes or actin ring in these osteoclasts. Distribution of WASP was observed throughout the cytoplasm of these osteoclasts.

Effects of Neomycin on the Interaction of WASP with Arp2 and PIP2

Next distribution of WASP/Arp2 (Fig. 5b, panels A–C) and WASP/PIP2 (Fig. 5b, panels D–F) was observed by immunostaining and confocal microscopy analyses (Fig. 5b). OPN stimulated colocalization of WASP (Fig. 5b, green) and Arp2 (red) in the actin ring (Fig. 5b, panel B) as compared with PBS-treated osteoclast (Fig. 5b, panel A). The yellow color (Fig. 5b, panel B, shown by white arrows) indicates colocalization of WASP and Arp2 at the periphery in the actin ring. Very minimal colocalization of these proteins was observed in the actin ring of neomycin/OPN-treated osteoclasts (Fig. 5b, panel C). Neomycin causes diffused distribution of both Arp2 and WASP in the cytoplasm of these osteoclasts. Similarly, a significant increase in colocalization (Fig. 5b, panel E, yellow and indicated by black arrow) of PIP2 (Fig. 5b, green) and WASP (Fig. 5b, red) was observed in OPN-treated osteoclasts (Fig. 5b, panel E) as compared with PBS-treated control cells (Fig. 5b, panel D). Consistent with the biochemical evidence of inhibition of PIP2 interaction with WASP by neomycin (Fig. 4), immunostaining analysis also exhibits inhibition of interaction of WASP (Fig. 5b, red) and PIP2 (Fig. 5b, green) in the actin ring (Fig. 5b, panel F). Diffused distribution of PIP2 was observed in the cytoplasm of these osteoclasts (Fig. 5b, panel F). Basal localization of WASP (Fig. 5b, panel R) was observed in the actin ring of these osteoclasts (Fig. 5b, panel F). Observations shown here substantiate the role of PIP2 in the activation of WASP and WASP-Arp2/3 complex formation.

Analysis of the Effects of OPN and Rho on the Interaction of Cdc42 with WASP

Cdc42 was shown to activate N-WASP and WASP in combination with PIP2 (14, 17). By having established the interaction of PIP2 with WASP in osteoclasts, the interaction of Cdc42 with WASP was then determined (Fig. 6a, panel A). WASP immunoprecipitates were immunoblotted with an antibody to Cdc42. OPN treatment (Fig. 6a, lane 4) and TAT-RhoVal14 transduction (lane 6) increases Cdc42 interaction with WASP. More intriguingly, neither of these effects occurs in osteoclasts pretreated with neomycin (Fig. 6a, lanes 2 and 5). In neomycin-pretreated osteoclasts, the levels of Cdc42 associated with WASP were found to be lower than the PBS-treated control (Fig. 6a, lane 3) or TAT-herpes simplex virus-thymidine kinase (a nonspecific protein control; lane 7)-transduced osteoclasts. Immunoprecipitation with a nonimmune serum is shown in Fig. 6a, lane 1. WASP level in each immunoprecipitate is shown in Fig. 6a, panel B. A decrease in binding of Cdc42 with WASP in osteoclasts treated with neomycin prior to OPN stimulation (Fig. 6a, lane 2) or RhoVal14-transduction suggests that PIP2 interaction with WASP may take place preceding Cdc42 interaction with WASP. Binding of PIP2 to WASP increased the effectiveness of Cdc42 binding to WASP. In view of the fact that only GTP-Cdc42 binds to WASP-Cdc42-binding domain, the associated Cdc42 ought to be in the form GTP-Cdc42 (14, 48).

Inhibition of interaction of GTP-Cdc42 with WASP in neomycin-pretreated osteoclasts raises the following two possibilities: 1) WASP is present in the inactive state; 2) PIP2 may be required for the activation of Cdc42 as shown by others (43, 49). Hence, to determine the second possibility, lysates made from osteoclasts treated with neomycin prior to OPN or RhoVal14 were used for GST-pull down analysis. Given that only GTP-Cdc42 binds to Cdc42 binding domain of WASP (WASP-CBD; see Refs. 14 and 48), GST-fused WASP-CBD protein was used for pull down analysis. Western analysis with an antibody to Cdc42 is shown in Fig. 6a, panel C. Immunoprecipitation with an antibody to Cdc42 was used as identification control (Fig. 6a, panel C, lane 10), and pull down with GST alone (vector protein) was used as negative control (lane 1). 100 µg of lysate from osteoclasts subjected to various treatments (Fig. 6a, panel D) was immunoblotted with an antibody to Cdc42 to determine the cellular levels of Cdc42. Our observations indeed demonstrated an increased pull down of GTP-Cdc42 in RhoVal14-transduced
Actin Ring Formation Depends on the Interaction of PIP2 and Cdc42 with WASP in Osteoclasts

Next, we performed experiments to determine the role of Cdc42 in actin ring formation by immunofluorescence analyses (Fig. 6b). Consistent with the observations shown by Linder et al. (50) in macrophages, increasing the intracellular levels of Cdc42Val14 in osteoclasts decreases the actin core present in podosomes (Fig. 6b, panel A, arrows). Although WASP staining was observed in podosomes and plasma membrane (in Fig. 6b, panel G), F-actin staining was not observed in the podosomes of these osteoclasts (Fig. 6b, panel R). These osteoclasts exhibited numerous microfibrillar-like extensions at the periphery with...
minimal or no distribution of actin. Addition of OPN to osteoclasts transduced with TAT-Cdc42Val12 not only increases F-actin core in podosomes but also actin polymerization in the peripheral microfibrillar extensions. These structures display colocalization of actin and WASP (Fig. 6b, panel B). Minimal colocalization of WASP and actin in the actin ring of osteoclasts transduced with TAT-Cdc42Val12 (Fig. 6b,
panel A) indicates that Cdc42 alone has negligible effect in the activation of WASP in osteoclasts. Pretreatment of osteoclasts with neomycin for 2 h prior to TAT-Cdc42Val12/OPN treatments blocked colocalization of actin and WASP at the periphery in the actin ring but had no effect on the filopodia-like extension formation. Although these osteoclasts exhibited filopodia-like extensions at the periphery (Fig. 6b, panel B), colocalization of F-actin and WASP was not observed in these structures. WASP distribution was mostly cytoplasmic in these osteoclasts. Osteoclasts transduced with TAT-RhoVal14 increases the number as well as clusters of podosomes formation (Fig. 6b, panel C). But actin ring formation is not equal to the levels observed in OPN-alone treated osteoclasts as shown in Fig. 5a. Cotransduction of constitutively active forms of TAT-Cdc42Val12 with RhoVal14 induced prominent cytoskeletal changes such as podosomes formation throughout the surface of osteoclasts, striking band of actin ring, and microspike extensions with adhesion structures (Fig. 6b, panel E). In the presence of neomycin, about 80–90% inhibition of colocalization of WASP and actin in podosomes as well as in the actin ring was observed. Long smooth filopodia-like extensions were observed as a replacement for microspike-like extensions with adhesion structures in these osteoclasts. Colocalization of actin and WASP is minimal in these extensions as compared with cells in Fig. 6b, panels B and E. Hence, these observations indicate that due to the failure of activation of WASP by PIP2, its interaction with Cdc42 as well as actin polymerization in the
actin ring area is inhibited, although diffuse distribution of WASP throughout the osteoclasts and in the smooth filopodia-like extensions were observed in osteoclasts pretreated with neomycin (Fig. 6b, panels C and F).

**Effects of siRNA to WASP on Actin Ring Formation in Osteoclasts**

We then introduced siRNAs into osteoclasts to reduce endogenous levels of WASP in osteoclasts. Transfection of WASP siRNA into osteoclasts by Lipofectamine 2000 (Invitrogen) produced 30–40% silencing of WASP mRNA, although introduction of siRNA to WASP into streptoclasts by Lipofectamine 2000 (Invitrogen) produced 30–40% silencing levels of WASP in osteoclasts. Transfection of WASP siRNA into osteoclasts pretreated with neomycin (Fig. 8, panel A, lanes 4 and 5) but not in scrambled (Sc) siRNA-treated (Fig. 7a, panel A, lane 3) osteoclasts. Streptolysin O treatment alone had no effect on the endogenous WASP levels (Fig. 7a, panel A, lane 2). Immunoblotting of the same blot with an antibody to GAPDH (Fig. 7a, panel B) was used as loading control.

Localizations of WASP and actin were determined in osteoclasts treated with siRNA to WASP (Fig. 7b). A significant decrease in actin ring matched the WASP level in osteoclasts treated with siRNA nucleotides (Fig. 7b, panels A–D and A’–D’) was observed. Actin ring as well as WASP distribution was barely detectable at 1 μM siRNA (Fig. 7b, panel D). Neither actin clumps nor bundles were observed in these osteoclasts. Cell shape or the distribution of actin and WASP was not affected by permeabilization with streptolysin O (Fig. 7b, panel A) or transfection of scrambled siRNA to WASP (1 μM; Fig. 7b, panel B). siRNA-mediated attenuation of WASP levels not only reduced the formation of actin ring but also bone resorption in osteoclasts (data not shown).

**Immunoblotting Analysis of Interaction of Arp2 with WASP**

The effect of various treatments on the formation of WASP/Arp2 complex was subsequently analyzed by immunoblotting of WASP immunoprecipitates with Arp2 antibody (Fig. 8). RhoVal14 transduction increases PIP2 interaction with WASP (Fig. 3) and augments F-actin content equal to the levels observed in OPN-treated osteoclasts (Fig. 9B). However, in RhoVal14-transduced osteoclasts, Arp2 (Fig. 8, lane 2) association with WASP is not increased to the levels observed in OPN (lane 5)-treated osteoclasts. Similar increase in Arp2 interaction with WASP was observed in osteoclasts transduced with TAT-Cdc42Val12 (Fig. 8, lane 10). Interaction of Arp2 with WASP was enhanced in osteoclasts treated with TAT-Cdc42Val12/TAT-RhoVal14 (Fig. 8, lane 4), TAT-Cdc42Val12/OPN (lane 11), and TAT-Cdc42Asn17/OPN (lane 12). The augmented effect observed in these osteoclasts is equal to levels observed in OPN-alone treated osteoclasts (Fig. 8, lane 5). OPN-induced Arp2 interaction is blocked by pretreatment with neomycin (Fig. 8, lane 6) as well as HA-TAT- (lanes 1), RhoAsna19 (lanes 3), and Cdc42Asn17 (lane 14)-transduced osteoclasts. Immunoprecipitation with a nonimmune serum is shown in Fig. 8, lanes 9 and 15. The above observations indicate that both Rho- and Cdc42-mediated events are required for WASP activation, interaction of...
Arp2/3 with WASP, and cortical actin polymerization. Immunoblotting of the same blot with an antibody to WASP (Fig. 8B) was used as loading control.

Actin Polymerization in Vitro

We then examined the role of PIP2 in the activation of WASP and the subsequent effect on actin polymerization mediated by the Arp2/3 complex. It has been demonstrated in vitro that WASP is activated by PIP2 and prenylated GTP-γS in order to arbitrate actin polymerization through its binding to Arp2/3 (51, 52). Hence, in vitro actin polymerization assay was performed in the presence of unlabeled and pyrene-labeled G-actin (42) (Fig. 9A). Purified Arp2/3 complex was used to a final concentration of 25 nM. An increase in actin polymerization was observed in lysate made from OPN-treated osteoclasts. This increase was blocked by neomycin treatment, and the effect was equal to the level observed in PBS-treated osteoclasts. The basal levels of Arp2/3-mediated actin polymerization in PBS- and neomycin/OPN-treated osteoclasts may be due to the WASP that is present in the masked unstimulated form. A similar inhibitory effect of neomycin was observed in the assay with purified proteins. The effects of Arp2/3-mediated actin polymerization in the presence of WASP-VCA and FL-WASP protein is more than the levels observed in assays performed with lysates made from OPN-treated osteoclasts. Maximum actin polymerization effect was observed with WASP-VCA protein, and addition of PIP2 micelle had no extra stimulatory effect on the effects mediated by WASP-VCA. Although WASP-VCA is more potent than FL-WASP, addition of PIP2 to FL-WASP increased polymerization to the level equivalent to WASP-VCA protein. The actin polymerization observed in reactions containing FL-WASP may be because some of the purified protein is present in active unmasked conformation. Addition of PIP2 to the reaction containing FL-WASP may activate the inactive masked WASP and actin polymerization as well. However, neomycin blocked PIP2-mediated activation of FL-WASP. Similar inhibition was observed in lysate made from neo/OPN-treated osteoclasts. Observations shown here as well as in Figs. 4–6 and 8 suggest that activation of WASP is mediated by direct binding of PIP2.

Measurement of F-actin Content

Next, the F-actin content in osteoclasts subjected to various treatments (Fig. 9B) was measured because this is compatible with the activation of WASP as well as the actin nucleating and polymerization function of Arp2/3. An increase in F-actin was observed in OPN, TAT-RhoVal14, TAT-Cdc42Asn17/TAT-RhoVal14, and TAT-Cdc42Val12/OPN-treated osteoclasts. Osteoclasts transduced with Cdc42Val12 can activate actin polymerization to a minimal extent, and it requires TAT-RhoVal14 to reach maximal activation. Pretreatment of osteoclasts with neomycin or C3 transferase significantly blocked the OPN- or Rho-induced F-actin levels in osteoclasts. Although OPN treatment increases F-actin content in osteoclasts transduced with TAT-Cdc42Val12, it does not have any effect in osteoclasts transduced with TAT-RhoAsn19. Osteoclasts transduced with TAT-Cdc42Val12, TAT-RhoAsn19, and TAT-Cdc42Asn17 did not have any effect on F-actin levels. PBS-treated or herpes simplex virus-thymidine kinase-transduced osteoclasts were used as controls.

DISCUSSION

Podosomes bring about motility and bone resorption functions of osteoclasts. We have demonstrated previously (5, 9, 35) the roles of gelsolin and the phosphoinositides binding domains of gelsolin in the podosome assembly/disassembly. Also, previous studies from our laboratory and other laboratories have demonstrated the role of several signaling molecules in osteoclast cytoskeletal organization, podosome assembly, actin ring formation, and bone resorption (27, 28, 35, 37, 53–57, 59, 62–64). It is not known at this time, how these signaling molecules regulate cytoskeletal remodeling toward actin ring formation. Also unknown are the target proteins involved in this course of action. WASP has been identified to have a regulatory role in the assembly and maintenance of podosomes in macrophages (50). WASP-null osteoclasts generated in vitro through the siRNA silencing technique failed to exhibit podosomes, actin ring, and actin plaques (Fig. 7). The most recent report by Calle et al. (23) has shown that in cultures of WASP-null osteoclasts, the formation of actin ring was markedly impaired. Although WASP-null osteoclasts were able to polymerize actin and form actin patches, highly dynamic podosomes or actin rings were not organized as in normal osteoclasts (23). Osteoclasts from gelsolin null (Gsn−/−) mice failed to exhibit distinct podosomes, but these osteoclasts demonstrated actin ring in the clear zone area (5). WASP distribution was observed in the actin ring of Gsn−/− osteoclasts (24). In the absence of WASP, gelsolin did not function to form the actin ring in WASP-null (−/−) osteoclasts. Failure of formation of actin rings in WASP−/− osteoclasts and the localization of WASP in the actin ring of Gsn−/− osteoclasts indicate that WASP could be the most probable candidate protein involved in the actin ring formation.

WASP functions as a scaffolding protein for Arp2/3, which is required for actin nucleation and polymerization. WASP binding with Arp2/3 takes place after full activation of WASP by PIP2 and Cdc42 (14, 15, 17, 18, 48, 65). Several laboratories have studied the regulatory molecules involved in the activation of WASP (14, 15, 48, 65–67). We have demonstrated previously that PIP2 regulates uncapping of gelsolin, actin polymerization, and podosome assembly in osteoclasts. Observations shown here have demonstrated colocalization of both PIP2 and WASP in the actin ring of resorbing osteoclasts. Cytotoxic characterization of RAW 264.7 osteoclast-like cells and bone marrow osteoclasts in which Arp2 was knocked down revealed fewer podosomes and no actin rings in resorbing osteoclasts, although many cells remained well spread (68). The above observations provide an explanation for the interaction of WASP with PIP2 and then Arp2/3 in the configuration of actin ring during bone resorption.

Participation of members of the WASP superfamily (WASP and Scar) in the formation of cortical actin polymerization to induce filopodia or membrane ruffling has been shown to be mediated by the members of Rho GTPases such as, Rho, Cdc42, and Rac (51, 69–71). An increase in the formation of PIP2 from PIP (substrate) was observed in in vitro kinase assay using lysates made from osteoclasts treated with OPN or transduced with RhoVal14. Osteoclast lysates treated with LY29004 or wortmannin blocked PI 3-kinase activity and not PI 4P 5-kinase activity. However, osteoclasts pretreated with C3 transferase prior to OPN addition or TAT-RhoVal14 transduction inhibited the formation of both PIP2 and PIP3. This indicates that both phosphatidylinositol 4-phosphate 5-kinase (PI4P 5-kinase) and PI 3-kinase activation are Rho-mediated. Moreover, inhibition of PIP3 synthesis and not PIP2 in wortmannin- or LY29004-treated osteoclasts indicates that the single phospholipids spot in the TLC analysis (Fig. 4A) is PI4(5)P2 (synthesized by PI4P 5 kinase) and not PI3(4,5)P2 (synthesized by PI3K). Physical association of the small GTPase Rho with a 68-kDa PI4P 5-kinase was observed in Swiss 3T3 cells (72). PI4P 5-kinase has been identified as direct regulator of PIP2 levels and CD44-ERM-actin complex formation (31, 44). Overexpression of PI4P 5-kinase, which synthesizes PIP2, was shown to induce actin-based movement of raft-enriched vesicles through WASP–Arp2/3 complex formation (17). An increase in the interaction of PIP2 with WASP in the Triton-insoluble fraction of OPN-treated or
Role of PIP2 and Cdc42 in Actin Ring Formation

RhoVal14-transduced osteoclasts (Figs. 1 and 3–5) are compatible with the functional profile favoring membrane targeting of WASP protein as well as its interaction with Arp2/3 complex. Inhibition of this interaction in C3 transferase-treated osteoclasts and a very small or no increase in the interaction of PIP2 with WASP in osteoclasts transduced with Cdc42Val12 supported the role of Rho GTPase in the activation of WASP.

Subsequently, the role of PIP2 in the activation of WASP was also confirmed by using neomycin. Neomycin, an aminoglycoside antibiotic, was identified to efficiently probe PIP2 on the cell membranes where binding of large molecules to PIP2 could be inhibited (73). Binding of neomycin to PIP2 was shown to affect the activation of ERM proteins regardless of cell type (31). Similarly, neomycin inhibited the effect of PIP2 on gelsolin function (34). Hence, we used this approach to determine the role of PIP2 in the membrane localization of WASP as well as actin ring formation. The results presented here identify PIP2 as regulator of podosomes assembly and actin ring formation in osteoclasts.

Membrane targeting of WASP, its interaction with Arp2/3, and actin ring formation is inhibited in neomycin-treated osteoclasts (Figs. 5, 6, and 8). WASP distribution was mostly cytoplasmic in these osteoclasts. As the number of podosomes is reduced in these osteoclasts, we propose that neomycin not only blocks WASP activation but also gelsolin function as well. This is consistent with our previous observations of the role of PIP2 in the gelsolin-mediated assembly of functional podosomes in osteoclasts (5, 9).

Although RhoVal14 transduction increases PIP2 interaction as well as membrane targeting of WASP (Figs. 3 and 4), it is not sufficient to form actin ring in osteoclasts (Fig. 5a). PIP2 is required for the activation of WASP- and neomycin treatment-blocked WASP activation in osteoclasts. Hence, it was not surprising that the level of GTP-Cdc42 coimmunoprecipitated with WASP immunoprecipitates is lesser in osteoclasts treated with either neomycin/RhoVal14 or neomycin/OPN than RhoVal14-transduced or OPN-treated osteoclasts (Fig. 6a, panel A). However, a decrease in binding of GTP-Cdc42 to GST/WASP-CBD in the GST-pull down analysis (Fig. 6a, panel B) using lysates from neomycin/RhoVal14 or neomycin/OPN was unanticipated if one envisaged that PIP2 has no effect on the activation of Cdc42. Most interestingly, activation of Cdc42 by PIP2 has been described recently by others (43, 49, 74). In addition to its role in actin polymerization (3, 14, 66), phosphoinositides are shown to have several other functions as follows: displacement of the guanine nucleotide dissociation inhibitor protein (75); activation of guanine nucleotide exchange factors by binding to their pleckstrin homology domain (76, 77); and stimulation of release of GDP from G-proteins (60). In the stimulation of guanine nucleotide exchange factor and release of GDP, PIP2 was shown to be more effective than P1 (60). Although the mechanism is not clear, it appears that PIP2 regulates activation of Cdc42 through its binding to Dbs, a Dbl family member that has been shown to have a specific regulatory role in the activation of Cdc42 and RhoA. Dbl family members are guanine nucleotide exchange factors for Rho GTPases and possess tandem Dbl family member that has been shown to have a specific regulatory role in the activation of Cdc42, Rac, and then Rho GTPases (61). The increase in actin ring formation in addition to filopodia-like extensions in osteoclasts transduced with both RhoVal14 and Cdc42Val12 indicates that PIP2 interaction with WASP is indeed a key step in the full activation and membrane targeting of WASP. Rho transduction increases podosomes formation throughout the surface of osteoclasts, but these cells do not exhibit microfilibrillar or microfilopodia-like structures at the periphery as observed in osteoclasts transduced with Cdc42Val12. Osteoclasts treated with OPN exhibit both peripheral microfilopodia-like projections and podosomes throughout the surface of osteoclasts. These structures demonstrate colocalization of WASP and actin. Formation of podosomes throughout the surface of osteoclasts and microfilibrillar structures at the periphery indicate the activation of both Cdc42 and Rho in OPN-stimulated osteoclasts.

Although stimulation of actin polymerization has been shown to be independent of PIP2 synthesis in polymorphonuclear leukocytes (60), an increase in actin polymerization was observed in response to PIP2 interaction with WASP in osteoclasts. The in vitro polymerization assay (Fig. 9A) provides a tool to measure the role of PIP2 in the WASP activation process. An increase in actin polymerization was observed in the presence of PIP2 vesicle and FL-WASP. Inhibition of actin polymerization in neomycin-treated osteoclasts and by neomycin in assays with purified protein (FL-WASP) and PIP2, mimics suggests that PIP2 interaction with WASP is required for its activation. This observation supported others who have shown that addition of PIP2 vesicle to WASP and Arp2/3 complex accelerated actin polymerization and produced an increase in actin filaments. Furthermore, addition of GTPγS-Cdc42 doubled the effect of PIP2. Nonlipidated Cdc42 was found to be ineffective because it does not bind vesicles (14).

Actin ring formation requires cooperative interaction of PIP2 and Cdc42 with WASP. The actin remodeling in the organization of podosomes is highly dynamic as opposed to actin ring formation during bone resorption. Hence, actin-remodeling processes during podosomes assembly/disassembly and actin ring formation are spatially separated. The present study provides evidence that Rho-mediated PIP2 interaction with WASP may contribute to the activation and membrane targeting of WASP. Subsequent interaction of Cdc42 and Arp2/3 with WASP may enhance cortical actin polymerization in the process of actin ring formation in osteoclasts. There seems to be no competition existing between PIP2 and Cdc42 in their binding to WASP. PIP2 is also required for the activation of Cdc42. The findings from this study are our first step to determine the regulatory mechanisms involved in actin ring formation. Further investigation on the spatially and temporally regulated functions of both WASP and gelsolin is necessary to identify their role in actin ring formation as well as podosomes assembly/disassembly during bone resorption and osteoclast motility.
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