Phosphorylation of a Wiscott-Aldrich Syndrome Protein-associated Signal Complex Is Critical in Osteoclast-associated Bone Resorption*

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The activities of different kinases have been correlated to the phosphorylation of Wiscott-Aldrich syndrome protein (WASP) by studies in multiple cell systems. The purpose of this study was to elucidate the regulatory mechanisms involved in WASP phosphorylation and the resulting sealing ring formation in osteoclasts. The phosphorylation state of WASP and WASP-interacting proteins was determined in osteoclasts treated with osteopontin or expressing either constitutively active or kinase-defective Src by adenovirus-mediated delivery. In vitro kinase analysis of WASP immunoprecipitates exhibited phosphorylation of c-Src, PYK2, WASP, protein-tyrosine phosphatase (PTP)-PEST, and Pro-Ser-Thr phosphatase-interacting protein (PSTPIP). Phosphorylation of these proteins was increased in osteopontin-treated and constitutively active Src-expressing osteoclasts. Pulldown analysis with glutathione S-transferase-fused proline-rich regions of PTP-PEST revealed coprecipitation of WASP, PYK2, c-Src, and PSTPIP proteins with the N-terminal region (amino acids 294–497) of PTP-PEST. Similarly, interaction of the same signaling proteins, as well as PTP-PEST, was observed with glutathione S-transferase-fused proline-rich regions of WASP. Furthermore, osteopontin stimulation or constitutively active Src expression resulted in serine phosphorylation and inhibition of WASP-associated PTP-PEST. The inhibition of PTP-PEST was accompanied by an increase in tyrosine phosphorylation of WASP and other associated signaling proteins. Experiments with an inhibitor to phosphatase and small interference RNA to PTP-PEST confirmed the involvement of PTP-PEST in sealing ring formation and bone resorption. WASP, which is identified in the sealing ring of resorbing osteoclasts, also demonstrates colocalization with c-Src, PYK2, PSTPIP, and PTP-PEST in immunostaining analyses. Our findings suggest that both tyrosine kinase(s) and the tyrosine phosphatase PTP-PEST coordinate the formation of the sealing ring and thus the bone-resorbing function of osteoclasts.

Osteoclasts are terminally differentiated, multinucleated, and highly motile cells responsible for bone resorption. The adhesion of osteoclasts to bone leads to the formation of the osteoclast clear zone, an actin-rich ring-like adhesion zone (sealing ring) circumscribing an area of bone resorption. Sealing ring formation has been considered to be a marker of osteoclast activation for bone resorption (1, 2). Bone resorption is mediated by the dynamics of an actin cytoskeleton ring. Distinct pathways and signaling molecules (c-Src, PYK2, c-Cbl, p130 Cas, leupaxin, and phosphatidylinositol 3-kinase) have been described to play roles in the organization of the sealing ring during bone resorption (3–10). Despite success in identifying the role of these kinases in osteoclast sealing ring formation in the clear zone of resorbing osteoclasts, the potential target protein of these kinases involved in the sealing ring formation is poorly understood. Calle et al. (11) have demonstrated that osteoclasts from WASP2 knock-out mice failed to demonstrate sealing ring, and these osteoclasts are bone resorption-disabled. Expression of WASP restores normal cytoarchitecture in these osteoclasts. More recently, we have demonstrated that sealing ring formation and bone resorption are enhanced by interaction of phosphatidylinositol 4,5-bisphosphate (PIP2) and Cdc42 with the WASP (12).

WASP integrates signals from Rho, Cdc42, and kinase(s) to bind to the Arp2/3 complex and stimulate Arp2/3-dependent actin polymerization (13–15). Phosphorylation of WASP has been reported in several cell systems. Tyrosine or serine phosphorylation of WASP increases the actin polymerization activity of WASP through the Arp2/3 complex (15–19). The phosphorylation of WASP at Tyr-291 is regulated by several kinases, including c-Src, FAK, Hck, and Nck (17, 18, 20–23). Efficient phosphorylation and dephosphorylation of WASP at amino acid Tyr-291 within the GTPase binding domain (GBD) of WASP is critical for osteoclast function and bone resorption.

2 The abbreviations used are: WASP, Wiscott-Aldrich syndrome protein; CA-Src, constitutively active Src; KD-Src, kinase-defective Src; FAK, focal adhesion kinase; F-actin, filamentous actin; GST, glutathione S-transferase; GBD, GTPase binding domain; GP domain, GBD and proline-rich domain; OPN, osteopontin; PAO, phenyl arsine oxide; PSTPIP, proline-, serine-, threonine-rich phosphatase-interacting protein; PTP-PEST, protein-tyrosine phosphatase-proline, glutamic acid, serine, threonine amino acid sequences; siRNA, small interference RNA; VCA, verprolin, cofilin acidic domain; aa, amino acids; SH, Src homology; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; cRNAi, control RNA interference; α-MEM, minimum Eagle’s α-medium; PIP2, phosphatidylinositol 4,5-bisphosphate; Hck, hematopoietic cell kinase; FL, full length.
WASP are both reliant on binding of Cdc42 (24) or PIP2 (12) with WASP. Phosphorylated GBD is able to bind SH2-containing tyrosine kinases (e.g. c-Src), thus destabilizing the inhibitory GBD-C-terminal VCA interaction and promoting Arp2/3 binding and actin polymerization (25). It was suggested that the ability of SH2 domains to bind and activate WASP could also have several functional implications. Phosphorylation of WASP increases the affinity of the C-terminal VCA domain of WASP for the Arp2/3 complex and is required for actin polymerization (21).

The protein-tyrosine phosphatase (PTP)-PEST was also shown to interact with WASP (26). PTP-PEST contains the typical tyrosine phosphatase catalytic domain flanked by proline-rich regions. These regions are capable of interacting with several signaling molecules, including paxillin, leupaxin, PYK2, p130 Cas, and Grb2 (10, 27–30). PTP-PEST interacts with WASP via a proline, serine, threonine phosphatase interacting protein (PSTPIP (26)). PSTPIP functions as a scaffold protein between PTP-PEST and WASP (20). It has a role in the regulation of cortical cytoskeleton reorganization via its SH3-mediated interactions with WASP (31). Moreover, this interaction allows PTP-PEST to dephosphorylate WASP (32). Although Fyn enhanced WASP-mediated Arp2/3 activation and was required for synapse formation, PTP-PEST combined with PSTPIP inhibited WASP-driven actin polymerization and synapse formation (20). Mutation of the tyrosine residue (Tyr\Delta Asp) that is present in the SH3 domain of the polyproline region of PSTPIP resulted in a loss of WASP binding in vitro and dissolution of colocalization in vivo (32). By regulating the tyrosine phosphorylation state of PSTPIP, PTP-PEST plays a key role in dephosphorylation of signaling proteins, such as p130 Cas and paxillin (33).

WASP is a binding partner for the c-Src SH3 domain (34). We have demonstrated the role of PIP2 and Cdc42 in the WASP-mediated sealing ring organization in response to integrin αvβ3 signaling (12). The mechanism by which signaling molecules regulate sealing ring formation in a complex spatial and temporal manner is unknown. It is important to identify the interaction between potential kinases/phosphatase(s) and WASP in the regulation of sealing ring formation during bone resorption. It is likely that interaction of phosphatase with WASP, in addition to its role in regulating the phosphorylation status of signaling proteins associated with WASP, may have a regulatory role in the stability of the sealing ring during bone resorption. A decrease or failure of sealing ring formation, in a number of conditions, is correlated to failure of osteoclast adhesion or proper signaling pathway mediated by the integrin αvβ3 (35-37). We have sought to further characterize the mechanism(s) by which the WASP-Arp2/3 complex becomes stabilized to localize to the sealing ring during the process of bone resorption by osteoclasts. Identification of mechanism(s) linking sealing ring formation and bone resorption will elucidate the distinctive properties of WASP and the associated signaling complex. Therefore, our aim in this study was to elucidate the mechanisms involved in the phosphorylation and dephosphorylation of WASP, and to decipher possible signaling pathways that control sealing ring formation and bone resorption in osteoclasts.

This study shows that osteoclasts treated with OPN or transfected with constitutively active Src (CA-Src) exhibited tyrosine phosphorylation of proteins, including PYK2, PTP-PEST, WASP, c-Src, and PSTPIP. Serine phosphorylation of proteins with molecular mass of 160–170 and 80–85 kDa was also observed. Basal level phosphorylation of these proteins was observed in control or kinase-defective Src (KD-Src)-transfected osteoclasts. The presence of PSTPIP in the WASP immunoprecipitates suggests that it may serve as a linker protein in the interaction of PTP-PEST with WASP. Serine phosphorylation of PTP-PEST in response to CA-Src expression and OPN stimulation suggests that PTP-PEST may have a regulatory role in the tyrosine phosphorylation state of proteins associated with WASP. An increase in phosphorylation of WASP and the associated signaling proteins enhanced the bone resorption function of osteoclasts through sealing ring formation. Small interference RNA (siRNA) to PTP-PEST or WASP (12) attenuates osteoclast bone resorption because of derangement of the actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Horseradish peroxidase-conjugated secondary antibodies for immunoblotting, rainbow molecular weight marker, and [32P]orthophosphate were obtained from Amer sham Biosciences. GAPDH antibody was obtained from Abcam Inc. (Cambridge, MA). Protein estimation reagent, molecular weight standards for proteins, and PAGE reagents were bought from Jackson ImmunoResearch (West Grove, PA). Antibodies to WASP, Arp2, and Arp3 were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). Actin polymerization kit and purified Arp2/3, G-actin, and pyrene-labeled G-actin were bought from Cytoskeleton, Inc. (Denver CO). Antibody to Src and recombinant Src protein (marker for kinase assay) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rhodamine phalloidin, phenyl arsine oxide, and other chemicals were purchased from Sigma.

**Preparation of Osteoclast Precursors from Mice**—C57/BL6 mice were used for osteoclast preparation. These mice were either purchased from Harlan Laboratory or generated in the animal house of the University of Maryland Dental School. Breeding and maintenance were carried out as per the guidelines and approval of IACUC. Osteoclasts were generated in vitro using 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 culturing 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 receptor activator of NF-κB ligand (55–75 ng/ml). After 3 days in culture, media were replaced with fresh cytokines. The multinucleated osteoclasts were seen from day 4 onward.

**Small Interfering RNA for PTP-PEST**—Two different siRNA sequences were purchased from Ambion, Inc. (Austin, TX).
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The first 1 and 2 sequences target to 5'-ggaagaaauacuauacg- catt-3' and 5'-uggcguaguauuuucuacctt-3' (siRNA ID 68984), and the second targets to 5'-ggguguagggccaaaaat-3' and 5'uuugccauacaccctctt-3' (siRNA ID 68890). PTP-PEST levels were reduced to a greater extent by siRNA ID 68890 than by siRNA ID 68890. Hence, we used siRNA ID 68984 to reduce endogenous PTP-PEST levels in osteoclasts.

Negative control RNAi (cRNAi) for PTP-PEST sequences and siRNA for GAPDH or β-actin were purchased from Ambion. Negative control RNAi sequences (cRNAi) for PTP-PEST siRNA sequences consisting of a scrambled sequence, which does not have specificity to any known cellular mRNAs, was used. Silencer positive control siRNA for GAPDH or β-actin was used to optimize siRNA experiments. Lipofectamine 2000 (Invitrogen) was used to transfect osteoclasts. Because the silencing was only 50% with Lipofectamine 2000 (Invitrogen) reagent, we used the permeabilization technique with streptolysin O to deliver the siRNA into osteoclasts, as described previously (12, 38). Osteoclasts were washed twice with permeabilization buffer (120 mM KCl, 30 mM NaCl, 10 mM HEPES, pH 7.2, 10 mM EGTA, and 10 mM MgCl2). Freshly prepared dithiothreitol (5 mM), ATP (1 mM), streptolysin O (0.5 units/ml), and siRNA (0.5, 1, and 2 μM) or cRNAi (2 μM) were added to the buffer at the time of permeabilization. Cells were incubated with the above-mentioned solution for 2–3 min, and rescaling was achieved by the addition of α-MEM containing 10% fetal bovine serum. Incubation was continued for 48 h. Control cells were permeabilized as above in the absence of siRNA. After 36–48 h, lysates were made and subjected to immunoblotting with an antibody to PTP-PEST.

Preparation of Osteoclast Lysate after Various Treatments—Constitutively active (CA)-Src and kinase-defective (KD)-Src that were generated essentially based on pAdEasy-1 system (39) were used for transfection. CA-Src contains only the Y527F point mutation to prevent tail binding to the SH2 domain, and KD-Src contains two point mutations, K297R to prevent ATP binding, and S538D to prevent tail binding to the SH2 domain. Virus was propagated as described previously (40). Adenovirus was added to osteoclasts at the 10–30 multiplicity of infection in the serum-free medium. Two hours after adenoviral infections, the medium was replaced with α-MEM supplemented with 10% fetal bovine serum (α-10). Expression of Src was evaluated by immunoblotting of the osteoclast lysate with a c-Src antibody. Expression was maximal at 36–48 h. Osteoclasts treated with PBS were used as controls. Some cultures were treated with OPN (25 μg/ml for 30–45 min), phenylarsine oxide (PAO, 100 μM for 45 min), and PAO/OPN at 37 °C. Following various treatments, osteoclasts were washed three times with cold PBS and lysed in a Triton X-100-containing lysis buffer (10 mM Tris-HCl, pH 7.05, 50 mM NaCl, 0.5% Triton X-100, 30 mM Na3PO4, 50 mM NaF, 100 μM Na3VO4, 5 mM ZnCl2, 1% aprotinin, and 2 mM phenylmethylsulfonyl fluoride). Cells were rocked on ice for 15 min and scraped off with a cell scraper. Cell lysates were centrifuged at 15,000 rpm for 15 min at 4 °C, and the supernatant was saved as a Triton-soluble fraction. The pellet was resuspended in RIPA buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1% aprotinin, and 2 mM phenylmethylsulfonyl fluoride) and centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant is the Triton-insoluble fraction. Protein contents were measured using the Bio-Rad protein assay reagent.

Purification of GST-fused WASP and PTP-PEST Proteins—Full-length (FL) WASP, GTPase binding domain and Proline-rich (GP) domain of WASP (41), and proline-rich (N- and C-terminal) regions of PTP-PEST (27) were created by cloning PCR-generated inserts into a pGEX vector (Amersham Biosciences). Proteins were expressed in Escherichia coli as glutathione S-transferase (GST) fusions as described previously (42). SDS-PAGE and Coomassie Blue staining tested the purity of the purified proteins.

Immunoprecipitation and Western Analysis—Equal amounts of lysate proteins were precleared with protein A-Sepharose, pre-soaked in lysis buffer containing bovine serum albumin and with nonimmune IgG coupled to Sepharose. The precleared supernatants were incubated with antibodies of interest, 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. Pulldown with GST-fused WASP proteins coupled to glutathione-Sepharose was also performed to determine that the proteins had the capacity to bind FL-WASP and WASP-GP region as described previously (42). The immune complexes were then eluted in electrophoresis sample buffer and subjected to SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane for Western blot analyses. To investigate the involvement of GP domain of WASP in the interaction of PSTPIP in osteoclasts, about 500 μg of protein was used for GST pulldown or immunoprecipitation with a WASP antibody. Immunoprecipitates or pulldown proteins were divided into two parts. The first part was immunoblotted with a phosphotyrosine antibody. This blot was stripped and immunoblotted with a GST antibody or WASP antibody. The second part was immunoblotted with a PSTPIP antibody. Immunoblotting was performed as described below. 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 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).

Immune Complex Kinase Assay Analysis—Equal amounts of protein lysates were immunoprecipitated with anti-WASP antibody or pulldown with GST-fused PTP-PEST proteins coupled to glutathione-Sepharose. About 50 μg of whole cell lysate and about 300 μg of lysate protein was used for GST-pulldown analysis. The immune complexes collected by the addition of protein A-Sepharose were used for immunoblotting analysis or kinase assay. For kinase assay, the Sepharose beads were collected by centrifugation and washed sequentially, three times each with the following buffers: lysis buffer, 20 mM Tris-HCl, pH 8.0, containing 0.2 M LiCl; finally with a buffer containing 20 mM HEPES, pH 7.4, 5 mM MgCl2, and 0.1 mM Na3VO4. The beads were resuspended in 20 μl of kinase buffer (20 mM HEPES, pH 7.4, 5 mM MgCl2, and 0.1 mM...
Na\textsubscript{3}VO\textsubscript{4}) containing 10 μCi of [\gamma\textsuperscript{32}P]ATP either in the presence or absence of casein (1 mg/ml) as an exogenous substrate. The mixture was incubated at 25 °C for 20 min, and the reaction was stopped by the addition of SDS sample buffer (43). The samples were boiled and subjected to SDS-PAGE and detected by autoradiography using Kodak X-Omat film.

**Immunocytochemistry**—Osteoclast precursors (10⁵ cells) were seeded on dentine slices and treated as indicated in Fig. 8 for 24–48 h. 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 (12). Background fluorescence was blocked by incubating cells with either 5% horse serum or 5% bovine serum albumin in PBS for 30–45 min at 4 °C. The cells were washed and incubated with primary antibodies of interest (WASP, PTP-PEST, and PST-PIP; 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 as described previously (42, 44). Negative controls were performed with non-immune mouse and goat sera for the double stainings. The cells on dentine slices were washed and mounted on a slide in a mounting solution (Vector Laboratories, Burlingame, CA) 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**—Purified Arp2/3 complex (RP01-A), WASP-VCA (VCG-03) proteins, and an actin polymerization kit (BK0003), were purchased from Cytoskeleton, Inc. Actin polymerization assay was performed using the lysates made from osteoclasts following various treatments. The assay was performed in a total volume of 100 μl. For the assay, cytosol (100–150 μg in 10–15 μl), Arp2/3 complex (25 nm), ATP (1 mM), and polymerization buffer provided in the kit were added to the assay mixture. In **vitro** actin polymerization assay was performed essentially according to the manufacturer’s instructions (Cytoskeleton, Inc.) and as described previously (12, 45). 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. All polymerization assays contained 1 μM unlabelled G-actin and 0.4 μM pyrene-labeled G-actin and 0.2 mM ATP in 100 μl of actin polymerization buffer provided in the actin polymerization kit (BK0003). Actin polymerization was measured for 10–15 min at 30-s to 1-min intervals at the excitation of 350 nm (20 S) and emission at the 405 nm in a luminescence spectrofluorometer (Fluoroskan Ascent Lab Systems type 374; software version 2.4.1) at room temperature. Statistical significance was calculated as described below under “Data Analysis.”

**Measurement of F-actin Content Using Rhodamine Phalloidin Binding**—Osteoclast precursors were prepared as described above. After 3 days in culture, media were replaced with fresh receptor activator of NF-κB ligand and mCSF-1. On day 4, osteoclasts were treated with PTP-PEST siRNA and cRNAi or Ad/CA-Src and Ad/KD-Src. Cells were incubated for 6–7 h, split into 24-well culture plates, and incubated for 36–48 h. Parts of the culture were treated with PBS (control), OPN, PAO, or PAO/OPN. After stimulation for 36–48 h, osteoclasts were rinsed with cold PBS and fixed with paraformaldehyde for 15 min, permeabilized, and incubated with rhodamine phalloidin (1:200; Sigma) in PBS for 30 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 with fluorimetry (Bio-Rad spectrophotometer). Osteoclasts untreated with rhodamine phalloidin were used to determine the background fluorescence of the cells. 10-Fold excess unlabelled phalloidin was used to determine the nonspecific binding. The nonspecific binding and background fluorescence was subtracted from the total binding to determine the specific binding (46, 47).

**Migration Assay**—Transwell chambers (Corning Glass, Costar) were used for migration assays as described previously (37). In brief, the undersurface of the polycarbonate membrane (pore size, 8 μm) was precoated with Vitrogen 100 (collagen type I; 30 mg/ml in PBS) for 2–3 h at room temperature according to the manufacturer’s instructions. Osteoclasts treated with PTP-PEST siRNA and cRNAi and untreated osteoclasts were scraped as described (37) and resuspended in α-MEM containing 1% serum and 2% bovine serum albumin. Osteoclasts (10⁴ in 100 μl of α-MEM) were added to the upper chamber. After the osteoclasts adhered to the wells, some wells were treated with PAO, PAO/OPN, PBS, and OPN. Osteoclasts treated with cRNAi or siRNA sequence to PTP-PEST were also treated with PBS or OPN. Each treatment was performed in quadruplicate. Migration assay was performed for 24–48 h at 37 °C before fixing with 95% methanol in PBS. Unmigrated cells on the upper side of the membrane were removed with a cotton tip applicator. The migrated cells were stained with acid hematoxylin (Sigma) for 6 min, washed well with water, and counted with use of an inverted microscope (×40). Statistical analysis was performed as described below under “Data Analysis.”

**Bone Resorption Assay**—Osteoclasts transfected with Src constructs or PTP-PEST sequences (cRNAi or siRNA) were added to 48-well containing dentine slices (2 × 10⁴ cells). After 2 h of adherence, cells were treated with OPN (10 μg/ml), PAO (100 nm), and PAO/OPN for 48 h. Medium was replaced with the respective treatment after 24 h. After 48 h, cells were scraped from dentine, and the slices were washed twice with water. Dentine slices were stained with acid hematoxylin (Sigma) and washed with water to remove excess stain. Pits were viewed under ×40 objective in a phase contrast microscope and photographed. Images were stored in TIF image format and processed by the Adobe Photoshop software program (Adobe Systems, Inc., Mountain View, CA).

**Data Analysis**—All values presented are expressed as the means ± S.E. of three or more experiments done at different times normalized to intra-experimental control values. Asterisks and ± signs were used to graphically indicate the statistical significance in the figures. A value of p < 0.05 was considered significant. For statistical comparisons, analysis of
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**RESULTS**

**Analysis of c-Src Association with WASP**—Several kinases, such as Hck, Nck, c-Src, and FAK, have been shown to interact with WASP in different cells (14, 17, 23, 34). To investigate the potential interaction of kinase(s) with WASP, we performed GST-pulldown assays (Fig. 1) with GST-fused FL and GP domain of WASP. Proteins coprecipitated with GST fusion proteins (Fig. 1, A–C, lanes 2 and 3) and total lysate (lane 1 in A–C) were subjected to SDS-PAGE and immunoblotted with the indicated antibodies. Pulldown with vector protein (GST, Fig. 1, A–C, lane 4) was used as negative control. Although both Nck (Fig. 1A, lane 1) and hematopoietic cell kinases (Hck) (B, lane 1) are present in osteoclasts, no detectable binding of Nck (A, lanes 2 and 3) or weak binding of Hck (B, lanes 2 and 3) to FL or GP domain of WASP was observed. However, a significant amount of binding of c-Src kinase to FL and GP domain of WASP (Fig. 1C, lanes 2 and 3) was observed. Pulldown with vector protein (Fig. 1C, lane 4) or WASP C-terminal (425–488 aa) protein (data not shown) failed to exhibit detectable binding of c-Src kinase.

To determine whether OPN could modulate the phosphorylation state of c-Src associated with WASP (Fig. 1, D and E), osteoclasts were treated with (+) or without (−) OPN. Immunoblotting was performed first with phosphospecific antibody to c-Src (Fig. 1D, pY418). Subsequently, the blot was reprobed with a c-Src antibody after stripping (Fig. 1E). Compared with PBS-treated control cells (Fig. 1D, lanes 1, 3, and 5), a significant increase in the phosphorylation of total c-Src (Fig. 1D, lane 2) and WASP-associated c-Src was observed in osteoclasts treated with OPN (Fig. 1D, lanes 4 and 6). Fig. 1E shows the amount of c-Src protein present in whole cell lysate (lanes 1 and 2) or bound to GST-WASP proteins (lanes 3–6). Pulldown with vector protein (GST) was used as a negative control (Fig. 1E, lane 7).

**Analysis of Phosphorylation of WASP and the Associated Proteins**—To corroborate the above data, osteoclasts were transfected with CA-Src and KD-Src-K297R cDNA by adenovirally mediated delivery (Fig. 2A). The ability of adenovirally mediated gene transfection to increase c-Src expression in osteoclasts was demonstrated (Fig. 2A, lanes 2 and 3), as compared with untransfected control cells (lane 1). The expression of c-Src was nor-
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tyrosine (Tyr(P))- and phosphoserine (Ser(P))-specific antibodies (Fig. 3).

An in vitro immune complex kinase assay was performed using the WASP immunoprecipitates made from osteoclast lysates as shown in Fig. 2B (two different exposures (1 day and 4 days) are shown). The effect of c-Src overexpression (Fig. 2B, lane 3) was compared with OPN stimulation (lane 2). The phosphorylation of c-Src and other proteins is greater in osteoclasts treated with OPN (Fig. 2B, lane 2) rather than in the control osteoclasts (lane 1). We observed the phosphorylation of WASP and three other proteins with molecular masses of 115 kDa (PYK2), 90–110 kDa (PTP-PEST), and 48–50 kDa (PTPPIP). An increase in the phosphorylation of these proteins was also observed in CA-Src-transfected cells as compared with KD-Src-transfected (Fig. 2B, lane 4) osteoclasts. An increase in the phosphorylation of c-Src increases its phosphorylating activity toward an exogenous substrate, casein (Fig. 2B, arrow). The coimmunoprecipitation of signaling proteins was confirmed by immunoblotting analyses of WASP immunoprecipitates with respective antibodies as shown in Fig. 3.

The addition of soluble OPN (Fig. 3A, lane 2) or transfection of CA-Src into osteoclasts (lane 3) increases the phosphorylation of PYK2, an unknown protein with a molecular mass 80–85 kDa (indicated by an asterisk), WASP, c-Src, and PTP-PEST. Basal level phosphorylation was observed in PBS-treated (Fig. 3, lane 1) and KD-Src-transfected (Fig. 3, lane 4) osteoclasts. Subsequently, we performed immunoblotting analyses with antibodies to respective proteins as indicated in Figs. 2 and 3A. This was done to characterize the proteins that were coimmunoprecipitated with WASP immunoprecipitates (Fig. 3B). Although the WASP level remained the same (B, panel II), the interaction of PYK2 (panel I), c-Src (panel III), and PTP-PEST (panel IV) with WASP was increased in OPN-treated and CA-Src-transfected osteoclasts. Basal level interaction of proteins with WASP was observed in KD-Src-transfected cells. It is possible that the tyrosine phosphorylation of WASP and associated proteins is in part dependent on the c-Src kinase activity.

We have previously demonstrated that PTP-PEST associated with gelsolin was phosphorylated on serine residues in response to OPN stimulation (42). Phosphorylation of Ser-39 in vitro decreases the activity of PTP-PEST (30). To examine if PTP-PEST interacts with WASP in osteoclasts, WASP immunoprecipitates were immunoblotted with an antibody to Ser(P) (Fig. 3C) and PTP-PEST (Fig. 3D, panel I). Fig. 3D shows the coprecipitation of PTP-PEST (panel I). OPN treatment (Fig. 3C, lane 3) and CA-Src expression (lane 4) has increased serine phosphorylation of PTP-PEST. Basal level phosphorylation was observed in PBS-treated (Fig. 3C, lane 2) or KD-Src (lane 5)-transfected cells. Table 1 shows the densitometric scans of

TABLE 1

Analysis of phosphorylation levels of different signaling proteins associated with WASP immunoprecipitates

Densitometric scans of three different blots expressed as percent control are shown as mean ± S.E. PBS-treated osteoclasts were used as control. * indicates p < 0.01; ** indicates p < 0.001 versus PBS-treated osteoclasts.

| Treatments | PYK2    | PTP-PEST | WASP     | c-Src    | PTPPIP   |
|------------|---------|----------|----------|----------|----------|
| PBS        | 94.04 ± 04 | 106.02 ± 09 | 99.86 ± 04 | 100.06 ± 10 | 99.4 ± 05 |
| OPN        | 147.03 ± 10* | 161.23 ± 06* | 183.09 ± 16** | 215.03 ± 13** | 189.02 ± 07** |
| CA-Src     | 173.05 ± 16* | 191.18 ± 12** | 219.38 ± 12** | 304.67 ± 34** | 206.78 ± 20** |
| KD-Src     | 96.02 ± 11 | 96.08 ± 03 | 99.43 ± 06 | 89.27 ± 07 | 102.08 ± 06 |
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Fig. 4. Specificity of binding of PTP-PEST and PSTPIP to GP domain of WASP. A and B, lysates made from osteoclasts treated with PBS (−) or OPN (+) were subjected to pulldown analysis with GST-fused GP domain of WASP (lanes 2 and 3) or GST alone (lane 1). Immunoblotting (IB) was performed with a phosphoserine antibody (A) or GST antibody (B). C–E, lysates (500 μg) made from osteoclasts treated with PBS (−) or OPN (+) were subjected to pulldown analysis with GST-fused WASP proteins (lanes 1 and 2) or immunoprecipitation (IP) with a WASP antibody (lanes 4 and 5). Pulludown with GST alone (lane 3) and immunoprecipitation with a species-specific nonimmune N (NI) serum (lane 6) were used as controls. Pulludown or immunoprecipitated proteins were divided into two parts. The first part was immunoblotted with a phosphotyrosine antibody (C). This blot was stripped and immunoblotted with a GST antibody (E, lanes 1–3) or WASP antibody (E, lane 4–6). Equal levels of GST proteins (lanes 1–3) or WASP proteins (lanes 4–6) were observed. The second part was immunoblotted with a PSTPIP antibody (D). Results shown are representative of three independent osteoclast preparations and experiments. HC, heavy chain.

three different independent experiments were expressed as percentage of control. The percentage was calculated by comparing the various treatments to PBS-treated osteoclasts for the indicated signaling proteins (Table 1). Phosphorylation of two other proteins with a molecular mass of ∼80–85 and 160–170 kDa was also observed (Fig. 3, A and C, indicated by asterisks). Serine phosphorylation of the 160–170-kDa protein was increased in OPN-treated (Fig. 3C, lane 3) as well as CA- and KD-Src expressing osteoclasts (lanes 4 and 5). An increase in the phosphorylation of the 160–170-kDa protein in KD-Src transfected cells suggests that other c-Src functional domains play a critical role for this effect. This also indicates the kinase activity may not be required for this phosphorylation. Serine phosphorylation of PTP-PEST and an unknown 80–85-kDa protein occurs because of an interaction of c-Src with WASP in osteoclasts. This event appears to be c-Src kinase-dependent. Although WASP levels were comparable (Fig. 3D, II), the interaction of PTP-PEST (Fig. 3D, panel I) with WASP was increased in OPN-treated and CA-Src transfected osteoclasts. Overall, it seems that binding of the indicated signaling proteins to WASP is dependent not only on the phosphorylation state of WASP but also on the binding partners.

Analysis of PTP-PEST Binding Proteins by GST-Pulldown Analysis—To examine the region of PTP-PEST that interacts with PSTPIP and other signaling proteins that are associated with WASP, a pulldown analysis with GST-fused PTP-PEST peptides was performed. GST-fused noncatalytic fragments (N- and C-terminal) of PTP-PEST were used for this analysis. The N-terminal fragment that corresponds to aa 297–494 of PTP-PEST contains the first two proline-rich clusters in aa sequences 311–404. The numbers given in parentheses indicate the proline-rich amino acids in each cluster: 350–355 (6 aa); 358–361 (4 aa); 363–372 (7 aa); 379–385 (7 aa); and 390–403 (14 aa). It is possible that these GST/GP-coprecipitated proteins interact with each other or have specificity to different proline-rich sequences within the GP domain of WASP.

The SH3 domain of PSTPIP has been shown previously to bind proline-rich regions of WASP (20). To determine the specificity of association of PSTPIP with WASP, we performed pulldown analysis with the GST-fused GP domain of WASP (Fig. 4C, and D, lanes 1 and 2). WASP immunoprecipitates (Fig. 4C, and D, lanes 4 and 5) were also used in parallel with GST pulldown analyses. Pulludown with GST alone (Fig. 4C, and D, lane 3) or immunoprecipitation with a nonimmune serum (lane 6) served as a negative control. Immunoblotting with a phosphotyrosine antibody demonstrated coprecipitation of PSTPIP with GP domain of WASP (Fig. 4C, lane 2) or in the WASP immune complex (Fig. 4C, lane 5). An increase in the tyrosine phosphorylation of PSTPIP was observed in OPN-treated osteoclasts (Fig. 4C, lanes 2 and 5) as compared with PBS-treated controls (lanes 1 and 4). Stripping and reprobing of this blot exhibited equal levels of GST proteins (Fig. 4E, lanes 1–3) or WASP protein in the immunoprecipitates (E, lanes 4 and 5). Immunoblotting with a PSTPIP antibody exhibited an equal level of PSTPIP protein in the pulldown analysis (Fig. 4D, lanes 1 and 2), whereas more PSTPIP coprecipitation was observed in the WASP immunoprecipitate of OPN-treated osteoclasts (Fig. 4D, lane 5) than in PBS-treated control (lane 4). Taken together, this set of experiments showed that the GP domain of WASP has the ability to form a complex with proteins such as c-Src (Fig. 1), PTP-PEST, and PSTPIP in vitro.
other two parts were subjected to an immunoblotting analyses (Fig. 5B) with antibodies to PYK2 and WASP. These blots were then stripped and reblotted with antibodies to Src and PTP-PEST, respectively.

The in vitro kinase assay demonstrated that the OPN stimulation of PYK2, WASP, c-Src, and PTP-PEST phosphorylation was associated with N-terminal PTP-PEST fragment (Fig. 5A, lane 2 in), as compared with PBS-treated control osteoclasts. Neither the phosphorylation (Fig. 5A, lane 3) nor the interaction of WASP or PTP-PEST (Fig. 5B, lane 2) was observed in the analyses with the C-terminal fragment of PTP-PEST (aa 441–775). An immunoblotting analysis (Fig. 5B) demonstrated an interaction of WASP, PYK2, PTP-PEST, and c-Src with the N-terminal region of PTP-PEST. The levels of PYK2, c-Src, WASP, or PTP-PEST that are associated with the N-terminal PTP-PEST were elevated in OPN-treated osteoclasts (Fig. 5B, lane 1 in panels I–IV) than in respective PBS-treated controls (lane 3). Moreover, the interaction of PYK2 (Fig. 5B, lane 2 in panel I) and c-Src (B, lane 2 in panel III) with the C-terminal fragment of the PTP-PEST was observed. It is possible that the proline-rich region of the C-terminal fragment may have an affinity to the SH3 domain of c-Src or PYK2. In vitro kinase assay and immunoblotting analyses specify the complex interaction of multiple proteins (e.g. as WASP, PTP-PEST, PYK2, and c-Src) with the N-terminal fragment of PTP-PEST. This is not observed with the C-terminal fragment of PTP-PEST despite the interaction of PYK2 and c-Src. The specificity of binding of PTP-PEST with the GP domain of WASP (Fig. 4C) or the N-terminal fragment of PTP-PEST further supports our assumption that it may function as a linker protein between PTP-PEST and WASP in osteoclasts (32). The serine phosphorylation of PTP-PEST in response to OPN treatment (Figs. 3 and 4) suggests a possible role for PTP-PEST in the modulation of the tyrosine phosphorylation state of signaling proteins associated with WASP.

Effect of PTP-PEST Silencing on Actin Polymerization and F-actin Content—Our analyses displayed an interaction of PTP-PEST with WASP (Figs. 2–5). We have previously shown that WASP has a significant role in sealing ring formation (12). The coprecipitation of PTP-PEST with WASP alludes to the role of PTP-PEST in controlling the tyrosine phosphorylation level of WASP and associated signaling proteins. We used siRNA strategy to reduce endogenous levels of PTP-PEST in osteoclasts. Osteoclasts were incubated with the PTP-PEST siRNA for 36 h at 37 °C. siRNA at doses of 0.5–2 μM decreased the PTP-PEST protein levels (Fig. 6A, lanes 2–4). The PTP-PEST protein levels were reduced by 80% or more at a final concentration of 2 μM siRNA (83 ± 8; n = 3; Fig. 6A, lane 4) as compared with the

FIGURE 5. Analysis of specificity of binding of phosphoprotein with proline-rich sequences of PTP-PEST. GST fusion peptides of PTP-PEST were used for pulldown analysis. Lysates (500 μg) made from osteoclasts treated with PBS (−) or OPN (+) were subjected to pulldown analysis with GST-fused PTP-PEST proteins. Amino acid sequences are indicated below in their respective lanes. Pulldown with GST alone (lane 4) was used as a negative control. A, in vitro kinase assay was performed with the GST-pulldown proteins. Src protein (Upstate Biotechnology, Inc.) was used as an identification marker (lane 1). An asterisk indicates coprecipitation of an unknown protein with a molecular mass 50–55 kDa. B, immunoblotting with anti-PYK2 (panel I), anti-WASP (panel II), anti-Src (panel III), and anti-PSTPIP (panel IV) demonstrate the levels of respective protein pulldown with GST-fused PTP-PEST sequences. The results shown are representative of three independent osteoclast preparations and experiments.

FIGURE 6. The effects of siRNA to PTP-PEST on actin polymerization and F-actin content in osteoclasts. A, effect of siRNA to PTP-PEST on protein levels of PTP-PEST in osteoclasts. Immunoblot analysis with an antibody to PTP-PEST is shown. Control cells permeabilized with streptolysin O (lane 1) were used as a control. Osteoclasts were permeabilized and incubated with the indicated concentration of siRNA (lanes 2–4) and cRNAi sequences (2 μM; lane 5) for 48 h. Equal amounts of lysate proteins were used for immunoblotting with an antibody to PTP-PEST. Loading was normalized by immunoblotting analysis of the same blot with an antibody to GAPDH after stripping (bottom panel). B, in vitro actin polymerization assay. Purified Arp2/3 complex was used to a final concentration of 25 nm. 100–150 μg (10–15 μl) of cytosol, ATP, as well as 1.0 μM unlabelled and 0.4 μM pyrene-labeled G-actin were used for the actin polymerization assay in vitro as described under “Experimental Procedures.” Results shown are means of three experiments, and each assay was performed in quadruplicate. C, measurement of F-actin content. F-actin content was measured by rhodamine phalloidin binding in osteoclasts treated as indicated in the figure. Cells were grown in 24-well tissue culture plates, and three to four wells were used for each treatment. The results presented are mean ± S.E. for three experiments. ***, p < 0.0001 versus KD-Src; **, p < 0.001 versus PBS treated osteoclasts.
levels of protein obtained after transfection with a scrambled cRNAi. The endogenous levels of PTP-PEST in control osteoclasts permeabilized with streptolysin O are shown in Fig. 6A, lane 1. Immunoblotting of the same blot with an antibody to GAPDH (Fig. 6A, lower panel) was used as a loading control.

To elucidate the roles of kinase(s) and the phosphatase PTP-PEST in actin polymerization, lysates were made from osteoclasts transfected with c-Src constructs (CA- and KD-Src) or PTP-PEST RNAi sequences (siRNA and cRNAi). Lysates made from osteoclasts treated with herbimycin A (a specific inhibitor of tyrosine kinases) and PAO (a potent phosphatase inhibitor) (48) were also used for actin polymerization assay (Fig. 6B). OPN treatment leads to enhanced actin polymerization. A remarkable increase was observed in CA-Src-transfected osteoclasts as compared with KD-Src-transfected cells. The addition of OPN to cRNAi-treated osteoclasts exhibits actin polymerization that is equal to the level observed in osteoclasts treated only with OPN. Even in the presence of OPN, herbimycin A, PAO, or siRNA to PTP-PEST-treated osteoclasts display actin polymerization that is equal to or lower than the level observed in PBS-treated osteoclasts (Fig. 6B). This shows that the activation and stability of WASP by tyrosine phosphorylation may increase its affinity to the Arp2/3 complex. This is critical for the subsequent actin polymerization process.

Consistent with the observation presented by the in vitro actin polymerization assay (Fig. 6B), an increase in F-actin content was observed in osteoclasts treated with OPN or transfected with CA-Src (Fig. 6C). Basal level F-actin content was observed in osteoclasts treated with PBS, PAO, and cRNAi. The addition of OPN to cRNAi-transfected osteoclasts (cRNAi/OPN) exhibited F-actin content that is equal to the level observed in OPN alone-treated osteoclasts. A decrease in the basal level of F-actin content was observed in osteoclasts transfected with KD-Src or treated with siRNA to PTP-PEST. The addition of OPN to PAO-treated cells (PAO/OPN) or siRNA to PTP-PEST-treated cells (siRNA/OPN) had no effect on F-actin levels. A decrease in actin polymerization (Fig. 6B) and actin content (Fig. 6C) in siRNA to PTP-PEST and KD-Src transfected cells may be due to the decreased phosphorylation state of WASP and the consequential loss of stability of WASP in active conformation.

Immunostaining Analysis of Distribution of WASP/c-Src, PTP-PEST, and PTP-PEST/PSTPIP in the Sealing Ring of Resorbing Osteoclasts—The above observations (Figs. 1–4) prompted us to investigate whether colocalization of c-Src, PSTPIP, and PTP-PEST with WASP occurs in the sealing ring of resorbing osteoclasts. Immunostaining analyses were performed in osteoclasts transfected with Src constructs (CA- and KD-Src; Fig. 7, B and C) or RNAi sequences (cRNAi or siRNA; D–F, K, and L) to PTP-PEST. The colocalization (yellow) of WASP/c-Src (Fig. 7A), WASP/PTP-PEST (D), and PTP-PEST/PSTPIP (J) was observed in the sealing ring of resorbing osteoclasts. The distribution of these proteins in the sealing ring of resorbing osteoclasts signifies their biological role in osteoclast function. Osteoclasts treated with cRNAi to PTP-PEST at a final concentration of 2 μM (Fig. 7, D and K) or transfected with CA-Src (B) exhibited sealing ring formation, as was observed in control cells (A and J). The distribution of the above-mentioned proteins was observed in the sealing ring of these osteoclasts (Fig. 7, B, D, and K). The failure to form a sealing ring in osteoclasts transfected with KD-Src construct (Fig. 7C) corroborates the previously established role of c-Src in osteoclast bone resorption (3, 49). A few small patches (Fig. 7, yellow, indicated by arrows) of Src (green) and WASP (red) were present in these osteoclasts. A greater diffused distribution of c-Src was observed in osteoclasts transfected with CA-Src (Fig. 7B) and KD-Src (C) than in the control osteoclasts (A).

A dose-dependent decrease in PTP-PEST (Fig. 7, green) immunostaining and sealing ring formation was observed in osteoclasts treated with PTP-PEST siRNA to a final concentration of 1 μM (E) and 2 μM (F). Small ring-like structure(s) and patches exhibiting colocalization (Fig. 7, yellow; indicated by arrows) of WASP/PTP-PEST were observed in osteoclasts treated with 1 μM siRNA to PTP-PEST (E). Immunostaining of PTP-PEST was dramatically reduced or absent in osteoclasts treated with 2 μM siRNA to PTP-PEST (Fig. 7F). WASP (Fig. 7, red) distribution was seen in patches (red). Osteoclasts treated with PAO (Fig. 7G) displayed a diffused cytoplasmic distribution of PTP-PEST (green) with numerous small patches that displayed the colocalization (yellow) of WASP (red) and PTP-PEST. These patches appear as small podosome ring-like structures as shown in the magnified image in Fig. 7H. The colocalization (Fig. 7, yellow) of PTP-PEST (green) and WASP (red) was observed in these structures. Osteoclasts treated with...
siRNA to PTP-PEST (Fig. 7L) exhibited small ring-like structures (indicated by arrows) stained for PSTPIP, but colocalization of PTP-PEST and PSTPIP (red) was not observed, as it had been in control (J) or cRNAi (K)-treated osteoclasts. Immunostaining with species-specific nonimmune serum is shown in Fig. 7L. The colocalization (Fig. 7, yellow) of c-Src, PTP-PEST, and PSTPIP with WASP in the sealing ring shows their interaction with WASP. The data indicates that the multifaceted interaction of these proteins is critical in the organization of the sealing ring during bone resorption.

**Bone Resorption and Migration Assays in Vitro—**To further assess the role of c-Src and PTP-PEST in osteoclast function, we performed in vitro bone resorption assays (Fig. 8) using osteoclasts transfected with Src constructs (CA-Src and KD-Src) or RNAi sequences (cRNAi and siRNA) to PTP-PEST. Also, PBS-, OPN-, PAO-, and PAO/OPN-treated osteoclasts were subjected to this assay. Quantitation of resorption pits generated in vitro by osteoclasts is shown in Fig. 8I. An inhibition of bone resorption was observed in osteoclasts transfected with KD-Src (Fig. 8D) or siRNA to PTP-PEST at 1.0 and 2 μM concentrations (F and G) and subsequently treated with OPN. In osteoclasts treated with PAO/OPN, numerous small resorption pits were observed, in concurrence with the small ring-like structure shown in Fig. 7G. These pits are diminutive and simple without any multiple overlappings as observed in osteoclasts treated with OPN alone (Fig. 8B). The pit area is smaller than the area observed in PBS-treated (Fig. 8A) or KD-Src transfected (D) osteoclasts. Similar to the effect noticed with OPN (B), osteoclasts transfected with CA-Src (C) or cRNAi/OPN (E) exhibited an increase in the pit area. The increase in pit area represents simultaneous processes of migration, the formation of sealing ring, and resorption.

The effects of various treatments on osteoclast migration were determined by a transwell migration assay (Table 2), as described under “Experimental Procedures” and discussed previously (37). The undersides of 8-μm transwell filters were coated with type 1 collagen. Osteoclasts treated with PBS or OPN alone were used as controls. A 3–5-fold increase in migration was observed in cells treated with OPN, cRNAi/OPN, and CA-Src. OPN had no effect on the migration of osteoclasts that are treated with PAO or siRNA to PTP-PEST (1 μM). Basal level migration was observed in PBS, PAO, PAO/OPN, and cRNAi-treated osteoclasts. In osteoclasts treated with siRNA and siRNA/OPN, migration was below the basal level migration. The ability of siRNA to PTP-PEST and PAO to inhibit cell migration and bone resorption demonstrates the role of PTP-PEST in osteoclast function.

**RESULTS**

**Actin polymerization and reorganization of actin cytoskeleton is promoted by the spatial organization of signaling, actin binding/nucleating, and scaffold proteins that have the capability to bind actin either directly or through protein-protein interactions. We have previously demonstrated that podosome assembly/disassembly and sealing ring formation are dependent on the interaction of phospholipid phosphatidylinositol 4,5-biphosphate with gelsolin and WASP, respectively (12, 37). Most recently, we have demonstrated a cooperative role of Rho and Cdc42 in WASP-mediated actin assembly in the sealing zone of resorbing osteoclasts through the αvβ3-mediated signaling mechanism (12). Src family kinases have been shown to activate WASP phosphorylation; this increases its affinity for the Arp2/3 complex (21, 50). WASP phosphorylation is func-
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tionally important in vivo, and it may play a role in stabilizing the unmasked conformation of WASP (15). Although c-Src and intergrin αvβ3 indicate the presence of a vital signaling complex in the osteoclast during bone resorption, we expect that WASP might be a binding partner for c-Src kinase. Immunoprecipitation of c-Src with WASP suggests that the interaction between these proteins may be involved in signaling pathways that regulate sealing ring formation during bone resorption. In osteoclasts, c-Src activation is essential for changes in the actin cytoskeleton during stimulation of bone resorption by OPN in osteoclasts (38). The increase in the activation of WASP by Rho GTPases and PIP2 may result from the following potential mechanisms. First, phosphatidylinositol 4-phosphate-5-kinase, which synthesizes PIP2, is a downstream effector of Rho GTPase (51). Second, in osteoclasts Rho GTPase activity is regulated by c-Src, as antisense oligodeoxynucleotides to c-Src reduced not only the activation of Rho GTPase but also PIP2 levels (44). The unmasking of WASP by Rho GTPases (12) leads to the complex interaction of signaling proteins that contain SH2/SH3/proline-rich domains with WASP in response to OPN/αvβ3-mediated signaling mechanism.

Binding studies using GST-SH3 fusion proteins have shown that the proline-rich region of WASP binds a variety of SH3-containing proteins, which include those of Src family kinases Hck, Nck, c-Src, as well as Abl (14, 16, 17, 23, 34). Although Hck and Nck are expressed in osteoclasts (Fig. 1), we found no interaction of these kinases with the WASP-GP domain. However, we observed a strong affinity to c-Src kinase. The data presented here (Figs. 1 and 3) along with observations by others suggest that WASP can bind to the SH3 domain of c-Src via proline-rich amino acids between residues 313 and 416 (16). The addition of Src SH2 is sufficient to disrupt the interaction of p-GBD and VCA. Cdc42 and the SH2/SH3 module of Lck cooperatively stimulate the activity of phosphorylated WASP (15). Although we observed the binding of SH2- and SH3-containing proteins in the GST-binding studies (Figs. 1 and 4), the precise mechanism by which the phosphorylation of WASP or its increased interaction with phosphoproteins is not completely known. It is possible that they may interact directly with WASP via proline-rich sequences in the GP domain of WASP that contains approximately six proline-rich clusters between aa sequences 311 and 404 or, alternatively, might interact indirectly via modular domains of other proteins.

The phosphorylation of c-Src associated with WASP was increased in OPN-treated cells. However, it is apparent that OPN treatment or CA-Src transfection (Figs. 1–3), in addition to increasing the phosphorylation of WASP, induces an affinity of WASP to a variety of signaling proteins (PTP-PEST, PSTPIP, PYK2, Src, p80–85 kDa, and 160–170-kDa proteins) and subsequently to Arp2/3 complex. This complex interaction of proteins with WASP augments not only actin polymerization (Fig. 6B) and F-actin content (Fig. 6C) but also sealing ring formation (Fig. 7) and bone resorption (Fig. 8) in osteoclasts. These effects were not observed in osteoclasts transfected with KD-Src.

PYK2 plays a central role in adhesion-dependent cytoskeletal organization and sealing zone formation during osteoclast bone resorption (6). Miyazaki et al. (49) demonstrated that the elimination of the c-Src-binding site on Pyk2 (Pyk2Y402F) markedly inhibited bone resorption by osteoclast-like cells, whereas kinase-dead Pyk2 had little effect. Also, kinase-dead Src, unlike kinase-dead Pyk2, markedly inhibited the bone-resorbing activity of wild-type osteoclasts. These observations suggest that osteoclastic bone resorption requires both c-Src kinase activity and the targeting of c-Src kinase by Pyk2 (49). An increase in phosphorylation of WASP in OPN-treated and CA-Src-transfected osteoclast indicates that OPN/αvβ3-mediated signaling, which involves c-Src kinase, has a major role in sealing ring formation. Several lines of evidence suggest that c-Src plays a role in regulating actin rearrangement and bone resorption in osteoclasts (4, 53, 54). Osteoclasts null for c-Src revealed impaired polarization and failure of bone resorption (4, 38, 55). Although other members of the Src family kinases account for redundancy in other cells, they do not compensate for the absence of c-Src in the Src null mice (55). An intermolecular interaction of proteins with PTP domain(s) may not only regulate the phosphorylation of WASP but may also be responsible for its stability in an active conformation.

The level of tyrosine phosphorylation of the cytoskeleton-associated protein PSTPIP was shown to be controlled by PTP-PEST (26, 31, 56). PSTPIP interacts with WASP through the SH3 domain, and it functions as a scaffold protein between PTP-PEST and WASP (32). In this study, data displayed an interaction of PSTPIP and PTP-PEST with the WASP-GP domain. Binding analyses with the WASP-GP domain or immunoprecipitation with WASP antibody exhibited the following: (a) an increase in serine phosphorylation of PTP-PEST (Figs. 3 and 4) (9); (b) tyrosine phosphorylation of PSTPIP, c-Src, and PYK2 (Figs. 1, 3, and 4) in response to OPN stimulation. PSTPIP was tyrosine-phosphorylated both endogenously and in v-Src-transfected BAF3 and COS cells (56), and the tyrosine phosphatase PTP-PEST was capable of dephosphorylating these tyrosine residues (26). Coimmunoprecipitation of Src with WASP and PSTPIP suggests that the interaction between these proteins may be involved in signaling pathways that regulate the phosphorylation of PSTPIP in sealing ring formation during bone resorption. PSTPIP may serve as a linker in the interaction of PTP-PEST with WASP.

PTP-PEST has been implicated in the modulation of tyrosine phosphorylation of proteins and cytoskeletal regulation (20). Although kinases such as c-Src, FAK, and PYK2 have been shown to have roles in osteoclast bone resorption, the role of a tyrosine phosphatase (e.g. PTP-PEST) in the regulation of phosphorylation of the above-mentioned proteins as well as sealing ring formation has not been previously elucidated. To clarify the complex interaction of proteins with PTP-PEST, we performed GST-pulldown analyses with proline-rich sequences (N- and C-terminal) in the catalytic domain of PTP-PEST. We found the binding of several signaling proteins consisting of modular domains that mediate protein-protein interactions. Although Src and PYK2 bind to both N- and C-terminal proline sequences in PTP-PEST, preferential binding of PSTPIP and WASP besides PYK2 and c-Src was observed with N-terminal proline sequences of PTP-PEST. An increased interaction of these proteins with the proline-rich region of PTP-PEST suggests that it has a role in the regulation of phosphorylation of proteins associated with WASP. The modulation of the phos-
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Phosphorylation states of these proteins by PTP-PEST may enhance protein-protein interactions through SH2, SH3, or proline-rich domains present in these proteins. Furthermore, the increase in serine phosphorylation of PTP-PEST in OPN-treated or CA-Src-transfected cells may represent its inactive state (42). The phosphorylation of PTP-PEST at two major sites (Ser-39 and Ser-435) transfected cells may represent its inactive state (42). The phosphorylation of PTP-PEST in OPN-treated or CA-Src-transfected cells may represent its inactive state (42). The phosphorylation of PTP-PEST at two major sites (Ser-39 and Ser-435) in HeLa cells treated with 12-O-tetradecanoyl-phorbol-13-acetate decreases its activity by reducing its affinity for substrates (30). Our results suggest that serine phosphorylation of PTP-PEST is one mechanism by which this protein may modulate the tyrosine phosphorylation of WASP and associated proteins. The role of PTP-PEST in osteoclast function is summarized in the working model provided in Fig. 9. However, it will be of interest to further elucidate how the serine phosphorylation of PTP-PEST regulates WASP function in osteoclasts.

To further understand the role of PTP-PEST in the regulation of actin remodeling and sealing ring formation, we used an siRNA strategy to reduce the levels of PTP-PEST and PAO inhibitor to reduce the activity in osteoclasts. We observed a striking inhibition in the phosphorylation of proteins, as well as actin polymerization and actin content. This resulted in inhibition of sealing ring formation and bone resorption in osteoclasts. PTP-PEST knock-out fibroblast cell line exhibited a marked decrease in migration and increase in number of focal adhesions (33). Constitutive hyperphosphorylation of focal adhesion proteins also increases the spreading rate of PTP-PEST−/− cells on fibronectin (33). The inhibition of phosphorylation of WASP and the associated signaling proteins take place by the cooperative function of kinase(s) and the phosphatase PTP-PEST. An increase in stable conformation of WASP by phosphorylation enhances its interaction with actin monomers and Arp2/3 complex. This multifaceted interaction is critical for actin assembly and sealing ring formation that contribute to bone resorption in osteoclasts. We propose that dephosphorylation of PTP-PEST on serine residue activates it and increases dephosphorylation of WASP and the associated signaling molecules. This leads to disruption of the sealing ring and termination of osteoclast bone resorption activity. PM, plasma membrane.

CONCLUSIONS

Phosphorylation of WASP and the associated signaling proteins is mediated by the cooperative function of kinases and the phosphatase PTP-PEST. Src regulates several actin-regulating proteins such as gelsolin, WASP, Arp2/3, and cortactin (12, 37, 52), which play important roles in podosome assembly/disassembly and sealing ring formation in osteoclasts. The inhibition of osteoclast bone resorption by PAO, KD-Src, as well as siRNAs to PTP-PEST and WASP (12) reduced osteoclast bone resorption because of the

FIGURE 9. Schematic representation of the role of PTP-PEST in osteoclast bone resorption and migration. Osteopontin/αvβ3-mediated phosphorylation of WASP and the associated signaling proteins take place by the cooperative function of kinase(s) and the phosphatase PTP-PEST. An increase in stable conformation of WASP by phosphorylation enhances its interaction with actin monomers and Arp2/3 complex. This multifaceted interaction is critical for actin assembly and sealing ring formation that contribute to bone resorption in osteoclasts. We propose that dephosphorylation of PTP-PEST on serine residue activates it and increases dephosphorylation of WASP and the associated signaling molecules. This leads to disruption of the sealing ring and termination of osteoclast bone resorption activity. PM, plasma membrane.
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inhibition of signaling that regulates actin cytoskeleton reorganization. An increase in stable conformation of WASP by phosphorylation enhances its interaction with actin monomers and Arp2/3 complex. This multifaceted interaction is critical for actin assembly and sealing ring formation, which contribute to bone resorption in osteoclasts. The deregulation of osteoclast function leads to pathological bone loss during osteoporosis caused by estrogen deficiency or cancer metastasis. A better understanding of the mechanism involved in sealing ring formation may hasten the development of novel anti-osteoporotic drugs. Novel strategies may target the signaling intermediates involved in sealing ring formation and bone resorption by osteoclasts.

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