Regulation of Sealing Ring Formation by L-plastin and Cortactin in Osteoclasts*

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The aim of this study is to identify the exact mechanism(s) by which cytoskeletal structures are modulated during bone resorption. In this study, we have shown the possible role of different actin-binding and signaling proteins in the regulation of sealing ring formation. Our analyses have demonstrated a significant increase in cortactin and a corresponding decrease in L-plastin protein levels in osteoclasts subjected to bone resorption for 18 h in the presence of RANKL, M-CSF, and native bone particles. Time-dependent changes in the localization of L-plastin (in actin aggregates) and cortactin (in the sealing ring) suggest that these proteins may be involved in the initial and maturation phases of sealing ring formation, respectively. siRNA to cortactin inhibits this maturation process but not the formation of actin aggregates. Osteoclasts treated as above but with TNF-α demonstrated very similar effects as observed with RANKL. Osteoclasts treated with a neutralizing antibody to TNF-α displayed podosome-like structures in the entire subsurface and at the periphery of osteoclast. It is possible that TNF-α and RANKL-mediated signaling may play a role in the early phase of sealing ring configuration (i.e. either in the disassembly of podosomes or formation of actin aggregates). Furthermore, osteoclasts treated with alendronate or αv reduced the formation of the sealing ring but not actin aggregates. The present study demonstrates a novel mechanistic link between L-plastin and cortactin in sealing ring formation. These results suggest that actin aggregates formed by L-plastin independent of integrin signaling function as a core in assembling signaling molecules (integrin αvβ3, Src, cortactin, etc.) involved in the maturation process.

Osteoclasts, the multinucleated and terminally differentiated giant cells, are involved in bone resorption. The adhesion of osteoclasts to the bone during bone resorption leads to the formation of the clear zone, an actin-rich ring-like adhesion zone circumscribing an area of bone resorption. Formation of a clear zone or sealing zone (also known as the sealing ring) has been considered to be a marker of osteoclast activation and is fundamental to the process of osteoclast bone resorption. Sealing ring formation is mediated by the dynamics of the actin cytoskeleton. Distinct pathways and signaling molecules have been shown to play roles in the organization of the sealing ring during bone resorption. Our previous observations in gelsolin null (Gsn null) osteoclasts demonstrated that deficiency of this protein blocks podosome assembly and motility. However, the cells still exhibit sealing ring and matrix resorption (1). Therefore, Gsn−/− osteoclasts are capable of resorbing bone, but the resorbed areas are small due to the absence of podosomes and the resulting hypomotile nature of osteoclasts (1). Observations in Gsn−/− osteoclasts also suggest that the organization of the sealing ring presumably reflect changes in the role of actin-binding proteins. Spatial configurations of actin filaments by actin-binding proteins (ABPs) account for the highly specific changes in cell shape during migration and bone resorption. ABPs assist in stabilizing and rearranging the organization of the actin cytoskeleton in response to external stimuli or during cell migration and adhesion. A few of the ABPs have been identified as having a role in osteoclast function (1–6).

Wiskott-Aldrich syndrome protein (WASP) is one of the ABPs, and it has a role in sealing ring formation and bone resorption in osteoclasts (7, 8). It is also a binding partner for c-Src family protein-tyrosine kinases (7, 9). Calle et al. (8) have demonstrated that osteoclasts from WASP knock-out mice failed to demonstrate a sealing ring, and these osteoclasts are less resorptive. Expression of WASP restores normal cytoarchitecture in these osteoclasts. Most recently, we have demonstrated that a phosphatase called PTP-PEST is involved in the dephosphorylation of Src at Tyr527 and phosphorylation of Src at Tyr418 in the catalytic site (10). This resulted in activation of Src and interaction of Src, cortactin, and Arp2/3 complex with WASP. WASP, which is identified in the sealing ring of resorbing osteoclasts, also exhibited interaction with Src, PYK2, cortactin, PTP-PEST, Pro-Ser-Thr phosphatase-interacting protein (PST-PIP), and Arp2/3 in immunostaining analyses (7). Furthermore, WASP integrates signals from Rho, Cdc42, and kinases to bind and stimulate actin polymerization and sealing ring formation in osteoclasts (5, 7, 10). Experiments with WASP peptides containing Pro-rich and Tyr(P)294-containing peptides demonstrated significant effects on osteoclast signaling and sealing ring formation (11). Modulation of the phosphorylation state of WASP by kinase(s) assists in integrating multiple signaling molecules that play a part in the assembly of...
Regulation of Sealing Ring Formation

the sealing ring. Integrin αβ3 signaling plays a key role in this process. We suggest this based on observations that osteoclasts treated with osteopontin, a ligand for αβ3, increase interaction of signaling proteins with WASP, formation of the sealing ring, and bone resorption (7).

Mounting evidence has demonstrated the ability of the integrin αβ3-mediated pathway to induce osteoclast activity and bone resorption. Formation of signal-generating complex consisting of Src, PYK2, focal adhesion kinase, p130Cas, c-Cbl, and PI3K associated with αβ3 has been shown to play roles in the organization of the sealing ring during bone resorption (7, 12–19) (for a review, see Ref. 20). An inhibitor to αv, RGD mimetics, and αβ3-blocking antibodies inhibit bone resorption in vitro and in vivo, suggesting that this integrin plays an important role in osteoclast function (21–23). Integrin αβ3-associated proteins integrate multiple signaling pathways and signaling molecules that partake in the assembly of the sealing ring during bone resorption and podosome assembly/disassembly during migration in osteoclasts.

The importance of TNF-α has been implicated in osteoclastogenesis and the bone loss that occurs in inflammatory diseases, such as rheumatoid arthritis and periodontitis (24–29). Fuller et al. (24) have shown that TNF-α potently directly activates osteoclasts, and actin rings were formed rapidly in response to minuscule concentrations of TNF-α. TNF-α was as potent as receptor activator of NF-κB ligand (RANKL) in osteoclast activation and even more effective in activation than osteoclast formation. It seems that TNF-α has the potentiality to act by itself and synergize with RANKL in osteoclast differentiation and bone resorption. Further investigations on the spatially and temporally regulated functions of actin-binding proteins and pathways are necessary to identify their role in actin cytoskeleton dynamics during bone resorption.

Osteoclasts may contain a variety of actin-binding proteins besides WASP, gelsolin, profilin, and Cap Z. However, it is not clear how these proteins specifically operate at the level of assembly of actin-related structures during bone resorption. The focus of this paper is to address how the sealing ring, a structure fundamental to the function of the osteoclast, is organized and regulated. Therefore, to identify the key proteins involved in sealing ring formation as well as to dissect the dynamic mechanisms that govern the formation of the sealing ring, we have used the novel proteomic approach to profile the dynamic mechanisms that govern the formation of the sealing ring.

In vitro and in vivo signaling plays a key role in this structure fundamental to the function of the osteoclast, is organized and regulated. Therefore, to identify the key proteins involved in sealing ring formation as well as to dissect the dynamic mechanisms that govern the formation of the sealing ring, we have used the novel proteomic approach to profile the dynamic mechanisms that govern the formation of the sealing ring.

**Regulation of Sealing Ring Formation**

- **Antibody to L-plastin (SC-16657), actin (SC-8432), GAPDH (SC-32233), p130Cas (SC-859), PYK2 (SC-1514), WASP (SC-13139), c-Src (SC-8056), anti-phosphotyrosine (PY20, SC-508), αv (SC-6617), and β3 (SC-6627) as well as siRNA to cortactin (SC-35093) and control RNAi (SC-44233) were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphoserine antibody was purchased from Zymed Laboratories Inc., Inc. (San Francisco, CA). Cortactin (H222, catalog no. 3503) antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies to GAPDH and TNF-α (anti-mouse; catalog no. 10950) were bought from Abcam Inc. (Cambridge, MA). Recombinant TNF-α and M-CSF proteins and TNFR1 (TNF-α receptor 1) antibody were purchased from R & D Systems (Minneapolis, MN). Cyclo-RGD peptide (catalog no. PCI-3661-P1) was purchased from Peptides International (Louisville, KY). Protein estimation reagent, molecular weight standards for proteins, and PAGE reagents were bought from Bio-Rad. Cy2- and Cy3-conjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA). HRP-conjugated secondary antibodies for immunoblotting were obtained from GE Healthcare.
- pEGFP-actin vector was bought from Clontech. Rhodamine-phalloidin and other chemicals were purchased from Sigma.

**Preparation of Osteoclast Precursors from Mice**—C57/BL6 mice were used for osteoclast preparation as described previously (7). These mice were either purchased from Harlan Laboratory or generated in the animal facility of the University of Maryland Dental School. Breeding and maintenance were carried out as per the guidelines and approval of the institutional animal care and use committee. Osteoclasts were generated in vitro using mouse bone marrow cells as described previously (7).

**Purification of Glutathione S-Transferase (GST)-fused RANKL**—pGEX vector containing RANKL cDNA was expressed in Escherichia coli as GST fusions as described previously (30). SDS-PAGE and Coomassie Blue staining tested the purity of the RANKL. Accurate protein content of the purified RANKL protein was determined by densitometric quantification of the protein bands separated by SDS-PAGE using known concentrations of BSA as a standard.

**Preparation of Osteoclast Lysate after Various Treatments**—After flushing the marrow cells for osteoclast differentiation, mouse long bones (free of cells inside and muscles outside) were washed extensively with PBS and kept in ethanol until use. Long bones were air-dried in hood and homogenized by a miniblender. Bone particles were sieved, and bone particles 60–80 μm in size were used for experiments. The multinucleated osteoclasts were seen from day 4 onward, as shown in Fig. 2. At this stage, osteoclasts were added with sterile native bone particles. 100 μg of bone particles were added to a well of 6-well cell culture plates. Three to four wells were used per condition.
Osteoclasts were treated with (+) and without (−) bone particles for 16–18 h in the presence of macrophage colony-stimulating factor (10 ng/ml) and RANKL (50–60 ng/ml).

Some cultures were treated with mouse TNF-α (20 ng/ml) and bisphosphonates (alendronate or pamidronate, 50 μM) at 37 °C. Bisphosphonate stocks (1 mM) were made in PBS or sterile H2O (31). Cells were treated with bisphosphonates in the presence of bone particles, M-CSF, and RANKL. Prior to the addition of TNF-α, osteoclasts generated with RANKL and M-CSF were washed extensively with serum-free alpha-minimum essential medium and incubated with the same for 30 min. Subsequently, adherent cells were detached with stripping solution (catalog no. 25056-CL; Cellgro by Media Tech, Inc., Herndon, VA) as described previously (32). Cells were replated in MEM containing 10% fetal bovine serum (FBS), M-CSF and TNF-α. Cultures treated with TNF-α do not contain RANKL. Based on dose-dependent studies, cells were neutralized with 40–50 ng/ml TNF-α antibody (goat polyclonal anti-mouse TNF-α; catalog no. ab10950, Abcam) and 3–5 μg/ml anti-mouse TNFR1 (MAB430) antibody. To enhance the blocking effect, cells were preincubated with the antibody of interest for 60–90 min prior to the addition of TNF-α and M-CSF. Incubation was continued for 12–14 h for the bone resorption assay and immunostaining analyses. Normal serum was used as a control for the antibody treatment. We have performed a trypan blue dye (Sigma T8154) exclusion test to determine the viability of osteoclasts after various treatments. Cells demonstrated clear cytoplasm with no inclusion of blue dye. Osteoclasts were viable after various treatments, such as bone particles, bisphosphonates, αv inhibitor, Src inhibitor, and anti-TNF-α.

Following various treatments, osteoclasts were washed three times with cold PBS and lysed in a radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1% aprotinin, 2 mM PMSF, 100 μM Na3VO4, and 1% aprotinin), as described previously (33). Cells were rocked on ice for 15 min and scraped off with a cell scraper. Cell lysates were centrifuged at 15,000 rpm for 5 min at 4 °C, and the supernatant was saved. Protein contents were measured using Bio-Rad protein assay reagent.

SDS-PAGE and Western Analysis—About 50–100 μg of protein was used for 10% SDS-PAGE. After electrophoresis, the gels were washed and stained with Coomassie Brilliant Blue for 2–3 h as described previously (34). About 100–150 μg of protein was used for immunoblotting analysis. The proteins were transferred to a PVDF membrane for Western analysis. Blots were blocked with 10% milk in PBS containing 0.5% Tween (PBS-T) for 2–3 h and then incubated with 1:1000 dilutions 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). Blocking and immunoblotting were performed with 5% BSA in PBS-T for phosphotyroisine antibody (PY20) (32).

Immunohistochemistry and Actin Staining—Osteoclast precursors (102 cells/coverslips) were cultured on dentine slices and incubated for different time periods as indicated in Figs. 1, 5, 6, and 7. 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 (5). 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 (cortactin, β3, or L-plastin) of interest (1:100 dilutions) in the blocking solution for 2 h at 4 °C. The primary antibodies were detected with either Cy2- or FITC-conjugated secondary antibody (1:100 dilutions). Cells stained for cortactin, β3, or L-plastin were stained for actin with rhodamine-phalloidin, as described previously (35). 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 Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

Actin Staining with Rhodamine-Phalloidin—Cells were fixed and permeabilized with 3.7% paraformaldehyde and 0.1% Triton X-100 for 10 min. Cells were then washed with PBS containing 5 mM EGTA (PBS/EGTA) and stained for actin with rhodamine-phalloidin (1:1000 dilution; Sigma) as described previously (2). Actin-stained cells were viewed and photographed on a Bio-Rad confocal laser-scanning microscope. Images were stored in TIF image format and processed by Adobe Photoshop (Adobe Systems Inc.). Sealing (actin) rings were counted in osteoclasts (~300–350), and significance was calculated as described below.

Bone Resorption Assay in Vitro—Bone resorption assay was performed as described previously (37). Osteoclasts were cultured on dentine slices and incubated for 16–18 h. Subsequently, dentine slices were scanned in confocal microscopy to determine the resorbed area. Images were stored in TIF format and processed by Adobe Photoshop (Adobe Systems Inc.).

Statistical Analysis—All values were mean ± S.E. of three or more experiments done at different times normalized to intraexperimental control values. A value of <0.05 was considered significant. For statistical comparisons, analysis of variance was used with Bonferroni corrections (INSTAT for IBM version 2.0, GraphPad software).

RESULTS

Analysis of Time-dependent Changes in Cytoskeletal Organization during Bone Resorption

During the process of bone resorption, osteoclasts undergo reorganization of actin cytoskeleton. Some of the earlier mechanisms required for generation and maturation of the sealing zone have not been studied. Therefore, we proceeded to establish the sequential changes in the morphology and organization of actin filaments in osteoclasts subjected to bone resorption in vitro. Osteoclasts plated on dentine slices were fixed at the indicated time and stained with rhodamine-phalloidin for actin. Fig. 1 illustrates time-dependent changes in the organization of actin cytoskeleton. Between 1 and 3 h, osteoclasts begin to spread on bone by adhering to the bone surface through the formation of secondary actin aggregates, which function as secondary adhesive
Regulation of Sealing Ring Formation

FIGURE 1. Analysis of actin distribution in osteoclasts subjected to bone resorption. Osteoclasts plated on dentine slices for 3 h (A and A’), 8 h (B), and 12 h (C) were stained with rhodamine-phalloidin to determine the changes in the distribution of actin during bone resorption. Osteoclasts are outlined in A’ to indicate stretching off of membrane at 2–3 h. Actin aggregates that most likely function as secondary adhesive structures are indicated by arrows in A’. An increase in the number of actin rings (B and C) was observed as a result of the increased surface area of osteoclasts at 8 and 12 h. Scale bars, 25 μm. The results are representative of four experiments with four separate osteoclast preparations.

Osteoclasts Migrated toward and Adhered to the Native Long Bone Particles

Osteoclasts subjected to bone resorption undergo considerable changes in morphology due to reorganization of actin cytoskeleton, as shown in Fig. 1. Therefore, we determined the changes in morphology of osteoclasts incubated with native bone particles (Fig. 2). Osteoclast precursors were observed on day 4 or 5 in culture (Fig. 2B). Native long bone particles of mice (60–80 μm; Fig. 2A) were added to cells at this stage (100 μg/well in a 6-well cell culture plate) for 16–18 h in the presence of RANKL (40–50 ng/ml) and M-CSF (10 ng/ml), as described under “Experimental Procedures.” Pictures were taken in an inverted phase-contrast microscope (Fig. 2, A–D). Migration of osteoclasts toward the particles was observed at 2 h (C), and attachment of osteoclasts to bone was observed at 4 h (D). Osteoclasts attached to bone particles were indicated by arrowheads (C and D). Similar observations were found in short term live cell analysis of osteoclasts expressing GFP-actin and incubated with dentine slices (supplemental Fig. S1, A and B).

Analysis of Expression Profile of Proteins in Osteoclasts Subjected to Bone Resorption

Lysates (~50 μg) made from osteoclasts incubated with (+) and without (−) bone particles for 16–18 h in the presence of RANKL and M-CSF were used for SDS-PAGE and Coomassie Blue staining (Fig. 3A). A dramatic increase in 80–85-kDa protein and a decrease in 65-kDa protein were observed (Fig. 3A, lanes 1 and 2). These proteins were identified as cortactin and L-plastin by immunoblotting analyses with respective antibodies (Fig. 3A, lanes 3 and 4). Lysates (500 μg) made from osteoclasts treated with or without bone particles were subjected to two-dimensional gel electrophoresis (supplemental Figs. S2 and S3). Two-dimensional gel electrophoresis and mass spectrometry (MS) analyses corroborate the above findings. The major spots in two-dimensional gel electrophoresis were digested, and the peptides were subjected to MS analysis. A trace yielded about 15–17 matching peptide masses, representing about 30–35% coverage for L-plastin (supplemental Table S1, under (−) Bone). Peptide sequences identified in MS are highlighted in red in supplemental Fig. S3A. A representative mass spectrum (FSLVGIAGQDLNEGNR) is provided in supplemental Fig. S3B. Similarly, a trace yielded about 25–31 matching peptide masses, representing about 19–22% coverage for cortactin (supplemental Table S1) in osteoclasts treated with bone particles (supplemental Table S1, under (+) Bone).

Because there is a decrease in the level of L-plastin at 16–18 h, we extended this analysis to determine the L-plastin levels at different time periods after the addition of bone particles in the presence of RANKL and M-CSF. Immunoblotting with an antibody to L-plastin was performed with ~50 μg of lysate protein.
Immunoblotting of the same blot with an actin antibody after stripping was used as a loading control. A decrease in the level of L-plastin was observed from 8 h onward (Fig. 3B, lanes 3–6) and remained unchanged until 20 h (lane 6). The decrease at 16–20 h corresponds with the decrease shown in Fig. 3A. These results suggest that changes in the levels of L-plastin occur at the early stage of bone resorption could be correlated with the dynamic nature of the sealing ring.

Analysis of Protein Profile in Osteoclasts Incubated with Different Substrates

Osteoclasts can attach to various substrates, such as glass, synthetic mineralized materials (e.g. osteologic discs), and mineralized bone (e.g. dentine, ivory, and natural bone). We have used previously (1, 10, 11) and at this time biologically derived hard tissues, such as devitalized native bone or dentine slices, as substrates for in vitro bone resorption assays. Researchers also use osteoclastic cells containing submicron calcium phosphate films (~0.6 μm) on transparent quartz or calcium phosphate films for in vitro bone resorption assays. Therefore, we explored whether the aforesaid substrates are biologically comparable and exhibit a very similar effect, as shown in Figs. 1–3. We have observed migration of osteoclasts toward native devitalized bone particles (Fig. 2C) and dentine (supplemental Fig. S1) but not toward osteologic discs. Therefore, osteoclasts were cultured on the surface of osteologic discs and incubated in the presence of RANKL (40–50 ng/ml) and M-CSF (10 ng/ml) for 16–18 h. SDS-PAGE was performed with lysates made from osteoclasts treated with the indicated substrates (Fig. 3C) for 16–18 h. Osteoclasts under the same conditions as above but untreated with any of the indicated substrates were used as controls (Fig. 3C, lane 1). Although the geometry of bone and dentine surfaces could be different, a very similar protein profile was observed in SDS-PAGE analysis (lanes 2 and 4). However, the protein profile of lysates made from osteoclasts incubated on osteologic discs (lane 3) does not correspond with these profiles (lanes 2 and 4). Osteologic discs lack bone matrix proteins and the geometry of dentine or bone particles. It is possible that synthetic mineralized matrices were quickly resorbed by mature osteoclasts within 4–6 h of incubation. We have observed osteoclasts attached on the quartz disc at this time period after the completion of resorption of the calcium phosphate matrix (data not shown). This could be the possible reason for a relatively similar protein profile in osteoclasts untreated with bone particles (lane 1) or plated on osteologic discs (lane 3).

Analysis of the Phosphoprotein Profile in Osteoclasts Incubated with Bone Particles

Two-dimensional gel electrophoresis and MS analyses of lysates made from osteoclasts incubated with bone particles demonstrated peptides for a few of the proteins (Src kinase, vacuolar H⁺-ATPase B2, vacuolar ATP synthase catalytic subunit A, L-plastin, cathepsin D and K, Arp2/3, cortactin, and gelsolin) identified as being involved in bone resorption. In order to gain insight into the signaling molecules involved in osteoclast bone resorption in general, we looked at the phosphorylation state of proteins on tyrosine and serine residues. We used ~250 μg of lysate protein for immunoblotting analyses (Fig. 3, D and E). Immunoblotting analysis with an antibody to phosphotyrosine (Fig. 3C) displayed an increase in the phosphorylation of p130cas, cortactin, WASP, and c-Src in osteoclasts treated with bone particles. Minimal changes in the tyrosine phosphorylation of PYK2 were observed (Fig. 3D, lanes 1 and 2). This status might not exclude the role PYK2 in osteoclast function. Phosphorylation may perhaps occur at the early stage of maturation of the sealing ring from actin aggregates. PYK2-dependent podosome belt formation in PYK2 null osteoclasts has been shown to occur by the kinase-defective mutant. It is also possible that PYK2 may function as a platform for...
Regulation of Sealing Ring Formation

FIGURE 4. SDS-PAGE and immunoblotting analyses of lysates made from osteoclasts subjected to various treatments. A and B, time-dependent effects of TNF-α on L-plastin and cortactin levels. Equal amounts of lysate proteins were used for immunoblotting analysis with an antibody (ab) to L-plastin (A) and cortactin (B). Actin was used as a loading control in A and B. C, equal amounts of lysate proteins from osteoclasts treated as indicated in the figure were subjected to SDS-PAGE (10%) and Coomassie Blue staining analyses. MCSF, macrophage colony-stimulating factor; (-) and (+), untreated and bone particle-treated; IgG, control nonspecific IgG control; TNFR1, anti-TNF-1 receptor. D and E, equal amount of lysate proteins from osteoclasts treated with various treatments, as indicated in the figure, were used for SDS-PAGE/Coomassie Blue staining (D) and immunoblotting analyses (E). Immunoblotting analyses with phosphocortactin (Tyr(P)\(^{567}\)) (E, top, p-Cortactin), cortactin (E, middle), and GAPDH (E, bottom) are shown. F-I, bone resorption assay in vitro. Osteoclasts were treated as indicated at the top of each panel (E–I). Pits were scanned under confocal microscopy. Resorption pits were seen as dark spots. Scale bar, 25 μm. These results represent one of three separate experiments performed with the same results.

recruitment and assembly of signaling proteins besides its function as a kinase (37). An increase in the tyrosine phosphorylation of c-Src and cortactin (Fig. 3D, lanes 1 and 2) corresponds with the protein levels in osteoclasts treated with bone particles (lanes 3 and 4). However, equal levels of WASP, PYK2, and p130\(^{Crk}\) proteins were observed in osteoclasts untreated and treated with bone particles (Fig. 3D, lanes 3 and 4). This observation confirms the previous observations of ours and others that an increase in the phosphorylation of several signaling proteins is necessary for osteoclast bone resorption (6, 7, 14, 18, 38).

Immunoblotting analysis with a phosphoserine antibody showed a decrease in the phosphorylation state of L-plastin in osteoclasts treated with bone particles (Fig. 3E, lane 2). This corresponds with the decreased protein level of L-plastin (Fig. 3E, lane 4). Immunoblotting analysis with a GAPDH antibody demonstrated the same levels of GAPDH protein (Fig. 3, A, D, and E). Overall, these results demonstrate a decrease in L-plastin and an increase in cortactin in osteoclasts incubated with native bone particles for 16–18 h in the presence of RANKL and M-CSF.

Analysis of the Effects of TNF-α on the Protein Profile in Osteoclasts Subjected to Bone Resorption

TNF-α is sufficient to induce osteoclast differentiation and bone resorption independently of the RANKL signaling pathway (19, 20, 39–42). Chronic inflammatory bone diseases, such as rheumatoid arthritis, periodontal disease, and aseptic periprosthetic osteolysis are characterized by bone loss around affected joints and teeth caused by increased osteoclastic bone resorption. This resorption is mediated largely by the increased local production of TNF-α (42). Therefore, we checked whether TNF-α could induce effects similar to that of RANKL in osteoclasts subjected to bone resorption with bone particles for the times indicated in Fig. 4 (A and B). An equal amount (~50 μg) of lysate protein made from osteoclasts incubated with (+) and without (−) bone particles in the presence of TNF-α and M-CSF was used. Immunoblotting analysis was performed with L-plastin (Fig. 4A) and cortactin (Fig. 4B) antibody. We have observed a time-dependent increase in L-plastin level at 2 and 4 h. A decrease was observed from 8 h onward, and the decrease was stabilized until 12–20 h and then increased at 24 h. Time-dependent changes in the levels of L-plastin protein correspond with the phosphorylation level of L-plastin on serine residues (data not shown). However, cortactin level remains the same in osteoclasts untreated for 24 h (B, lane 1) and treated (lanes 2 and 3) with bone particles for 2 and 4 h. A gradual increase in the level of cortactin was observed from 8 h onward, and this increase was stabilized until 24 h.

As shown in Fig. 4, TNF-α (Fig. 4C, lane 4) reproduced the effects mediated by RANKL (lane 2) in the presence of bone particles for 16–18 h. An increase in the levels of cortactin with a corresponding decrease in L-plastin was observed under these conditions (lanes 2 and 4) as compared with osteoclasts treated with M-CSF only in the presence of bone particles (lane 3) or both M-CSF and TNF-α in the absence of bone particles for 16–18 h (lane 1). Osteoclasts were pretreated with an antibody to TNF-α (lane 6) or TNF receptor (lane 7) for 60–90 min prior to the addition of bone particles, TNF-α, and M-CSF as described under “Experimental Procedures.” Osteoclasts treated as above with antibody failed to show a decrease in L-plastin level. The level remains the same as in control osteoclasts untreated with bone particles but treated with TNF-α and M-CSF (Fig. 4C, lane 1). However, a small increase in the level of cortactin was observed in these cells (lanes 6 and 7). Treatment of osteoclasts with species-specific IgG (lane 5) serves as a control for TNF-α antibody treatment (lane 6). The response to TNF-α antibody was not observed in osteoclasts treated with IgG. Thus, the failure of anti-TNF-α/TNFR1 treatment to alter L-plastin levels suggests that TNF-α/TNFR1 signals mediate the early phase of sealing ring formation.

Analysis of the Effects of Bisphosphonates on the Protein Profile in Osteoclasts Subjected to Bone Resorption

Bisphosphonates inhibit bone resorption by osteoclasts (43–46). We recently reported that bisphosphonates could modulate the function of osteoclasts by inhibiting the tyrosine phos-
Regulation of Sealing Ring Formation

Phosphorylation of cortactin by regulating the activity of PTP-PEST and Src kinase (10). Consistent with this observation, immunoblotting analysis with a phosphospecific antibody to cortactin Tyr421 demonstrated a significant decrease in the Tyr(P)421 blotting analysis with a phosphospecific antibody to cortactin phosphorylation of cortactin by regulating the activity of PTP-PEST (39, 42). A decrease in the phosphorylation of cortactin is in osteoclasts treated with alendronate (31) suggest that bisphosphonates could modulate the function of osteoclasts by inhibiting integrin αvβ3 downstream signaling involved in tyrosine phosphorylation of cortactin (10). This could be separate from the Rho GTPase pathway (44).

Analysis of Time-dependent Changes in Actin Organization in Osteoclasts Subjected to Bone Resorption

The experiments shown in Figs. 3 and 4 indicate a clear association of L-plastin and cortactin to osteoclast bone resorption. We sought to determine the spatiotemporal organization of L-plastin and cortactin in osteoclasts plated on dentine slices by immunostaining and confocal analyses (Fig. 5). It is of particular interest to determine whether the changes in the levels and localization of L-plastin and cortactin are in fact associated with the reorganization of actin structures in resorbing osteoclasts. Immunostaining of osteoclasts plated on dentine slices were performed with L-plastin (Fig. 5, A–F) and cortactin (Fig. 5, G–L). Costaining with rhodamine-phalloidin was performed to determine the actin localization. Higher magnification images of osteoclasts exhibiting changes in actin organization are shown. Changes in the actin distribution were determined at different time points in ~300 osteoclasts and provided as a graph in Fig. 5M. We have observed a spatiotemporal change in the sealing ring organization and composition (i.e. localization of L-plastin and cortactin) in a time-dependent manner as described below.

L-Plastin/Actin Distribution—Besides diffuse distribution, L-plastin localization was also observed as patches or aggregates (indicated by arrowheads) within 1 h (Fig. 5A) of plating mature osteoclasts on dentine. Colocalization of L-plastin and actin was observed in some of these aggregates (indicated by the arrows in Fig. 5, A and B). These aggregates develop into larger sizes in a time-dependent manner from 2 to 4 h (Fig. 5, B and C). Multiple big and small actin aggregates were observed at 4 h. Colocalization (yellow) of L-plastin and actin was observed in these aggregates (Fig. 5C). Actin aggregates fuse together by 6 h (Fig. 5D), at which time localization of L-plastin in these structures is reduced, but diffuse distribution of L-plastin was observed throughout the osteoclast (Fig. 5D). A gradual decrease in the localization of L-plastin (Fig. 5, E and F) accompanies cortactin localization in actin aggregates and maturation of sealing ring formation. Actin aggregate formation corroborates the actin staining shown in Fig. 1. These data indicate that the actin aggregates or patches are possible precursors or pre-sealing zone for the mature sealing ring.
Regulation of Sealing Ring Formation

FIGURE 6. Analysis of the effects of depletion or knockdown of cortactin on sealing ring formation and bone resorption. I, immunoblotting analysis of cortactin levels in osteoclasts treated with control RNAi (Sc; 200 nM) and siRNA (Si; 100 and 200 nM) to cortactin (A). Immunoblotting analysis with GAPDH is shown in B. II, the effects of ScRNAi (A and D) and siRNA (B, C, and E) to cortactin on sealing ring formation. Osteoclasts were stained for cortactin (green) and actin (red). Cortactin distribution is shown in A–C. The effects of scrambled RNAi and siRNA on multiple osteoclasts are shown in D and E. Osteoclasts were stained for actin (red) with rhodamine-phalloidin. Dentine is shown by the reflected light (green in D and E). Resorption pits were found underneath osteoclasts (D, D’, E, and E’). Actin distribution (D’ and E’) and resorption lacuna (D’ and E’) are shown separately in gray. Actin aggregates and superficial pits are indicated by asterisks in E and E’. Sealing rings are indicated by arrowheads (D and D’) and arrows (E and E’). Scale bar, 25 μm. III, bone resorption assay in vitro. Osteoclasts treated with scrambled ScRNAi and siRNA were cultured on dentine for 24 h. Pits were scanned in a confocal microscopy. Resorption pits were seen as dark spots. Experiments were repeated three times with three different osteoclast preparations. These results represent one of three separate experiments performed with the same results.

Cortactin/Actin Distribution in Sealing Ring—Distribution of cortactin in actin aggregates is very minimal or not observed at 4 h (Fig. 5G). Localization of cortactin was observed from 5 to 6 h onward (Fig. 5H) with a concomitant decrease in the localization of L-plastin (Fig. 5D). An increase in the localization of cortactin corresponds with the maturation of actin aggregates to the sealing ring between 8 and 10 h (I and J). A gradual decrease in the localization of cortactin was observed from 12 h onward (K), and at 14 h, cortactin was not observed in the mature actin ring, although diffuse distribution of cortactin was observed in these cells (L). The time-dependent changes in actin organization in osteoclasts expressing GFP-actin (supplemental Fig. S1) correspond with the changes shown in fixed cells (Fig. 5). Individual frames of the osteoclasts at 2–4, 4–6, and 10–12 h time periods are shown. Actin aggregates were formed at 4–6 h, and maturation of actin aggregates into a functional sealing ring was observed at 10–12 h (supplemental Fig. S1, Sr in C’). Cells spread well on the bone at this time (supplemental Fig. S1C), and a resorption pit (supplemental Fig. S1, 2 in C’) was found underneath the sealing ring (supplemental Fig. S1C).

Analysis of the Role of Cortactin in Sealing Ring Formation and Bone Resorption

To address the possible role of cortactin in the maturation of actin aggregates to sealing ring, we knocked down or depleted cortactin using siRNA sequences. Untreated (Fig. 6I, A, lane 1) and scrambled RNAi (ScRNAi)-treated osteoclasts were used as controls. Osteoclasts were incubated with siRNA (A, lanes 3 and 4) or ScRNAi (lane 2) for 18–20 h at the indicated doses as described previously (7). A dose-dependent decrease in the cortactin level was observed in osteoclasts treated with siRNA to cortactin and incubated for 16–18 h at 37 °C (A, lanes 3 and 4). The decrease was maximal at 200 nM siRNA (~90–95%; lane 4) as compared with the levels of protein in ScRNAi-transfected osteoclasts (A, lane 2). The viability of osteoclasts was tested by a trypan blue exclusion test. Immunoblotting with a GAPDH antibody was used as a loading control (Fig. 6I, B).

After transfection with siRNA and ScRNAi for 18–20 h, osteoclasts were cultured on dentine slices for 10–12 h. Osteoclasts transfected with ScRNAi displayed mature sealing rings (Fig. 6II, A and D). Localization of cortactin (6II, A)
Regulation of Sealing Ring Formation

Localization of Integrin β3 in Sealing Ring

L-plastin phosphorylation has been shown to associate with integrin activation (47). Osteoclasts cultured on dentine slices for 12–14 h were processed for double staining with actin/cortactin (I, A and B) and actin/integrin β3 (C). Confocal microscopic images of osteoclasts stained for actin (A–C, red), cortactin (A’, green), and integrin β3 (C’, green) are shown. Yellow color represents colocalization of cortactin and integrin β3 with actin (overlay panels A’ and C’). A higher magnification view of the area indicated by an arrow in A’ is shown in B and B’. More than eight actin rings (outlined in B’) were observed in this osteoclast. Resorption pits are indicated by asterisks, and an osteoclast that exhibits single or multiple sealing rings is indicated by an arrowhead (A, C, and C’). Scale bars, 10 μm (B) and 25 μm (C’). II, confocal analysis of localization of actin with cortactin, integrin β3, and L-plastin in osteoclasts treated with siRNA and ScRNAi to cortactin. Osteoclasts were treated with ScRNAi (A) and siRNA to cortactin (B and C). Confocal microscopic images of osteoclasts stained for actin (A–C, red), cortactin (A, green), integrin β3 (B, green), and L-plastin (C, green) are shown. Yellow color represents colocalization of cortactin and integrin β3 with actin (overlay panels A and B). Scale bar, 25 μm. The results represent one of the three separate experiments performed with three different osteoclast preparations.

either small actin rings (indicated by arrows in Fig. 6II, E and E’) or actin aggregates (Fig. 6II, B’ and C’; indicated by asterisks in E). A decrease in bone resorption in vitro was observed (Fig. 6II, E”) in a dose-dependent manner (Fig. 6III, C and D). The presence of actin aggregates and the failure of sealing ring formation in siRNA-treated cells suggest that cortactin-mediated actin polymerization is involved in the maturation of the sealing ring.

and actin (A’) was observed in the sealing ring of resorbing osteoclasts. The in vitro bone resorption assay displayed an increase in the pit area or complexity with untreated and scrambled RNAi-treated osteoclasts (Fig. 6II, A and B). A significant decrease in the staining of cortactin was observed in a dose-dependent manner in siRNA to cortactin-treated cells (Fig. 6II, B and C). The effects of scrambled RNAi and siRNA on multiple osteoclasts are shown (Fig. 6II, D and E). Osteoclasts transfected with siRNA to cortactin displayed indicated by an arrow (Fig. 7I, A”), is shown at higher magnification in Fig. 7I, B and B”. Colocalization of actin with cortactin was observed in patches in a confocal microscopy scan of the osteoclast taken at the level of dentine (Fig. 7I, B and B”). More than eight actin rings (outlined in Fig. 7I, B”) were observed in this osteoclast. The sealing rings formed were efficient and functional, which is recognized from the matching resorption pit underneath the osteoclast (indicated by an asterisk in Fig. 7I, B, B”, and C”).
Regulation of Sealing Ring Formation

The Effect of Cortactin Depletion on the Formation of Sealing Ring

After treating with ScRNAi (Fig. 7II, A) and siRNA (Fig. 7II, B and C) to cortactin for 18–20 h, osteoclasts were cultured on dentine slices for 8–12 h. Cells were double-stained for actin/ cortactin, actin/integrin β3, and actin/L-plastin. Osteoclasts transfected with ScRNAi displayed colocalization of actin and cortactin in sealing rings (7II, A). As shown in Fig. 6, actin aggregates were found in osteoclasts transfected with siRNA to cortactin (Fig. 7II, B and C). These actin aggregates demonstrated colocalization of integrin β3 (Fig. 7II, B) but not L-plastin (Fig. 7II, C). L-plastin staining was observed throughout the osteoclasts but at a very minimal level. The reduced staining corresponds with the reduced L-plastin levels at 8–20 h in osteoclasts incubated with bone particles for 4–20 h (Fig. 3B). Failure of maturation of the sealing ring in cortactin-depleted osteoclasts (Fig. 6) despite the localization of integrin αv (data not shown) and β3 in actin aggregates (Fig. 7II, B) suggests that the maturation process is dependent on cortactin. Other actin-binding protein(s) could not recompense its role in the maturation process.

Analysis of Actin Distribution in Response to Treatment with TNF-α

Several studies report interesting outcomes for the role of TNF-α in osteoclast differentiation and function (24, 25, 39, 48, 49). Cyclical changes in L-plastin and cortactin protein levels were observed in a time-dependent manner (Fig. 4, A and B). Next, we proceeded to determine if TNF-α could reproduce the effects mediated by RANKL in the chronological processes of organization of the mature sealing ring. Therefore, osteoclast precursors were cultured on top of dentine slices and incubated for 2–3 h at 37 °C for adhesion. After adhesion for 2–3 h, cells were replaced with fresh medium containing TNF-α and M-CSF, and incubation at 37 °C was continued for 4, 8, and 12 h. Cells were fixed and stained with rhodamine-phalloidin to determine the distribution of actin. Consistent with previous studies (24), we have shown here that TNF-α has a role in osteoclast sealing ring formation during bone resorption. We have observed time-dependent changes in the organization of actin filaments. TNF-α treatment simulates the formation of actin aggregates at 3–4 h (Fig. 8I, A). Between 8 and 10 h, the size of actin aggregates increased in addition to the formation of some actin rings. Osteoclasts spread well and form multiple actin or sealing rings at 12–14 h (Fig. 8I, C). Actin rings (indicated by the arrowheads in Fig. 8I, B and C) formed at 8 h (B) and 12 h (C) are capable of resorbing bone, and pits were found underneath the sealing ring (green panels in B and C). TNF-α (Fig. 4C) reproduced the effects mediated by RANKL in changes in the levels of L-plastin and cortactin (Fig. 4C) as well as organization of the sealing ring in a time-dependent manner.

FIGURE 8. I, confocal analysis of time-dependent changes in actin organization and bone resorption in response to TNF-α. Osteoclasts plated on dentine slices were treated with TNF-α for 2–3 h (A), 8 h (B), and 12 h (C). Confocal microscopic images of osteoclasts stained for actin are shown. Distribution of actin (red) and scans of dentine slices (green) are shown separately. Several resorption pits were observed after incubation with TNF-α for 12–14 h. II, confocal analysis of the effects of various treatments on sealing ring formation and bone resorption. Osteoclasts were treated with a neutralizing antibody to TNF-α (B), Src (C), and αv inhibitor (D) in the presence of TNF-α. Osteoclasts treated together with a species-specific IgG and TNF-α were used as control (A) for neutralizing antibody to TNF-α antibody treatment (B). Sealing rings are indicated by arrowheads in A. Punctate podosome-like structures are indicated by crooked arrows in B and C (red panel). Actin patches (B and C, red panel) and aggregates are indicated by arrows (D, red panel). Bone resorption is considerably reduced in osteoclasts treated with a neutralizing antibody to TNF-α (B) and inhibitors to Src and αv (C and D) in the presence of TNF-α (green panels). Scale bar, 25 μm. The results represent one of the three separate experiments performed with three different osteoclast preparations.
Studies with anti-TNF-α and anti-TNFR1 suggest that TNFRI can elicit signals related to the formation of actin aggregates because osteoclasts treated with these antibodies failed to show changes in the levels of L-plakin (Fig. 4C, lanes 6 and 7). Therefore, to further elucidate the possible role of TNF-α signaling in osteoclast cytoskeletal reorganization during bone resorption, osteoclasts were treated with the neutralizing antibody to TNF-α (Fig. 8II, B), Src inhibitor (Fig. 8II, C), and αv inhibitor (Fig. 8II, D) plus TNF-α for 12–16 h. Osteoclasts treated with a species-specific non-immune IgG in the presence of TNF-α were used as a control (Fig. 8II, A) for the antibody treatment shown in Fig. 8II, B. Osteoclasts were stained for actin with rhodamine-phalloidin (Fig. 8II). Consistent with the observation shown in Fig. 8I, C, TNF-α not only increased the spreading of osteoclasts but also the formation of several sealing rings in one osteoclast. Nonspecific IgG had no effect on the changes elicited by TNF-α (Fig. 8II, A).

A neutralizing antibody to TNF-α, Src inhibitor, and αv inhibitor reduced the effects of TNF-α at different levels. These osteoclasts displayed a considerable decrease in bone resorption (Fig. 8III, B–D, green panel). Osteoclasts treated with a neutralizing antibody to TNF-α (Fig. 8II, B) and an Src inhibitor (8II, C) in the presence of TNF-α demonstrate comparable effects. Under both conditions, punctate and small patchy actin structures were observed in these osteoclasts (B and C). Punctate structures resemble podosomes and are distributed throughout the osteoclasts and at the periphery (8II, B and C; indicated by crooked arrows). Actin patch-like structures are smaller in size. Therefore, both podosome- and actin patch-like structures may compromise cell adhesion on bone but not actin aggregate or sealing ring formation. TNF-α signaling might be essential for the disassembly of podosomes and formation of actin aggregates. It is possible that Src may be a part of a TNF-α-activated signaling pathway. In contrast, osteoclast treated with αv inhibitor exhibited actin aggregates in the presence of TNF-α and M-CSF. αv inhibitor indeed reduced the maturation of actin aggregates to the sealing ring. Therefore, bone resorption is considerably reduced in these osteoclasts (Fig. 8III, D, green). The presence of actin aggregates (8III, D, red; indicated by arrows) in these osteoclasts suggests that it occurs upstream and also independent of integrin signaling. Formation of actin aggregates in osteoclasts treated with an αv inhibitor suggests that the maturation of the sealing ring is under the aegis of integrin αvβ3 signaling.

DISCUSSION

There is a linear correlation between the size of the osteoclasts and the number of nucleus (50). The area of the resorption pit formed by each osteoclast can be correlated with the number of nuclei and size of the osteoclast. This is because those multinucleated giant osteoclasts can cover a large area on the bone and consequently form big resorption pits (51). Big osteoclasts make multiple contacts by membrane extension and demonstrated marginal or peripheral crawling, as shown in this paper (Figs. 1 and 5–8). Therefore, they are capable of forming multiple sealing rings, which resulted in the formation of multilocular pits (Figs. 4 and 6–8). Small osteoclasts are capable of making either simple (single) pits or contiguous pits linearly (Fig. 7C, indicated by an arrowhead) (32). The latter is due to sequential events of cell adhesion, resorption, and migration of osteoclasts in one direction. It is not known whether an osteoclast can make multiple sealing rings during bone resorption in vivo. However, it is likely, based on time lapse live analyses performed by several investigators, including our own using various mineralized matrices, such as dentine (supplemental Fig. S1) and bone slices (52) as well as discs coated with apatite-collagen complexes (53, 54).

In an early study, osteoclasts have been shown to develop different patterns of contact characteristics, such as rings, crescents, and patches. Formation of these structures reflects the progress of the cell in the process of bone resorption. Osteoclasts are uniquely motile in that they continue to resorb bone beneath one area of the cell while another area is moving and reorganizing a sealing ring (55). Our study also shows that osteoclasts make actin aggregates or patches during the extension of the membrane. These secondary actin adhesive aggregates or patches confer extensive adherence and unique motile behavior as well as membrane extension (Figs. 7 and 8). These are consistent with the observations shown by others in the formation of actin-rich adhesion structures prior to the formation of the sealing ring (52). These actin aggregates may function as secondary adhesive structures and assist in the spreading of osteoclasts (Fig. 1, A–C). The spreading of osteoclasts with traction by the secondary actin adhesive structures increases the surface area. This may also possibly decrease the distance between osteoclasts and bone to facilitate proximity of ruffled border to bone. Actin aggregate formation seems to be necessary for the ensuing development of the sealing ring (Figs. 5–8) regardless of the number of sealing rings. Actin aggregates were 8–10-fold larger (Figs. 1 and 5–8) than podosome- or actin patch-like structures observed in osteoclasts treated with a neutralizing antibody to TNF-α (Fig. 8II, B and C). We consider that these actin aggregates or patches may be precursors for the sealing ring.

It is generally thought that the sealing ring is derived from the fusion of podosomes (14, 56, 57). However, several findings suggest that the sealing ring on bone has a different three-dimensional organization not derived from podosomes. The spatial arrangement of the presealing zone (referred to as actin aggregates) demonstrated neither clusters nor rosettes of podosomes. We found that osteoclasts plated on gelatin substrate (a non-mineralized matrix) organize either rosettes or clusters of podosomes at the migration front. These podosomes were capable of degrading the gelatin matrix because of the presence of MMP9 in these structures (36). Also, degradation of the matrix underneath the osteoclasts was not observed. These structures are different from typical podosome structures that are observed in osteoclasts during adhesion and migration (1, 36). Based on the time-dependent analysis (Fig. 5) and previous reports of others (53, 58), we suggest that sealing rings may not be the derivative of podosomes. We suggest this based on our previous observations in osteoclasts generated from gelsolin null (Gsn−/−) mice. A dearth of gelsolin blocks podosome assembly and motility but not sealing ring formation and bone resorption (1). The resorbed areas by these osteoclasts are simple and non-contiguous due to the hypomotile nature of oste-
Regulation of Sealing Ring Formation

Osteoclasts (1). Observations in Gsn<sup>−/−</sup> OCs also suggest that the organization of the sealing ring necessitates the role(s) of different actin-regulatory proteins.

We have observed time-dependent changes in the levels of L-plastin and cortactin in osteoclasts subjected to bone resorption (Figs. 2–4). It is interesting to speculate that actin aggregates are formed by L-plastin due to its actin bundling property (Fig. 5). This occurs at the early phase of bone resorption. Actin aggregates may function as an assembly center in the localization of integrin and other signaling molecules that are critical in the maturation of actin aggregates to functional sealing rings. L-plastin was described to be homologous with the F-actin-bundling protein fimbrin (59), and it is present in the podosomes of osteoclasts (2, 60). However, its function in osteoclasts is highly unknown. Analysis in L-plastin null osteoclasts (LPL<sup>−/−</sup>) demonstrated normal osteoclast differentiation and peripheral podosomes. However, significant decreases in the formation of the adhesive contact region (actin aggregates) reduces not only the functional sealing ring formation but also the membrane extension or marginal unique motile quality (data not shown). Our results provide the first evidence of the role of L-plastin in the formation of actin aggregates in osteoclasts.

Cortactin is an important substrate for Src kinase (61). Cortactin has been implicated in osteoclast podosome assembly/disassembly and bone resorption (6, 10, 62, 64). siRNA to cortactin blocked sealing ring formation, but these osteoclasts displayed actin aggregates (Figs. 6 and 7II). Although siRNA to cortactin inhibits the maturation of the sealing ring, it had no effect on the function of L-plastin, formation of actin aggregates (Figs. 6 and 7II), and localization of integrin αvβ3 (Fig. 7II, B). Taken together, these data suggest that cortactin-mediated actin polymerization occurs at the later stage of sealing ring formation. The roles of several signaling molecules and integrin signaling in the formation of the sealing ring are currently identified. However, the role of integrin signaling in the maturation of the sealing ring has not been studied well. Phosphorylation of L-plastin on serine residues is associated with the integrin activation (65). Localization of integrin αvβ3 (Fig. 7, I and II) in the actin aggregates may play a role in the maturation of actin aggregates to the sealing ring.

Up-regulation of signaling proteins, such as c-Src, Rho, Rho kinase, and cortactin, was observed in osteoclasts subjected to bone resorption (Fig. 3, supplemental Fig. S2, and supplemental Table S1). Interestingly, osteoclasts subjected to the following treatments blocked maturation of the sealing ring and bone resorption but displayed actin aggregates. The treatments are: echistatin (66); bisphosphonates (31); αv inhibitor (Fig. 8); phenyl arsine oxide (an inhibitor to PTP-PEST) (10); and siRNA to cortactin (Fig. 6) (6), PTP-PEST, or WASP (7, 10). Consistent with our observations, WASP null osteoclasts also demonstrated actin patches and reduced bone resorption (8).

Integrin β3 (Fig. 7II), WASP, and Src localize to the actin aggregates of osteoclasts treated with siRNA to cortactin (Fig. 7II) or PTP-PEST (7) and bisphosphonates (10). The failure of formation of mature sealing ring in these osteoclasts suggests different levels of defective downstream integrin signaling mechanism. Cortactin integrates signals from Src kinase to form a trimolecular complex (cortactin-WASP:Arp2/3), which activates actin polymerization (67–69). Reducing the levels of either one of the proteins seems to have an impact on osteoclast bone resorption (8).

The comparable actin staining in osteoclasts treated with Src inhibitor and neutralizing antibody to TNF-α suggests that Src also functions (Fig. 8) at the early stage of sealing ring formation (i.e. organization of the presealing zone or actin aggregates) in a TNF-α-dependent manner. There is clear evidence that human and mouse TNF-α stimulated the expression of c-Src in osteoclasts (70). Furthermore, we have shown previously a diffuse distribution of actin in osteoclasts expressing kinase-defective (KD) Src in the presence of RANKL. Neither podosomes nor actin aggregates were found in these osteoclasts (7). These observations raise the following questions. Does TNF-α regulate disassembly or turnover of podosomes and RANKL regulate the assembly of actin aggregates in osteoclasts? Does Src have a role at the early and late phase of sealing ring formation? Activation of Src by different signaling pathway may lead to varied downstream activation mechanisms and functional outcomes at the early and late phase of the sealing ring. As suggested by others, TNF-α could synergize with RANKL to increase osteoclast activation to a large extent (24). It is possible that the TNF-α/RANKL pathway could occur at the initial phase of sealing ring formation followed by the activation of integrin αvβ3 signaling toward the maturation phase of the sealing ring. The role of TNF-α and RANKL-mediated pathways and the exact step in which Src kinase fits in this pathway to regulate the formation of actin aggregates via L-plastin remain to be determined.

Inhibition of integrin αvβ3 signaling with a αv inhibitor or knockdown of β3 integrin with an siRNA (data not shown) had negligible effect on either the phosphorylation of L-plastin at the serine 9 residue or the formation of actin aggregates. Treatment of osteoclasts with a αv inhibitor did not block the localization of integrin in actin aggregates. However, localization of Src and cortactin in actin aggregates was blocked. Therefore, these osteoclasts demonstrated defective downstream signaling. Integrin expression and ligand binding affinity were not changed in Src<sup>−/−</sup> osteoclasts despite the consequences of defective downstream signaling (71). Integrin β3 knock-out mice displayed an osteosclerotic phenotype, suggesting that osteoclast activity is not completely affected (72). However, targeted disruption of Src leads to the osteopetrotic phenotype due to a defect in the activity of osteoclasts (73). This possibly suggests a multiplicity of roles for Src at different levels in osteoclast function, including (a) assembly/disassembly of podosomes for adhesion and migration; (b) formation of actin aggregates, which function as a center for orchestrating integrin signaling; and (c) maturation of actin aggregates to fully functional sealing ring.

Bone consists of organic (collagen and a variety of non-collagenous proteins) and inorganic components. Surface micro-geometry of implants plays a role in tissue/cell-implant surface interactions. However, our understanding of its effects at cellular levels or responses of bone cells to implants is incomplete. Podosomes were observed in cells plated on glass coverslips. Either actin aggregates or sealing rings were found in osteoclasts...
Regulation of Sealing Ring Formation

Our aim is to dissect the molecular mechanisms behind the cyclical processes of sealing ring formation. Taken together, our results suggest that TNF-α, RANKL, and integrin αvβ3-mediated pathways play roles in sealing ring formation and bone resorption. L-plastin has a role in the earliest stages of sealing ring formation. Phosphorylation of L-plastin and its activation occur independently of integrin αvβ3 signaling (data not shown). Src kinase may play a role in the formation of actin aggregates through the TNF-α/RANKL pathway. The TNF-α/RANKL pathway could occur at the initial phase of sealing ring formation, subsequently followed by the activation of integrin αvβ3 signaling toward the maturation phase of the sealing ring. Although integrin signaling has been shown to play a role in the formation of the sealing ring, its role in the maturation of actin aggregate to sealing ring has not yet been studied. The presence of actin aggregates in osteoclasts treated with αv inhibitor, siRNA to cortactin, Src inhibitor, and bisphosphonates (which block PTP-PEST and Rho GTPase activity) suggests that the formation of actin aggregates occurs independently of these molecules. Src kinase plays a crucial role at each level. A novel mechanistic link between plastin and cortactin localization and function exists in sealing ring formation. The formation of a spatially and dynamically regulated functional sealing ring and optimal bone resorptive capacity relies on the integrin-dependent pathway, which may involve the formation of the trimo-lecular complex consisting of cortactin-WASP-Arp2/3.

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REFERENCES

1. Chellaiah, M., Kizer, N., Silva, M., Alvarez, U., Kwiatkowski, D., and Hruska, K. A. (2000) J. Cell Biol. 148, 665–678
2. Babb, S. G., Matsuda, P., Sato, M., Correia, I., and Lim, S. S. (1997) Cell Motil. Cytoskeleton 37, 308–325
3. Hurst, I. R., Zuo, J., Jiang, J., and Holliday, L. S. (2004) J. Bone Miner. Res. 19, 499–506
4. McMichael, B. K., Kotadiya, P., Singh, T., Holliday, L. S., and Lee, B. S. (2006) Bone 39, 694–705
5. Chellaiah, M. A. (2005) J. Biol. Chem. 280, 32930–32943
6. Tehrani, S., Faccio, R., Chandrasekar, I., Ross, F. P., and Cooper, J. A. (2006) Mol. Biol. Cell 17, 2882–2895
7. Chellaiah, M. A., Kopf, M., Lasky, L., and Linder, S. (2007) J. Biol. Chem. 282, 10104–10116
8. Calle, Y., Jones, G. E., Jagger, C., Fuller, K., Blundell, M. P., Chow, J., Chambers, T., and Thrasher, A. J. (2004) Blood 103, 3552–3561
9. Banin, S., Truong, O., Katz, D. R., Waterfield, M. D., Brickell, P. M., and Gout, I. (1996) Curr. Biol. 6, 981–988
10. Chellaiah, M. A., and Schaller, M. D. (2009) J. Cell Physiol. 220, 382–393
11. Ma, T., Samanna, V., and Chellaiah, M. A. (2008) J. Mol. Signal. 3, 4
12. Chellaiah, M., Fitzgerald, C., Alvarez, U., and Hruska, K. (1998) J. Biol. Chem. 273, 11908–11916
13. Duong, L. T., Lakkakorpi, P. T., Nakamura, I., Machwate, M., Nagy, R. M., and Rodan, G. A. (1998) J. Clin. Invest. 102, 881–892
14. Lakkakorpi, P. T., Nakamura, I., Nagy, R. M., Parsons, J. T., Rodan, G. A., and Duong, L. T. (1999) J. Biol. Chem. 274, 4900–4907
15. Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Barcelay, C., Antoine, E., Levy, J., Gallet, J., Bowtell, D., Horne, W. C., and Barson, R. (2001) J. Cell Biol. 152, 181–195
16. Tanaka, S., Amling, M., Neff, L., Uehling, E., Levy, J. B., and Baran, R. (1996) Nature 383, 528–531
17. Hartman, G. D., and Duggan, M. E. (2000) Expert Opin. Investig. Drugs 9, 1281–1291
18. Miyazaki, T., Sanjay, A., Neff, L., Tanaka, S., Horne, W. C., and Baran, R. (2004) J. Biol. Chem. 279, 17660–17666
19. Gupta, A., Lee, B. S., Khadeer, M. A., Tang, Z., Chellaiah, M., Abu-Amer, Y., Goldknopf, J., and Hruska, K. A. (2003) J. Bone Miner. Res. 18, 665–685
20. Teitelbaum, S. L. (2007) Am. J. Pathol. 170, 427–435
21. Carron, C. P., Meyer, D. M., Engleman, V. W., Rico, J. G., Runinski, P. G., Ovnberg, R. L., Westlin, W. F., and Nichols, G. K. (2000) Endocrinol. 165, 587–598
22. Horton, M. A., Dorey, E. L., Nesbitt, S. A., Samanen, J., Ali, F. E., Stadel, J. M., Nichols, A., Greig, R., and Helfrich, M. H. (1993) J. Bone Miner. Res. 8, 239–247
23. Duong, L. T., Lakkakorpi, P., Nakamura, I., and Rodan, G. A. (2000) Matrix
