Rho-A Is Critical for Osteoclast Podosome Organization, Motility, and Bone Resorption*

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Rho plays a regulatory role in the formation of actin stress fibers and focal adhesions, and it is also involved in integrin-mediated signaling events. To study the role of Rho in αβ₃/gelsolin-dependent signaling, the HIV-Tat peptide, hemagglutinin (HA)-tagged RhoVal¹⁴ (constitutively active) and RhoAsn⁻¹⁹ (dominant negative) were transduced into avian osteoclasts. Protein transduction by HA-Tat was highly efficient, and 90–100% of the cells were transduced with HA-tagged proteins. We demonstrate here that RhoVal¹⁴ transduction (100 nM) stimulated gelsolin-associated phosphatidylinositol 3-kinase activity, podosome assembly, stress fiber formation, osteoclast motility, and bone resorption, mimicking osteoclast stimulation by osteopontin/αβ₃. The effects of RhoVal¹⁴ transduction stimulation were time-dependent. C3 exoenzyme blocked the effects of RhoVal¹⁴ and induced podosome disassembly, loss of motility, and inhibition of bone resorption. Transduction of Rho Asn⁻¹⁹ produced podosome disassembly, and blocked osteopontin stimulation. These data demonstrate that integrin-dependent activation of phosphoinositide synthesis, actin stress fiber formation, podosome reorganization for osteoclast motility, and bone resorption require Rho stimulation.

Rho, which plays a critical and regulatory role in the formation of actin stress fibers and focal adhesions, is also involved in integrin-mediated signaling events (1) and has been implicated in osteoclast function. Rho stimulates the activity of Rho kinase (ROCK) and the profilin-binding protein, Dia1, to augment the onset of stress fiber formation (2–6). Furthermore, the effects of Rho family members on the actin cytoskeleton are mediated in part by phosphoinositide kinases (7). Chihara et al. (8) has reported that microinjection of constitutively active Rho kinase into fibroblasts induced the formation of focal adhesions under conditions wherein stress fibers were disrupted, demonstrating dual functions of Rho kinase in stress fiber formation and focal adhesion assembly.

Rho also plays an important role in osteoclast function. The actin ring structure typical of resorbing osteoclasts is disrupted by the C3 exoenzyme (9), which specifically ADP-ribosylates (inactivates) the Rho protein (10). The C3 enzyme also inhibits bone resorption (9). Osteoclasts are highly motile cells, which depend on rapid changes in their actin cytoskeleton to accomplish their ordered cycles of movement and attachment during bone resorption (11–14). However, osteoclasts do not have the focal adhesion attachment structures characteristic of other cells (15). Instead, osteoclasts attach to culture or matrix surfaces through structures called podosomes, which are dot-like aggregations of actin that cluster in a ring around the cell periphery (16–20). Changes in podosome assembly/disassembly allow osteoclast migration, adhesion, and bone resorption. Peripheral rows of podosomes fuse to form the actin rings that circle the area under the osteoclast, where active bone resorption occurs (11, 21–23).

Although podosomes and focal adhesions are similar, important functional differences exist. Unlike focal adhesions, podosomes are highly dynamic structures with a 2–12-min life span compared with 30 min for the former (18). Podosomes contain numerous proteins observed in focal adhesions of other cells, however, the arrangement of these proteins differ between the two structures. In podosomes, the actin microfilaments are surrounded by talin and vinculin, forming the rosette structure observed by immunostaining of these proteins (11, 21). We have reported data suggesting that gelsolin is the actin capping/severing protein of the podosome (19, 24).

Osteoclasts isolated from bone marrow cells of mice rendered gelsolin-deficient by genetic recombination (Gen−/−) have a markedly different distribution of actin, with no detectable podosomes (20). Furthermore, the integrin-mediated recruitment of signaling molecules required for motility and bone resorption in response to osteopontin (OP) is absent in gelsolin null osteoclasts (20). Likewise, osteoclasts derived from Src−/− mice fail to express podosomes (25). The inhibition of pp⁶⁰⁰-Src expression by the use of antisense oligodeoxycytidinenucleotides demonstrated the direct upstream role of pp⁶⁰⁰-Src in gelsolin-mediated actin cytoskeleton reorganization, podosome assembly, and bone resorption in avian osteoclasts (19). Here, we have investigated whether Rho is required in the formation of podosomes and integrin-mediated signaling in response to OP.

We demonstrated that C3 exoenzyme eliminated podosome assembly. Using HIV-TAT-mediated delivery (26) of Rho proteins into osteoclasts, we demonstrated that constitutively active Rho transduction was not only sufficient for podosome assembly but that it mimicked the effects of OP by stimulating actin stress fiber formation, motility, and bone resorption. RhoVal¹⁴ transduction into osteoclasts regulated gelsolin-associated kinase activities further mimicking the actions of OP/αβ₃. Dominant negative RhoAsn⁻¹⁹ blocked the effects of OP

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1 The abbreviations used are: OP, osteopontin; α-MEM, α-minimal essential medium; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; HA, hemagglutinin; PtdIns, phosphatidylinositol.
stimulation downstream of gelsolin-associated c-Src. Therefore, Rho activation is not only required for podosome assembly and integrin-mediated signal transduction, leading to osteoclast motility and stimulation of bone resorption, but is sufficient for these events.

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**

\[ {\gamma}^{32}P\]ATP and rainbow molecular weight markers for proteins were obtained from Amersham Pharmacia Biotech. Protein A-Sepharose, mouse IgG, anti-gelsolin antibody, phospholipid standards, and most of the chemicals were purchased from Sigma. Protein assay reagent kits and EDTA-free protease inhibitor cocktail (aprotinin, leupeptin, and pepstatin) were purchased from Bio-Rad. Recombinant Src was obtained from UBI (Lake Placid, NY). Rhodamine phalloidin was obtained from Molecular Probes (Eugene, OR). Rho\textsuperscript{V14} and Rho\textsuperscript{GTP} cDNAs were kindly provided by Dr. Alan Hall (MRC Laboratory for Molecular Cell Biology, Department of Biochemistry, University College of London, London, United Kingdom). GST-C3 construct was kindly provided by Dr. Larry Feig (Department of Biochemistry, Tufts University, Boston, MA).

**Preparation of Osteoclast Precursors**

Avian osteoclast precursors were prepared as described previously (24, 27). Briefly, osteoclast precursors were isolated from bone marrow of egg-laying hens maintained on Ca\textsuperscript{2+}-deficient diets. Partially purified preparations of mononuclear cells were recovered from the interface of Ficoll/Hypaque gradients. Nonadherent cells were separated from the adhered population fraction after 18 h of culture. The nonadherent cells were sedimented, resuspended in fresh media (5 \times 10^5 cells/ml), and cultured in the presence of cytokine arabinoside (5 mg/ml). Multinucleated osteoclast precursor cells formed between 3 and 6 days in culture, and the preparations were 70–90% pure multinucleated tartrate-resistant acid phosphatase positive cells.

**Protein Transduction into Avian Osteoclasts**

**Protein Purification**—Constitutively active Rho (V14), Cdc42 (V12), and inactive Rho (N19) were cloned in frame into a bacterial expression vector, pTAT-HA, to produce TAT fusion proteins. The vector pTAT-HA has an N-terminal 6-histidine leader followed by the 11-amino acid TAT protein transduction domain flanked by glycine residues, a hemagglutinin (HA) tag, and polylinker (26). Herpes simplex virus thymidine kinase protein was used as a control (42 kDa) (TAT-TK). The purification protocol is adapted from the published procedure using a Ni-NTA column (26). Briefly, bacterial pellets were resuspended in a buffer containing 100 mM NaCl, 20 mM Hepes (pH 8.0), and 8 mM urea, sonicated, and centrifuged at 12,000 rpm for 10 min at 4 °C. Imidazol was added to the supernatant to a final concentration of 10–20 mM and purified in Ni-NTA column as described (26). Addition of 5 mM urea to the sonication buffer allows for the isolation of insoluble proteins in bacterial inclusion bodies and efficient transduction into cells. Bound proteins were eluted with stepwise addition of 5–10 mM each of 100, 250, and 500 mM and 1 mM imidazol in the above buffer. Urea was removed by rapid dialysis by using the Slide-A-Lyzer cassette (Pierce Chemical Co.) or by the use of desalting PD-10 columns (Sephadex G-25) (Amersham Pharmacia Biotech). Desalting was done per the manufacturers instructions. Purification of C3 was done essentially as described (28).

**Cell Permeabilization**—Osteoclast permeabilization with streptolysin O was done as described (19). Osteoclasts were washed twice with permeabilization buffer (120 mM KCl, 30 mM NaCl, 10 mM Hepes, pH 7.2, 10 mM EGTA, 10 mM MgCl\textsubscript{2} (43)). freshly prepared dithiothreitol (5 mM), ATP (1 mM), and 0.5 unit/ml streptolysin O and C3 exoenzyme (100 ng/ml) (Biomol Research Laboratories, Inc., Plymouth Meeting, PA) were added to the buffer at the time of permeabilization and incubated for 2–5 min at 37 °C. Resealing was achieved by the immediate addition of α-MEM containing 10% fetal bovine serum and washed with serum-free medium. The incubation was continued for 2 h in serum-free medium and stimulated with OP (25 µg/ml) for 15 min at 37 °C. All the parallel experiments were treated the same as above but in the absence of C3 exoenzyme. GST-C3 exoenzyme was also purified in one laboratory using the published protocol (28) and used in these experiments. GST-C3 blocked the Rho effect at 200–250 ng/ml with an incubation time of 2–3 h at 37 °C.

**Transduction of Proteins into Osteoclasts**—After cells were kept in serum-free α-MEM for 2 h, Tat proteins were added to cells to a final concentration of 100 nM in serum-free media. Dose and time-dependent uptake of proteins were determined. Doses in the range of 50–500 nM were used. Maximal uptake and response was seen at 100–150 nM concentration. Uptake was seen within 15 min of incubation with TAT proteins.

**Lyase Preparation, Immunoprecipitation, and Western Blot Analysis**

Following treatments as described above, cells were washed three times with ice-cold PBS and lysed in a Triton-containing lysis buffer (10 mM Tris-HCl, pH 7.05, 50 mM NaCl, 0.5% Triton X-100, 30 mM pyrophosphate, 5 mM sodium fluoride, 0.1 mM Na\textsubscript{3}VO\textsubscript{4}, 5 mM ZnCl\textsubscript{2}, and 2 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged (15,000 rpm, 15 min, 4 °C), and protein concentrations in the supernatants were determined using the Bio-Rad protein assay kit. Equal amounts of protein lysates were used for immunoprecipitations. Immunoprecipitations and Western blotting were done as described (19, 24).

**Immune Complex Kinase Assay Analysis for pp60c-Src or PI 3-Kinase**

Equal amounts of protein lysates were immunoprecipitated with anti-gelsolin. Immune complexes collected by the addition of protein A-Sepharose were used for kinase assays. The Sepharose beads, after washing several times with different buffers, were assayed for pp60c-Src or PI 3-kinase activities as described previously (19, 24).

**Fluorescent Labeling of Proteins**

Osteoclasts were cultured on glass coverslips or dentine slices. Fluorescent labeling was done with either HA antibody or Rhodamine phalloidin after osteoclasts were treated with HA-Tat proteins as described above. Following the indicated periods, immunostaining was performed as reported previously (29). Briefly, osteoclasts were fixed with 3% paraformaldehyde and permeabilized with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM CaCl\textsubscript{2} containing 0.1% Triton X-100 for 1 min. The cells were washed with PBS and incubated with antibodies for 2 h at room temperature. Antibody dilutions used were 1:100. Cells were washed and recounted with Cy2-conjugated anti-mouse IgG as the secondary antibody. Actin staining was done with Rhodamine phalloidin as described previously (19). Cells were then washed twice to three times with PBS and mounted on a slide in a mounting solution (Vector laboratories, Inc., Burlington, CA). The cells were viewed on a Zeiss LSM 410 confocal laser scanning microscope (Thornwood, NY) and photomicrographs were obtained. Images were recorded and analyzed as described (30). The time-dependent distribution of Tat-Rho\textsuperscript{V14} protein in HA-stained osteoclasts were visualized using a Zeiss Axiowert S100 microscope fitted with a spot camera (Diagnostic Instruments, Inc., Alexandria, VA).

**Transwell Migration Assay**

Assays for cell migration were performed in Transwell migration chambers (8-µm pore size) (31). Undersides of membranes were coated with 1% BSA or OP at 37 °C in serum-free medium for 4–14 h; membranes were air dried and washed twice with PBS. Membranes were rinsed with α-MEM medium prior to addition of cells. After 3–4 days in culture, chicken osteoclasts were washed twice with PBS and incubated at 37 °C with cell dissociation buffer (Sigma) for 20 min. Cells were removed by gentle scrapping and resuspended in α-MEM containing 1% serum and 2% BSA (50,000 cells/ml). Cells were added to the upper chamber in the above mentioned medium (100 µl) and allowed to adhere for 1–2 h. After cells attached to the membrane, TAT fusion proteins were added to a final concentration of 100 nM in the upper chamber in a serum-free α-MEM. α-MEM containing 1% serum and 2% BSA (600 µl) was added to the lower chamber. Cell migration was allowed to proceed at 37 °C in a tissue culture incubator for 12–14 h. Cells were then removed from the undersides of the membrane with a cotton swab, and cells that migrated to the undersides were stained as follows. Wells were fixed with an alcohol/formaldehyde/acetic acid mixture (20:2:1) for 15 min. Filters were stained with hematoxylin stain (Sigma), rinsed well with water, and dried. Dried filters were cut out and mounted with permount solution (Thomas Scientific, Swedesboro, NJ) on a glass slide. Cells were viewed under \times40 objective in an inverted microscope and counted (Zeiss microscope) as mentioned (31).

**Bone Resorption Assay**

After 3 days in culture, avian osteoclasts were washed with PBS, and treated with cell dissociation buffer for 15 min at 37 °C. Cells were...
New podosomes were larger in size and distinguishable from the original podosome structures (arrows, Fig. 1B) by their donut shape. In cells treated with C3 for 2 h, podosomes dissolved and actin became disorganized in a structure involving the central part of the cell (Fig. 1C, right). OP addition to C3 pretreated cells (C3/OP) produced actin coalescence into short, irregular actin cables (Fig. 1D).

Transduction of Rho Proteins into Osteoclasts

To further analyze the effects of Rho activity, we used HIV Tat-mediated delivery (transduction) of Rho proteins into osteoclasts. Constitutively active RhoVal-14 and dominant-negative RhoAsn-19 cDNAs were cloned into the bacterial expression vector, pTAT-HA, to produce Tat fusion proteins. The following HA-TAT fusion proteins were generated: RhoVal-14 (25–30 kDa), RhoAsn-19 (25–30 kDa), Cdc42Val-12 (25–30 kDa), and a control HSV-TK protein (TAT-TK, 42 kDa). Purification and transduction of denatured proteins into osteoclasts were performed according to our reported methods (28). After transduction of Tat proteins into osteoclasts, cells were incubated with a HA antibody to measure the uptake of proteins. More than 90% of the cells were transduced with Tat fusion proteins. Confocal microscopy analysis of osteoclasts transduced with Tat proteins for 60 min confirmed the uptake of transduced proteins (Fig. 2). A diffuse staining was seen in Tat-TK transduced cells (Fig. 2B); punctate staining was seen in cells transduced with Val-14 or RhoAsn-19 proteins which included structures resembling podosomes and localization in structures surrounding large vacuoles (Figs. 2, C and D). Negligible HA background staining was seen in the control, untransduced, osteoclasts (Fig. 2A). As shown in Fig. 2E for RhoVal-14, there was a time-dependent distribution of the HA-TAT Rho fusion proteins. By 15 min, there was a peripheral distribution of HA in areas consistent with podosomes. At 30 and 60 min, Rho was more diffusely distributed, and it was present in newly formed donut-shaped structures consistent with newly organizing podosomes. By 3 h, the Rho-stained podosomes were dot-like structures consistent with their maturation, and Rho was localized to large vesicular-like structures surrounding large vacuoles. At 6 and 9 h, Rho was diffusely localized in their rounded motile cells. By 12 h, the osteoclasts had respread, and Rho was present in many of the podosomes as the clear zone was re-established. Western analyses (not shown) of lysates from HA-RhoVal-14 or HA-RhoAsn-19 transduced osteoclasts for the times discussed above revealed a 33-kDa protein band. By 50 h HA immunostaining was diffuse and in vesicular bodies associated with protein degradation.

Effects of RhoVal-14 and RhoAsn-19 on the Cytoskeleton

The effects of transduced proteins on the reorganization of the actin cytoskeleton were studied by staining of cells cultured on glass with Rhodamine phalloidin (Figs. 3 and 4). Cells transduced with Tat protein (8–10 kDa) (Fig. 3A) or control Tat-TK proteins (Fig. 3B) showed enrichment of F-actin in the peripheral dot-like podosome structures similar to PBS-treated cells (Fig. 1A). A time-dependent increase in stress fiber formation was observed in Tat/RhoVal-14 transduced osteoclasts. By 15 and 30 min of RhoVal-14 transduction, stress fiber formation increased along with the disappearance of the peripheral podosome organization (Fig. 3, C and D). These results are similar to the effects of OP (19, 20). After 60 min (Fig. 3E) numerous new dot-like larger podosome structures were seen either at the cell periphery or in the cell center, regions where stress fibers terminate. After 3 h of Rho transduction, the podosome structures began to reorganize at the periphery (Fig. 3F) and stress fibers disappeared by 6–8 h (Fig. 3G). Podo-
somes appeared to be denser and larger in RhoVal-14 transduced cells (Fig. 3, E-G) than control cells (Fig. 3, A and B). Stress fiber formation or podosome reorganization were not observed in osteoclasts transduced with dominant negative Rho mutant, RhoAsn-19 (Fig. 3, H and I). Transduction of RhoAsn-19 into osteoclasts for 15 min (Fig. 3H) or 30 min (Fig. 3I) led to dissolution of F-actin in podosomes, which were replaced with membrane ruffles at the cell periphery.

Co-treatment of RhoVal-14 transduced cells with C3 exoenzyme inactivated Rho by the formation of ADP-ribosylated Rho, and blocked Rho-stimulated stress fiber formation. Addition of OP to C3/RhoVal-14-treated cells (Fig. 4C), failed to form the stress fibers stimulated by RhoVal-14 (Fig. 4B). Also, transduction of this ADP-ribosylated inactive Rho into osteoclasts mimicked the effect of RhoAsn-19 in dissolution of podosome structures (Fig. 4E). No response to OP was seen in cells treated with C3/RhoVal-14 or RhoAsn-19 (Fig. 4, C and E). Since Cdc42 has been shown to activate actin stress fibers in some cells (1), we transduced osteoclasts with constitutively active Cdc42Val-12. Transduction with Tat/Cdc42Val-12 stimulated the formation of thin actin fibrils within the cell and filopodia outside the cell periphery (Fig. 4D). The actin clumping, shown in Fig. 4D was unique to Cdc42-transduced cells. No distinct organization of podosome-like structures was seen in Cdc42Val-12-transduced cells.

**Effects of Rho Transduction on Gelsolin-associated Kinase Activities**

**Effects on pp60c-Src Kinase**—To explore the possible connection of Rho with gelsolin-associated pp60c-Src/PI 3-kinase-mediated regulation of cytoskeletal reorganization in more detail,
lysates made from cells treated with various TAT fusion proteins were subjected to in vitro immune complex kinase assays for gelsolin-associated pp60c-Src and PI 3-kinase. As shown in Fig. 5, TAT-RhoVal-14 protein transduction for 15 min (lane 1) or 16 h (lane 2) increased pp60c-Src kinase autophosphorylation and its activity in phosphorylating an exogenous substrate, casein, as compared with cells transduced with TAT protein only (lane 5). Co-incubation of C3 exoenzyme with RhoVal-14 for 15 min blocked prior to transduction the pp60c-Src kinase autophosphorylation (lane 3), which recovered by 16 h (lane 4). Also, the basal level of gelsolin-associated Src autophosphorylation was decreased in cells treated with C3/HA-TAT (lane 6) as compared with HA-TAT-alone treated cells (lane 5). Other gelsolin-associated protein kinases were not affected by C3 exoenzyme as shown by casein phosphorylation in lanes 3, 4, and 6. To analyze the effect of C3 on OP-stimulated kinase activities, cells were permeabilized as described previously (19) and treated with C3 exoenzyme for 2–3 h prior to OP addition as described under “Experimental Procedures.” No significant inhibition of pp60c-Src kinase autophosphorylation or activity associated with gelsolin was seen in C3 pretreated and OP-stimulated cells (lane 8). The increase in phosphorylation of gelsolin-associated pp60c-Src was 221.6 ± 20.7% over baseline and casein was 272.4 ± 10.8% (mean ± S.E., n = 3) in OP-treated cells, as demonstrated by quantitation of three different experiments by densitometry of the autoradiograms expressed as percent of control. In C3/OP-treated cells the increase in phosphorylation of pp60c-Src and casein was 205 ± 25.35 and 225 ± 21.2% (mean ± S.E., n = 3) respectively. Vehicle-treated control cells were considered 100%. The increase in phosphorylation of pp60c-Src and casein was 335 ± 67 and 320 ± 77% (mean ± S.E., n = 3), respectively, in TAT

**FIG. 3. Time course of the RhoVal-14 transduction effect on actin cytoskeleton.** Confocal microscopy analysis of osteoclasts stained for actin after transduced with the following Tat-fusion proteins. A, Tat only; B, Tat-TK; C, Tat-RhoVal-14 (15 min); D, Tat-RhoVal-14 (30 min); E, Tat-RhoVal-14 (60 min); F, Tat-RhoVal-14 (3 h); G, Tat-RhoVal-14 (6–8 h); H, Tat-RhoAsn-19 (15 min); I, Tat-RhoAsn-19 (30 min). The donut shaped podosomes as indicated by arrows in E and F. Scale bar, 50 μm. RhoVal-14 transduction increases stress fiber formation in a time-dependent manner. Numerous small ring-shaped podosomes were seen after 1–3 h of Rho (E and F) transduction. Stress fibers or new organization of podosomes were not seen in RhoAsn-19 transduced cells (H and I). Treatments are shown in duplicate.

**FIG. 4. Analysis of actin distribution in osteoclasts treated with C3/RhoVal-14, RhoAsn-19, and Cdc42.** Confocal microscopy analysis of osteoclasts stained for actin after treated with TAT-TK (A), RhoVal-14 (B), C3/RhoVal-14/OP (C), Cdc42 (D), RhoAsn-19 (E), and RhoAsn-19/OP (F) is shown. Podosome organization at the periphery is seen in TAT-TK transduced cells (A). RhoVal-14 transduction stimulated stress fiber formation (B); OP addition to C3/Val-14-treated cells does not have any effect (C); RhoAsn-19: E demonstrates dissolution of actin structures in the podosome and OP treatment (RhoAsn-19/OP); F has no effect. Scale bar, 50 μm.
Rho<sub>Val-14</sub>-treated cells and HA-TAT-transduced cells were considered 100%.

pp<sup>60c-src</sup> kinase and casein phosphorylation were also measured (Fig. 6) in cells transduced with Rho<sub>Asn-19</sub> and Cdc42<sub>Val-12</sub> for 15 min. Cells transduced with Rho<sub>Asn-19</sub> (lane 1), Cdc42<sub>Val-12</sub> (lane 7), or C3/Rho<sub>Val-14</sub> (lane 3) did not increase pp<sup>60c-src</sup> activation or phosphorylation as compared with C3/RhoVal-14-transduced control cells (lane 6). OP addition for 15 min to Rho Asn-19 (lane 5), C3/Val-14 (lane 3), Cdc42 (lane 8), or OP stimulation of the cells treated with the above mentioned proteins (Lanes 2, 4, and 8, respectively) did not increase pp<sup>60c-src</sup> phosphorylation or activity. Phosphorylation of pp<sup>60c-src</sup> and casein is indicated by arrows. These results represent one of three experiments performed.

**Phosphorylation of pp<sup>60c-src</sup> and casein is marked by arrows.**

**FIG. 5.** The effects of HA-Tat protein transductions on gelsolin-associated pp<sup>60c-src</sup> kinase activity. In vitro kinase assays were performed in lysates made from osteoclasts treated as follows. Rho<sub>Val-14</sub> (lane 1), Rho<sub>Val-14</sub>/OP (lane 2), C3/Rho<sub>Val-14</sub> (lane 3), C3/Rho<sub>Val-14</sub>/OP (lane 4), Rho<sub>Val-14</sub>/OP (lane 5), Cdc42 (lane 7), Cdc42/OP (lane 8), Tat-TK (lane 9), Rho<sub>Val-14</sub>/OP (lane 10), Src positive control from UBI (lane 11). Lysates were immunoprecipitated with either anti-gelsolin antibody (lanes 1–10) or non-immune serum (lane 11) and subjected to immune complex kinase assays. OP stimulation of Rho<sub>Val-14</sub>-transduced cells (lane 6) did not stimulate pp<sup>60c-src</sup> activity or phosphorylation as compared with Rho-alone treated cells (lane 5). Asn-19 (lane 1), or C3/Val-14 (lane 3), Cdc42 (lane 8), or OP stimulation of the cells treated with the above mentioned proteins (Lanes 2, 4, and 8, respectively) did not increase pp<sup>60c-src</sup> phosphorylation or activity. Phosphorylation of pp<sup>60c-src</sup> and casein is indicated by arrows. These results represent one of three experiments performed.

**FIG. 6.** The effects of OP on gelsolin-associated pp<sup>60c-src</sup> kinase activity after transduction with various proteins. In vitro kinase assays were performed in lysates made from osteoclasts treated as follows. Rho<sub>Asn-19</sub> (lane 1), Rho<sub>Asn-19</sub>/OP (lane 2), C3/Rho<sub>Val-14</sub> (lane 3), C3/Rho<sub>Val-14</sub>/OP (lane 4), Rho<sub>Val-14</sub>/OP (lane 5), Rho<sub>Val-14</sub>/OP (lane 6), Cdc42 (lane 7), Cdc42/OP (lane 8), Tat-TK (lane 9), Rho<sub>Val-14</sub>/OP (lane 10), Src positive control from UBI (lane 11). Lysates were immunoprecipitated with either anti-gelsolin antibody (lanes 1–10) or non-immune serum (lane 11) and subjected to immune complex kinase assays. OP stimulation of Rho<sub>Val-14</sub>-transduced cells (lane 6) did not stimulate pp<sup>60c-src</sup> activity or phosphorylation as compared with Rho-alone treated cells (lane 5). Asn-19 (lane 1), or C3/Val-14 (lane 3), Cdc42 (lane 8), or OP stimulation of the cells treated with the above mentioned proteins (Lanes 2, 4, and 8, respectively) did not increase pp<sup>60c-src</sup> phosphorylation or activity. Phosphorylation of pp<sup>60c-src</sup> and casein is indicated by arrows. These results represent one of three experiments performed.
coated with PBS or OP (10 mg/ml). TAT proteins were added to cells in the upper chamber after the cells were attached to the membrane and then migration assays were continued for 12–14 h. A 2–2.5-fold increase in migration was observed on OP-coated membrane in cells treated with HA-TAT, TAT-TK, and PBS as compared with PBS-coated membrane (Table I). A 2–3-fold increase in migration toward OP-coated membranes was observed with OP or RhoVal-14-treated cells. No significant response to OP-coated membranes was seen in cells transduced with RhoAsn-19, or in C3/RhoVal-14-transduced cells. In Cdc42Val-12-transduced cells, the basal level of migration of osteoclasts toward PBS was equal to RhoVal-14 but OP-coated membranes did not stimulate this.

**In Vitro Bone Resorption Assay**—The effects of TAT-protein transduction on osteoclastic bone resorption were measured. OP (Fig. 9B) and RhoVal-14 (Fig. 9C) stimulated the bone resorative activity of osteoclasts, RhoAsn-19 had no effect (Fig. 9D) when compared with OP-transduced cells (Fig. 9A). The data shown in Fig. 9 are representative of three separate experiments.

**DISCUSSION**

We have shown that osteopontin binding to the osteoclast integrin $\alpha_v\beta_3$ stimulates gelsolin-associated pp$^{60c}$-Src, leading to increased gelsolin-associated PI 3-kinase activity and PtdIns-3,4,5-P$_3$ levels. This increase in kinase activity facilitates actin filament formation, osteoclast motility, and bone resorption (19). Signal generation by $\alpha_v\beta_3$ is produced by the activation of protein complexes including FAK, pp$^{60c}$-Src, and PI3-kinase, which are associated with $\alpha_v\beta_3$ (33). The data presented here provide the first evidence that osteoclast podosome assembly and associated PI 3-kinase activity are blocked by C3 exoenzyme, demonstrating the role of Rho-GTPase in the regulation of OP-induced signal transduction and cytoskeletal reorganization.

As shown here and previously, OP induced actin stress fiber formation in a time-dependent fashion and increased osteoclast F-actin levels (19, 24). Gelsolin, an actin-binding protein present in the osteoclast podosome, is critical for actin assembly/disassembly (34–37). During actin assembly, when OP binds to the osteoclast, $\alpha_v\beta_3$ stimulates gelsolin-associated phosphoinositides and uncapping of barbed end actin, resulting in actin filament formation (24). We have recently shown that OP stimulates pp$^{60c}$-Src and PI 3-kinase associated with gelsolin leading to the production of polyphosphoinositides (PtdIns-P$_2$, PtdIns-P$_3$) which are involved in the regulation of gelsolin function (19). Because PtdIns-P$_2$ and PtdIns-P$_3$ are increased in OP-stimulated osteoclasts, the respective kinases, PI4P 5-kinase and PI 3-kinase, may also have a role in promoting actin filament formation. PI4P 5-kinase, which phosphorylates PI4P to generate PtdIns-P$_2$, has also been implicated in integrin
signaling processes (38, 39). Furthermore, Rho has been shown to regulate a PI4P 5-kinase in mammalian cells (38), and to associate physically with PI4P 5-kinase in Swiss 3T3 cells (40). Phosphoinositides (PtdIns-4,5-P_2, PtdIns-3,4,5-P_3, and PtdIns-3,4-P_2) and PI 3-kinase are possibly involved in actin reorganization (41–44). Experiments by Hartwig and colleagues (45) gave the first indication of how Rho-like GTPases can control actin polymerization. Rac induced rapid PtdIns-P_3 synthesis and Rac induction of actin filament uncapping was inhibited by PtdIns-P_3 binding peptides derived from gelsolin (45).

Several studies have provided evidence that Rho plays a key role in actin stress fiber and focal adhesion formation (10, 46–50). In this report, we have shown that addition of OP to osteoclasts resulted in actin stress fiber formation, increased cell migration, and bone resorption in avian osteoclasts. Since actin filament formation and podosome dynamics are the driving force of cell motility, we focused our studies on the role of Rho in actin filament assembly/disassembly and podosome formation.

We demonstrated that Rho controls the polymerization of actin, turnover of podosomes, and motility of osteoclasts. Tat/RhoVal-14 transduction stimulated PI 3-kinase activity associated with gelsolin, and stimulated actin stress fiber formation in a time-dependent manner. Tat/RhoVal-14 transduction increased osteoclast motility and bone resorption in a manner similar to induction by OP/α_5β_3. The appearance of new donut-shaped podosomes in the center and periphery of osteoclasts along with the slow disappearance of actin stress fibers was observed after 1–3 h of Tat/RhoVal-14 transduction. After 6 h, the cells began to respread and podosomes were relocated to the perimarginal region. Osteoclasts cultured on bone slices demonstrated similar organization of podosomes either as a single entity or a cluster of podosomes organized as a small ring, which are presumed to be precursors of actin rings (51).

The time-dependent organization of podosomes in RhoVal-14-transduced cells suggests that Rho and/or its effector proteins regulate the turnover of podosomes. C3 exoenzyme blocks either RhoVal-14 or the OP effects and induces dissolution of actin structures in podosomes. The transduction of Tat/RhoVal-14 produced podosome disassembly and blocked OP stimulation. These observations provide additional evidence for the involvement of Rho in podosome assembly/disassembly processes. Our results with RhoVal-14 transduction mimic the OP effect in osteoclast function. Although Tat has been shown to have angiogenic properties by induction of in vitro migration and invasion of cytokine-stimulated endothelial cells on a matrix support (52), this report demonstrates that in the osteoclast, Tat peptide alone has no effect on F-actin-enriched podosome structure, actin stress fiber formation, or osteoclast motility.

Functionally active osteoclasts form podosomes (53) and several studies have shown that the structure of podosomes is extremely sensitive to several agents. Herbimycin A treatment stimulated distribution of podosomes from the periphery to the central region of osteoclasts after 4 h which disappeared within 16 h (54). PI 3-kinase has been implicated in the regulation of various cellular processes including membrane ruffling, motility, and vesicle trafficking (55–58). Administration of wortmannin, a potent PI 3-kinase inhibitor (59), C3 exoenzyme (9), or microinjection of Rho-GDI (60) disrupted actin rings and inhibited their pit forming activity. The role of pp60c-Src in osteoclast function has been widely studied in Src−/− mice (54, 61–63). Osteoclasts from Src−/− mice have a markedly different distribution of actin, with no peripheral podosome arrangement (25). Tyrosine kinase-mediated signals involved in the cytoskeletal organization regulated by Rho p21 were reported in other cell types (43, 47, 64). Rho has also been shown to play a role in regulating cell motility in epithelial cells and neutrophils (65).

We have demonstrated that signaling molecules such as, pp60c-Src, PI 3-kinase, and RhoA are essential for cytoskeletal reorganization, podosome formation, cell adhesion, motility, and bone resorption. Although pp60c-Src activation is upstream...
of Rho in OP-treated cells, an increase in pp60c-Src kinase phosphorylation and activity was observed after 2 and 16 h (overnight) of RhoVal-14 transduction. This may be due to podosome assembly and recruitment of integrins to the podosomes. These integrins induce actin stress fiber formation, activation of kinases, and phosphorylation of signaling proteins in the podosomes to facilitate motility and bone resorption. Rho mediates stress fiber formation and generates tension, thereby inducing aggregation of the integrins at the ventral surface of the cells, which in turn stimulates the formation of focal adhesions and tyrosine phosphorylation of focal adhesion proteins. These results are similar to those proposed by Burridge and Chrzanowska-Wodnicka (66). Activation of kinases associated with gelsolin by OP or RhoVal-14 transduction and inhibition of these kinase activities, along with the absence of podosomes in the antisense oligodeoxynucleotide to Src-treated cells, indicate that signaling molecules associated with gelsolin are crucial in osteoclast motility and bone resorption (19). Several studies indicate the involvement of αβ3, in both migration and organization of the sealing zone (17, 67, 68).

By using time-lapse recording and photography, Kancheisa et al. (18) demonstrated that the podosome structures moved, changed size, and appeared and disappeared. By measuring the rate of migration and bone resorption of osteoclasts using time-lapse recording and scanning electron microscopy, they found that osteoclasts resorb bone matrix at rates up to 400 μm/h and migrate across the surface of bone slices up to 100 μm/h. Thus, the reorganization of the cytoarchitecture of osteoclasts must be a fast, requirement in keeping with the ability of podosomes to disappear, form, and reorganize in minutes (12). We demonstrate here that stress fiber formation/podosome disassembly and stress fiber disassembly and podosome assembly represent the sequence of events observed in RhoVal-14-transduced cells. Conversely, constitutively active Cdc42Val-12 did not affect podosome assembly, or kinase activities in osteoclasts Cdc42Val-12 did stimulate podosome disassembly and filopodia formation but this did not stimulate motility. Cdc42 was shown to activate Rho in stress fiber formation (1) or disassembly of actin stress fibers in two different Swiss 3T3 cell lines (69). Transduction of Cdc42Val-12 into osteoclasts had no effect on Rho activation. Therefore, signaling cascades may be different for Rho and Cdc42 GTPases in osteoclasts.

The molecular mechanism underlying Rho-induced stress fiber and podosome formation remains to be established. A number of downstream effectors have recently been shown to interact with Rho. For example, Rho kinase mediates the formation of actin stress fibers and focal adhesions (70). PtdIns-P2 collaborates with Rho kinase to facilitate the formation of stress fibers (71). Our previous observation demonstrated a time-dependent OP stimulatory effect on PtdIns-P2 association with gelsolin (24). The precise role of PI4P5K in Rho-mediated signaling in osteoclasts needs to be defined. Also, PtdIns-P3 was able to disrupt the association of PI 3-kinase with the tyrosine-phosphorylated proteins (72). The increase in the synthesis and association of phosphoinositides with Triton-soluble gelsolin by OP, functions in the recruitment of signaling molecules containing SH2 domains to the podosomes by protein-phospholipid interactions. This interaction may allow PI 3-kinase and PI 3-kinase to play an important role in the Rho-mediated signaling cascade involved in osteoclast functions. Since PI4P 5-kinase and PI 3-kinase are the known downstream effectors of Rho (38, 39, 42), the increase in the levels of PtdIns-P2 and -P3 associated with gelsolin in OP-treated cells raises the intriguing possibility that gelsolin is the protein that mediates the actions of constituents of the podosomal signaling network such as, pp60c-Src, FAK, PI 3-kinase, and Rho proteins.

Gelsolin is unique because it associates with podosomes and not with adhesion plaques (53). Osteoclasts from gelsolin null mice (73) failed to express podosomes and were hypomotile. They also failed to respond to OP, resulting in a significant decrease in the rates of bone resorption (20). Therefore, gelsolin and the associated signaling molecules including Rho play a crucial role in osteoclast function. Future research should answer questions concerning the networks of proteins with interactions with gelsolin along with the biochemical pathways that mediate the regulation of podosome assembly/disassembly and osteoclast function.

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Rho-A Is Critical for Osteoclast Podosome Organization, Motility, and Bone Resorption
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