Downregulation of Small GTPase Rab7 Impairs Osteoclast Polarization and Bone Resorption

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During skeletal growth and remodeling the mineralized bone matrix is resorbed by osteoclasts through the constant secretion of protons and proteases to the bone surface. This relies on the formation of specialized plasma membrane domains, the sealing zone and the ruffled border, and vectorial transportation of intracellular vesicles in bone-resorbing osteoclasts. Here we show that Rab7, a small GTPase that is associated with late endosomes, is highly expressed and is predominantly localized at the ruffled border in bone-resorbing osteoclasts. The decreased expression of Rab7 in cultured osteoclasts by antisense oligodeoxynucleotides disrupted the polarization of the osteoclasts and the targeting of vesicles to the ruffled border. These impairments caused a significant inhibition of bone resorption in vitro. The results indicate that the late endocytotic pathway is involved in the osteoclast polarization and bone resorption and underscore the importance of Rab7 in osteoclast function.

Osteoclasts are multinucleated cells derived from the monocyte-macrophage lineage that are responsible for degradation of mineralized matrix (1). Osteoclastic bone resorption is essential for normal bone growth and remodeling as well as for tooth eruption. Disturbances in osteoclast differentiation or function result in either osteoporosis, which is characterized by increased bone resorption or various forms of osteopetrosis due to the defective osteoclastogenesis, or the inability of mature osteoclasts to resorb bone.

After attachment to mineralized bone matrix, osteoclasts undergo a defined sequence of events whereby they shift from a nonpolarized to a highly polarized resorbing state (2). Intracellular vesicles are vectorially transported to and fused with the bone-facing plasma membrane to form the ruffled border (3, 4), a highly convoluted membrane domain circumscribed by an actin-rich, ring-like tight attachment structure called the sealing zone (5). During this process, a large amount of vacuolar H⁺-ATPase (v-H⁺-ATPase) is inserted into the ruffled border membrane where it subsequently pumps protons to the resorption lacuna, resulting in the dissolution of the mineral phase of the bone matrix (6, 7). A variety of proteases including cathepsin K are secreted into the resorption lacuna to digest the organic bone matrix (8, 9). The degradation products are then taken up by endocytosis at the ruffled border and transported via a transcytotic pathway to the functional secretory domain at the top of the osteoclast basal membrane opposing to the bone surface (10–12). Although an important role of intracellular membrane trafficking in osteoclast function has been established, much less is known about the nature and regulation of these trafficking pathways in actively resorbing osteoclasts.

Within the past 10 years, small GTPases of the Rab family have emerged as key regulators of intracellular membrane trafficking (13, 14). More than 50 Rab proteins (including isoforms) have been identified in mammalian cells (15). Rab proteins are located at the cytoplasmic face of distinct membrane compartments along the biosynthetic/secretory and endocytic pathways, where they regulate defined steps of vesicular transport through the recruitment of specific effector proteins and cooperation with other regulatory machinery (16–18). Rab7 is a small GTPase that associates with late endosomes (19). Ypt7, the yeast homologue of Rab7, has been shown to regulate transport to vacuoles (20, 21) and is also required for the homotypic fusion between vacuoles (22). Recent data suggest that in mammalian cells Rab7 is involved in the transport from early to late endosomes (23) or from late endosomes to lysosomes (24).

Although the ruffled border faces extracellular matrix, it has been shown to display some distinct features of late endosomal/lysosomal membrane (4). In this study, we have examined the role of Rab7 in osteoclast polarization, ruffled border formation, and bone resorption activities.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of Osteoclasts**—Osteoclast cultures were prepared as described previously (25). Briefly, osteoclasts were scraped from the long bones of 1–2-day-old rat or mouse pups into a minimal essential medium containing 20 mM Hepes, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum, pH 6.9. The disaggregated cells were plated onto glass coverslips or bovine cortical bone slices. After attachment at 37 °C in 5% CO₂, 95% air for 30 min, non-adherent cells were washed away, and the remaining cells were cultured for 48 h and were used for experiments. All cell culture reagents were purchased from Life Technologies, Inc.

**Immunofluorescence and Laser Confocal Scanning Microscopy**—For the localization analysis of Rab7, cells were washed once with phosphate-buffered saline (PBS) and permeabilized with 0.05% saponin in triphosphatase; F-actin, filamentous actin; Lamp2, lysosome-associated membrane protein2; ODN, oligodeoxynucleotide; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction.

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‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF286535.

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Fig. 1. Intracellular distribution of Rab7 in osteoclasts. A, cells were cultured on glass coverslips for 16 h and then were incubated with anti-Rab7 antibody. Images show one optical section of Rab7 labeling in a non-resorbing osteoclast (a) and in mononuclear cells (b). B, double staining of Rab7 and Lamp2 in mouse osteoclasts. C, osteoclasts were cultured on bovine bone slices for 48 h. Double labeling of Rab7 with F-actin (d–c) and β3-integrin (d–f) demonstrates that Rab7 is predominantly localized at the ruffled border membrane in resorbing osteoclasts. Boxed region in (f) is enlarged (g) to demonstrate the localization of Rab7 and β3-integrin at the ruffled border. Localization of Rab7 and v-H+-ATPase in resorbing osteoclasts is shown in D. Bars: A, B, and D, 10 μm; C, c and f, 10 μm; C, g, 2 μm.

80 mM K-Pipes, pH 6.8, 5 mM EGTA, 1 mM MgCl2 for 5 min (26). Cells were fixed with 3% paraformaldehyde in PBS for 20 min. Free aldehyde groups were quenched with 50 mM NH4Cl in PBS for 10 min. The bone slices were incubated in PBS, 0.2% bovine serum albumin, 0.05% saponin (PBSBS) for 5 min to block nonspecific binding. The cells were then incubated with primary antibodies in PBSBS for 45 min. Primary antibody binding was visualized using fluorescent dye-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) in PBSBS for 45 min. Filamentous actin (F-actin) was stained with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate-labeled phalloidin (Sigma-Aldrich) at 0.3 μg/ml. To detect the v-H+-ATPase, the cells were fixed and penetrated with methanol for 5 min and acetone for 30 s, both at −20 °C. Immunofluorescence analysis was performed using a Leica fluorescence microscope and a Leica TCS-SP confocal laser-scanning microscope equipped with argon-krypton lasers (Leica Microsystems Heidelberg GmbH).

Rabbit polyclonal anti-Rab7 antibody was a gift from Dr. S. R. Pfeffer (Stanford University). Goat polyclonal anti-Rab7 antibody was purchased from Santa Cruz Biotechnology, Inc. Mouse monoclonal anti-rat β3-integrin (F11) antibody was obtained from Dr. M. A. Horton (University College London). Rabbit polyclonal anti-B2 subunit of v-H+-ATPase antibody was a gift from Dr. J. P. Mattsson (AstraZeneca). Rat anti-mouse Lamp2 monoclonal antibody developed by Dr. J. T. August was obtained from the Developmental Studies Hybridoma Bank.

Uptake of Transferrin—Osteoclasts cultured on bone slices were incubated in α-minimal essential medium with 20 mM Hepes at 37 °C for 30 min. The cells were then incubated in 200 nM iron saturated human transferrin in α-minimal essential medium with 20 mM Hepes and 0.1% bovine serum albumin for the indicated time. The cells were then fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Immunostaining with polyclonal anti-human transferrin antibody was then carried out.

Electron Microscopy—Osteoclasts cultured on bovine bone slices were fixed with 5% glutaraldehyde in 0.16 mM collidine buffer, pH 7.4, for 3 h. Bone slices were then decalciﬁed in 5% EDTA, 0.1% glutaraldehyde for 3 days. Cells were then postfixed in 2% OsO4, 3% potassium ferricyanide for 2 h and dehydrated in ethanol and embedded in Epon LX 112. Ultrathin sections were stained shortly thereafter with lead citrate and examined using JEOL 100SX transmission electron microscope.

Bone Resorption Assay—After 48 h of oligonucleotide (ODN) treatment, the cells were ﬁxed with 3% paraformaldehyde in PBS for 20 min. F-actin was stained with fluorescein isothiocyanate-labeled phalloidin at 0.3 μg/ml in PBS, and the nuclei were stained with Hoechst 33258 (Molecular Probes, 1 mg/ml stock diluted 1:800 in PBS). The numbers of multinucleated cells and actin ring-containing resorbing osteoclasts were counted using a Leica fluorescence microscope with appropriate filters.

The staining of resorption lacuna and subsequent quantitation of the total resorbed area was performed as described previously (27). Briefly, the cells were removed from the bone slices by sonication in PBS followed by wiping the surface of the bone slices with a soft brush. After a brief rinse in PBS, the slices were incubated with 20 μg/ml peroxidase-conjugated wheat germ agglutinin (Sigma-Aldrich) for 30 min. After washing in PBS, 3,3′-diaminobenzidine (0.52 mg/ml in PBS containing 0.1% H2O2) was added onto the bone slices for 15 min. Quantitation of the resorbed areas on each bone slice was performed with a color camera equipped with an MCID/M2 image analysis system (Imaging Research, Inc.). The resorption activity of osteoclasts was also followed by measuring the amount of carboxyl-terminal telopeptide of type I collagen (CTX) according to the manufacturer’s instructions (Osteometer Biotech, Inc.).

Rapid Amplification of cDNA Ends (RACE)—Highly puriﬁed osteoclasts were obtained by the procedure introduced by Collin-Osdoby et al. (28) with slight modiﬁcations. Briefly, mouse anti-rat β3-integrin
antibody (F11) was absorbed to the sheep anti-mouse antibody-labeled Dynabeads M450 (Dynal, Inc.) at 4°C overnight. The beads were washed with 0.1% bovine serum albumin, 1% fetal bovine serum in PBS and incubated for 5–10 min with the cells scraped from the bone marrow of long bones of neonatal rat pups. With this technique, more than 90% of the obtained cells were osteoclasts, as determined by the tartrate-resistant acid phosphatase staining.

Poly(A) RNA was purified using the QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech). For 3' RACE, mRNA was first reverse-transcribed into cDNA using an oligo-dT primer from the 5'3' RACE kit (Roche Molecular Biochemicals). A gene-specific primer, 5'-TACAAAGCACAATAGGAGCGGACTT-3', and a polymerase chain reaction (PCR) anchor primer included in the kit were used for subsequent amplification of Rab7. 35 cycles of PCR were performed at 94°C for 60 s, 60°C for 60 s, and 72°C for 120 s. For 5' RACE, a gene-specific primer, 5'-CTCTTTGTGGCCACTTGTCT-3', was used as the reverse transcription primer to synthesize cDNA. An oligo-dT anchor primer (included in the kit) and another gene-specific primer, 5'-GCCTGGATGAGAAACTCGTC-3', were used in 35 cycles of PCR at 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s. The RACE products were purified from a 1% agarose gel and sequenced.

Oligodeoxynucleotide Treatment—Rab7 antisense (5'-AGAGGT-CATCCTGGGACTTC-3'), sense (5'-GCTTCAAGGATGACCTCT-3'), and scrambled (5'-ATTATGGGTGCGAAAC-3') ODNs were selected from the sequence site that overlapped the translation initiation codon of the Rab7 cDNA from rat osteoclasts (GenBank accession number F286535). High performance liquid chromatography-purified 5'- and 3'-phosphorothioate-modified ODNs were purchased from Amersham Pharmacia Biotech. ODNs were added to the culture medium immediately after the osteoclasts were plated onto the bone slices. Fresh ODNs were added after 24 h. The cells were incubated for 48 h with antisense, sense ODNs, or with the vehicle alone.

Reverse Transcription-PCR—Poly(A) RNA was purified from six samples of each group by using the QuickPrep micro mRNA purification kit (Amersham Pharmacia Biotech). For 3' RACE, mRNA was first reverse-transcribed into cDNA using an oligo-dT primer from the 5'3' RACE kit (Roche Molecular Biochemicals). A gene-specific primer, 5'-GCCTGGATGAGAAACTCGTC-3', was used in 35 cycles of PCR at 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s. The RACE products were purified from a 1% agarose gel and sequenced.

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kit, and cDNA was synthesized using SUPERSCRIPT preamplification system (Life Technologies, Inc.). One-tenth of these cDNAs was then amplified with rat Rab7-specific primers (upstream, 5'-TACAAGCCCACAATAGGAGCCTCTTGAC-3', and downstream, 5'-ATTGATGGCCTCTTGGCACTGGTCT-3'). Rat β-actin-specific primers (upstream, 5'-CGAGCGACCGCATCC-3', and downstream, 5'-CTTGATGGGTTTGAAGCTGTAG-3') were used as an inner control of the amplification. After 25 cycles of 95 °C (60 s), 60 °C (60 s), and 72 °C (60 s), 10 μl of the reaction product was analyzed on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

**Immunoblotting**—Cells from six bone slices of each group were scraped and lysed in standard SDS sample buffer (Bio-Rad). Protein extracts were electrophoresed on 15% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane. The filters were then incubated in 5% milk and probed with primary antibodies for 1 h. The filters were washed and incubated with the corresponding biotin-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and horseradish peroxidase-conjugated strepavidin (Dako), respectively, and the proteins were visualized using the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech). For reprobing,
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Expression and Subcellular Localization of Rab7 in Osteoclasts—Rab proteins exist in all eukaryotic cells and form the largest branch of the Ras superfamily of small GTPases. Some Rab proteins or isoforms are preferentially expressed in certain tissues or cell types and regulate specific transport events. For instance, Rab3a is abundant in neurons and neuroendocrine cells (29), whereas Rab17 is induced in polarized epithelial cells (30). Rab37, the most recent member, is specifically expressed in mast cells and is selectively localized to the secretory granules (31). In an attempt to define the spectrum of Rab proteins expressed in osteoclasts, we first used degenerate PCR to amplify the fragments of Rab cDNAs from a highly purified rat osteoclast population. 17 different partial cDNA sequences of Rab proteins (32, 33). They have also been applied successfully in mammalian cell culture systems in selecting and expressing specific transport events.

We then examined the subcellular distribution of Rab7 in osteoclasts using immunofluorescence and laser confocal scanning microscopy. Punctate labeling of vesicular compartments confined to the perinuclear areas was observed in non-resorbing osteoclasts and in other cell types cultured on glass coverslips (Fig. 1A). In other cells, Rab7 was seen to colocalize with Lamp2, a late endosomal/lysosomal marker, in osteoclasts (Fig. 1B). In addition, Rab7 was not observed to localize in Golgi apparatus and early endosomes, as examined by labeling with clathrin adaptor protein 3 or early endosomal marker, EEA1 (data not shown). Taken together, these data indicate that Rab7 is associated with late endosomal/lysosomal compartments in osteoclasts. The number of Rab7-positive endosomes in osteoclasts was appreciably higher than in other cells (Fig. 1A). Strikingly, when osteoclasts were cultured on bone slices, the number of Rab7-positive vesicles in the cytoplasm of resorbing osteoclasts decreased, whereas a bright uniformly labeled band was found to localize at the membrane inside the actin ring with a pattern reminiscent of the ruffled border (Fig. 1C, a–c). The localization of Rab7 at the ruffled border was further demonstrated by the clear co-localization of Rab7 with either β3-integrin (Fig. 1C, d–g) or vH^-ATPase (Fig. 1D) at this specialized membrane domain of resorbing osteoclasts. Given the known role of Rab7 in membrane trafficking and fusion, these data suggest an important role for Rab7 in formation of the ruffled border.

Specific Inhibition of Rab7 Expression by Antisense ODNs—We next tested the potential function of Rab7 in bone-resorbing osteoclasts by using antisense ODN targeted against rat osteoclast Rab7. This approach was chosen as it has been used successfully in mammalian cell culture systems in selectively inhibiting expression of a variety of genes, including Rab proteins (32, 33). They have also been applied successfully for suppression of specific gene expressions and for inhibition of bone resorption in cultured osteoclasts (34–36).

The levels of Rab7 expression in vehicle control, sense and antisense ODN-treated cells were analyzed by Western blotting. After a 48-h incubation with 5 μM ODNs, antisense ODNs...
markedly reduced the total amount of Rab7 compared with the untreated cultures and the cultures treated with the sense ODNs. To confirm further the specificity of antisense effects, we also used scrambled ODNs as a control. The amount of cellular proteins loaded in each lane was determined by the staining of actin on the same blot (Fig. 2A). The antisense effect was specific for the Rab7, since the level of rab11 expression was not affected by Rab7 ODN treatment (Fig. 2B). Three other independent experiments showed the similar results.

The effect of antisense ODNs on the level of Rab7 mRNA was examined by reverse transcription-PCR with β-actin as an internal control. The PCR reaction was performed in 25 cycles where saturation was not yet attained as determined from our preliminary experiments. As shown in Fig. 2C, a significant decrease of Rab7 mRNA in antisense ODN-treated cells could be seen. We then further confirmed the down-regulation of Rab7 in antisense-treated osteoclasts by direct immunofluorescent staining. Fig. 2D demonstrates that staining intensity of Rab7 in antisense ODN-treated osteoclasts is clearly less than in controls and sense ODN-treated cultures. These data indicate that the antisense ODNs used in this study were specifically targeted to Rab7 mRNA and resulted in a decrease of Rab7 protein.

Inhibition of Bone Resorption by Rab7 Antisense ODNs—Once the inhibitory effect of the antisense ODNs on Rab7 expression was confirmed, we then attempted to establish a link between Rab7 and osteoclast function. After a 48-h incubation with different concentrations of ODNs, the number of adherent osteoclasts on each bone slice was not significantly different from vehicle control cultures (Fig. 3A). No obvious cytotoxic effects on osteoclasts were observed as appraised by trypan blue and Hoechst 33258 stainings (data not shown). After the treatment with 5 μM antisense ODNs, osteoclast pit formation was greatly decreased as compared with the controls (Fig. 3B). Quantification showed that both the total resorbed area on each bone slice and the number of resorption pits were significantly reduced by the antisense treatment (Fig. 3, C and D). However, the average size of the individual pit was not significantly different among the cultures (Fig. 3E). Inhibition of bone resorption by antisense ODNs was also confirmed by measuring the release of carboxyl-terminal collagen fragments into the culture medium (Fig. 3F). Two other independent experiments yielded similar results.

Impaired Polarization and Ruffled Border Formation due to Rab7 Down-regulation—We next attempted to determine whether the inhibition of osteoclastic bone resorption by Rab7 antisense ODNs was due to impairments in osteoclast polarization and vesicular transportation. As shown in Fig. 4, A and B (a–c), in vehicle and sense ODN-treated cultures, more than 90% of the osteoclasts revealed well developed acting rings and were associated with resorption lacunae beneath the cells. Dramatically, after the treatment with 5 μM antisense ODNs, less than 30% of the osteoclasts contained the intact actin rings (Fig. 4A). The less significant inhibition of the actin ring formation was observed at 10 μM rather than 5 μM antisense ODN treatment. This may be, for instance, due to the inhibition of RNase H activity by higher concentrations of phosphorothioate modified ODNs, as has been shown earlier (37). Quantification of several experiments demonstrated that in 20–30% of osteoclasts exposed to antisense ODNs, significant fraction of F-actin was in the cytoplasm. Actin was also seen clustered at the plasma membrane facing the bone, but the actin ring and pit formation was disturbed (Fig. 4B, d–g). This pattern suggests that the osteoclast polarization and actin organization were prevented at a certain stage of the resorption cycle (38). In contrast, only 1–2% of the osteoclasts in control cultures existed at the early stage of the resorption cycle after a 48-h culture. In these cells, no visible accumulation of the actin in the cytoplasm was observed.

The v-H⁺-ATPase was mainly localized at the ruffled border
of resorbing osteoclasts in control and sense-treated cultures (Fig. 5A). In contrast, the v-H\(^+\)-ATPase was observed in the cytoplasm and mistargeted to the basal membrane in 50–60% of osteoclasts in antisense ODN-treated osteoclasts (Fig. 5B, a–f). To further characterize the effects of Rab7 antisense ODNs on intracellular membrane trafficking in osteoclasts we used transferrin as a marker to follow the vesicular trafficking to the ruffled border. Consistent with our previous observation (4), we found that in control cultures transferrin was endocytosed from the basal membrane of the resorbing osteoclasts and was transported to the ruffled border (Fig. 6A). When Rab7 was down-regulated by antisense ODNs, the transport of transferrin to the ruffled border was blocked although we could not observe any inhibition of its uptake and entry into the early endosomes (Fig. 6, B and C).

Finally, the ultrastructural changes in osteoclast morphology induced by the down-regulation of Rab7 were examined by transmission electron microscopy. A highly enfolded ruffled border could be seen inside the sealing zone in most of the osteoclasts from the control cultures (Fig. 7A). However, in antisense-treated osteoclasts, the impaired ruffled border formation was observed along with an extended clear zone (Fig. 7B).

**DISCUSSION**

We show here that Rab7 is highly expressed and associates with late endosomes in osteoclasts. Furthermore, it is predominantly localized at the ruffled border membrane of bone-resorbing osteoclasts. The down-regulation of cellular Rab7 in cultured osteoclasts disrupted the polarization and the targeting of vesicles to the ruffled border. These impairments resulted in a significant inhibition of osteoclastic bone resorption in vitro. Given the important role of Rab7 in late endocytic pathway and the localization of several late endosomal membrane proteins at the ruffle border (3, 4, 39), the results suggest that the late endocytic pathway is involved in the formation of the ruffled border and the osteoclastic bone resorption.
Previous in vitro studies have shown that a large number of acidic vesicles exist in non-resorbing osteoclasts. During the activation of the osteoclast, these intracellular vesicles disappear and simultaneously a highly enfolded ruffled border membrane appears (4). Thus, it is generally assumed that the ruffled border is formed by the fusion of these acidic vesicles with the plasma membrane. However, the identity of these vesicles and the molecular machinery involved in this exocytotic process remain unclear. Interestingly, the distribution pattern of the late endosomal/lysosomal small GTPase, Rab7, has a similar shift from the cytoplasmic vesicles to the ruffled border membrane in actively resorbing osteoclasts. Thus, our data indicate that the ruffled border is formed by the fusion of late endosomal/lysosomal compartments with the plasma membrane during the early phase of the resorption cycle. Recently, a similar process has been identified also in other cell types (40).

Two isoforms of Rab3, Rab3A and Rab3B, have also been shown to be expressed in osteoclasts (41). Given the crucial role of Rab3 in the regulated secretion in neurons and neuroendocrine cells, Rab3 has been suggested to play a role in the regulation of both osteoclast differentiation and function (41, 42). Rab3A in osteoclasts has been shown to be involved in the ruffled border formation in the osteoclasts. This further supports the notion that the ruffled border membrane, although a part of the plasma membrane, functions as late endosomal compartment.

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